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Detection and characterisation of carbapenem-resistant Gram-negative bacilli infections in Ghana

Francis Samuel Codjoe

A thesis submitted in partial fulfilment of the requirements of
Sheffield Hallam University
for the degree of Doctor of Philosophy

September 2016

Table of contents	ii
List of Figures	vi
List of Tables.....	vii
Acknowledgements	ix
Declaration	xi
Dedications.....	xii
Abbreviations	xiii
Abstract	xvii

Contents page

1. Introduction

1.1 Background	1
1.2 Gram-negative bacteria and infections.....	2
1.2.1 Common antimicrobials for Gram-negative bacterial infections.....	4
1.3 Antimicrobial resistance in Gram-negative bacteria.....	4
1.3.1 Clinical significance and risk of ESBLs	5
1.3.2 Other types of ESBLs	7
1.4 Carbapenem antimicrobials.....	10
1.4.1 Carbapenem mode of activity and structure-function relationship	10
1.4.2 Carbapenem usage and side effects.....	13
1.5 Development of carbapenem resistance.....	14
1.5.1 Intrinsic resistance of Gram-negative bacilli	15
1.5.2 Acquired resistance of Gram-negative bacilli.....	16
1.5.3 Risk factors for acquisition of CRE infection.....	20
1.6 Gram-negative bacteria carbapenemase resistance	22
1.6.1 The significance of carbapenemases	22
1.7 Molecular classification of carbapenemase enzymes	25
1.7.1 Class A carbapenemases	25
1.7.2 Class B carbapenemases	27
1.7.3 Class D carbapenemases	28
1.8 Laboratory detection of carbapenem-resistant organisms.....	30
1.8.1 Phenotype based methods	31

1.8.2 Problems in phenotype based detection	37
1.8.3 Genotype based techniques	38
1.9 Epidemiology of carbapenemase-producing organisms	41
1.10 Treatment options for CRE infections	51
1.11 Prevention of CRE infections	54
1.12 Problems and clinical relevance of carbapenemase production.....	56
1.13 Future solutions to curb global CRE threat.....	58
1.14 Justification of the study in Ghana.....	60
1.15 Hypothesis.....	65
1.16 Main objective.....	65
1.16.1 Specific objectives	65
 2. Materials and methods	
2.1 Materials.....	66
2.2 Ethical Approval	66
2.3 Sample sites	66
2.3.1 Korle Bu Teaching Hospital	68
2.3.2 Effia Nkwanta Hospital	68
2.3.3 AngloGold Mines hospital	69
2.3.4 Ho Regional hospital.....	69
2.4 Bacterial isolates	69
2.5 Microbiological assays	70
2.5.1 Identification of bacterial isolates	70
2.5.2 Antimicrobial resistance testing.....	71
2.5.3 Determination of minimum inhibitory concentration by the E-test method.....	71
2.6 Phenotypic based methods for carbapenem-resistant organisms	72
2.6.1 Modified Hodge test.....	72
2.6.2 Boronic acid-disc synergy test	73
2.6.3 Sensitivity and specificity calculation for modified Hodge test and boronic acid-disc synergy test.....	74
2.6.4 Nitrocefin assay for β -lactamase enzyme identification	75
2.6.4.1 Principle of nitrocefin assay.....	75

2.6.4.2 Preparation of β -lactamase extracts	75
2.6.4.3 Microplate assay for determination of β -lactamase concentration	76
2.7 Genotypic characterisation of carbapenemase-producers	77
2.7.1 Genomic DNA extraction on CR organisms.....	77
2.7.2 Preparation of primer sets	77
2.7.3 PCR reaction mix preparation.....	78
2.7.4 PCR amplification conditions	79
2.7.5 PCR products for electrophoresis.....	80
2.7.6 Loading and electrophoresis of amplified PCR products	80
2.7.7 Visualisation of bands on agarose gel.....	80
2.7.8 Purification of PCR products for sequencing	80
2.7.9 Sequencing of purified PCR products.....	81
2.7.10 Determination of extended spectrum β -lactamase genes in total DNA extracts	81
2.7.11 Outer membrane protein analysis of CR isolates.....	81
2.7.11.1 Separation of outer membranes of <i>Escherichia coli</i> , <i>Acinetobacter</i> species and other organisms	82
2.7.11.2 Outer membranes separation from <i>Pseudomonas</i> species	83
2.7.11.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).....	84
2.8 Plasmid analysis of carbapenem-resistant organisms	85
2.8.1 Plasmid DNA extractions.....	85
2.8.2 Purified plasmid DNA and concentration analysis	85
2.8.3 Detection of common β -lactamases and carbapenemase genes in plasmid DNA extracts.....	86
2.8.4 Plasmid transfer experiment.....	86
2.8.5 Plasmid curing and restriction enzyme digestion experiment.....	87
2.9 Molecular typing of carbapenem-resistant isolates by ERIC-PCR	88
2.9.1 Electrophoresis and computer analysis of ERIC-PCR data	88
2.9.2 Sequence data and analysis of phylogenetic trees.....	89
2.9.3 ERIC-PCR genetic relatedness analysis.....	89

3. Phenotypic and genotypic characterisation of carbapenem-resistant isolates

3.1 Background and Aims.....	90
3.2 Method summary	92
3.3 Results	93
3.3.1 Carbapenem-resistant isolates demographics and characteristics.....	93
3.3.2 Resistance pattern of CR isolates	94
3.3.3 ESBL determination by cefpodoxime and nitrocefin assay.....	99
3.3.4 Prevalence of carbapenemase detection based on phenotypic tests.....	100
3.3.5 Phenotypic and genotypic correlation of carbapenem-resistant isolates	104
3.3.6 Distribution and prevalence of carbapenemase-producing genes	105
3.3.7 Analysis of outer membrane proteins by SDS-PAGE	110
3.3.8 Sequenced data and phylogenetic analysis of carbapenemase positive genes	116
3.3.9 Phylogenetic relationships of carbapenemase genes.....	117
3.4 Discussion	121

4. Plasmid analysis of carbapenem-resistant Gram-negative bacteria

4.1 Background and Aims.....	139
4.2 Method summary	141
4.3 Results	141
4.3.1 Plasmid DNA concentration analysis	141
4.3.2 Plasmid DNA analysis by gel electrophoresis	142
4.3.3 Plasmid transfer analysis.....	144
4.3.4 Resistance pattern of cured plasmid-isolates to carbapenems	149
4.3.5 Resistance profile of cured isolates by E-test assay.....	151
4.3.6 Plasmid DNA restriction pattern of cured carbapenemase-positive gene carriers	162
4.4 Discussion	164

5. Genetic relatedness of carbapenem-resistant isolates from Ghana using ERIC-PCR technique

5.1 Background	173
5.2 Method summary	176

5.3 Results	176
5.4 Discussion	186
 6. Overall discussion, conclusion and future work	
6.1 Overall discussion	192
6.2 Conclusion	203
6.3 Future work	204
References	210
Appendices	268

List of figures

Figure 1.1 Three β -lactam agents depicting the functional groups.....	11
Figure 1.2 Resistance due to β -lactamase, PBP and efflux pump mechanisms in <i>Pseudomonas aeruginosa</i>	19
Figure 2.1 Map of Ghana depicting sample sites.....	67
Figure 3.1 Carbapenem-resistant isolates and E-test MIC against carbapenems.....	98
Figure 3.2 Positive and negative modified Hodge test on Mueller-Hinton agar plate	100
Figure 3.3 Two boronic acid-disc synergy tests on Mueller-Hinton agar plate.....	104
Figure 3.4 Distribution of carbapenemase resistance genes among hospitals and type of specimens	108
Figure 3.5 Distribution of carbapenemase-positive genes in both sexes, among age groups and carbapenem-resistant isolates.....	109
Figure 3.6 Outer membrane proteins of control strains and selected carbapenem-resistant isolates on SDS-PAGE.....	112
Figure 3.7 Phylogenetic trees for OXA-48 positive gene with OXA-48 genes of known sequenced organisms.....	118
Figure 3.8 Phylogenetic trees for VIM-1 positive gene with VIM-1 genes of known sequenced organisms.....	119
Figure 3.9 Phylogenetic trees for NDM-1 positive gene with NDM-1 genes of known sequenced organisms.....	120

Figure 4.1 Plasmid DNA profiling on agarose gel electrophoresis	144
Figure 4.2a Amplified carbapenemase genes from plasmids.....	147
Figure 4.2b Amplified carbapenemase genes from plasmids	148
Figure 4.3 Plasmid profiling of two β -lactamase genes on gel electrophoresis.....	149
Figure 4.4 Comparison between resistance patterns of an uncured plasmid-borne isolate and corresponding cured susceptibility testing.....	150
Figure 4.5 Undigested and digested restriction enzyme profiling from cured carbapenemase-positive gene carriers	163
Figure 4.6 Profiling of restriction enzyme digestion from cured carbapenemase-positive gene carriers	163
Figure 5.1 Representative gels showing banding ERIC-PCR from diverse carbapenem-resistant isolates.....	177
Figure 5.2 Dendrogram generated from ERIC-PCR genomic DNA products for both carbapenemase non-PCR positives and carbapenemase-positive gene carriers	180
Figure 5.3 Dendrogram generated from ERIC-PCR genomic DNA products for carbapenemase-positive gene carriers.....	184

List of tables

Table 1.1 Beta lactamases classifications, ESBL activity and representative Enzymes	8
Table 1.2 Carbapenemases by classification, activity and organisms	30
Table 1.3 Breakpoint values (MIC, mg/l) for carbapenems according to guidelines in Europe (EUCAST) and the United States (CLSI).....	33
Table 1.4 Interpretation of inhibitor-based phenotypic synergy tests.....	36
Table 2.1 Primer sets for amplification of carbapenemase and extended spectrum β -lactamase genes.....	78
Table 2.2 Composition of PCR reaction mix	79
Table 3.1 Distribution pattern of carbapenem-resistant organisms by age groups, sex and source of specimens.....	95
Table 3.2 Distribution pattern of carbapenem-resistant organisms from selected hospitals in Ghana	96
Table 3.3 Resistance pattern of carbapenem-resistant isolates against	

carbapenems	97
Table 3.4 Detection of β -lactamase activity using nitrocefin assay	101
Table 3.5 Distribution of presumptive carbapenemase producers in phenotypic and genotypic assays among carbapenem-resistant isolates	106
Table 3.6 Prevalence of carbapenemase resistance genes and the tested assays	107
Table 3.7 Important outer membrane proteins of <i>P. aeruginosa</i> strain	110
Table 3.8 Analysis of outer membrane proteins among <i>Pseudomonas</i> isolates on SDS-PAGE.....	113
Table 3.9 Analysis of outer membrane proteins among isolates of <i>Acinetobacter baumannii</i> on SDS-PAGE	115
Table 3.10 Analysis of outer membrane proteins among the Enterobacteriaceae isolates on SDS-PAGE.....	116
Table 4.1 Quantification of plasmid DNA in carbapenem-resistant isolates using the Nano-Drop Spectrophotometer (ND-1000).....	143
Table 4.2 Beta-lactamase genes amplified from plasmid DNA identified in carbapenem-resistant isolates.....	145
Table 4.3 Distribution of carbapenemase genes carried on plasmids in carbapenem-resistant Gram-negative isolates.....	146
Table 4.4a Amplified carbapenemase genes from plasmids.....	152
Table 4.4b Amplified carbapenemase genes from plasmids.....	160
Table 5.1 Genetic relatedness for both carbapenemase non-PCR positives and carbapenemase-positive gene carriers.....	181
Table 5.2 Genetic relatedness among the carbapenemase-positive gene carriers....	185

Acknowledgements

The journey to embark on studying for my PhD really, began when the demand for terminal degree became compulsory for the lectureship job in 2010 at the University of Ghana. Indeed, I would like to express my sincere gratitude to the Head of BMRC, Sheffield Hallam University, Professor Nicola Woodroffe for granting me the opportunity to study to the PhD level in my Alma mater and with a lot of appreciation to Mrs Marguerite Lyons of the BMRC for her undeniable skills in communication throughout my PhD studies.

Great appreciation and affection go to my Principal supervisor, Dr Keith Miller of the BMRC, Sheffield Hallam University for his knowledgeable experience, interest, affection, directness and zeal of guidance to successfully complete the research study. My gratitude also goes to my second supervisor, Professor Thomas J. Smith for the support and critically reviewing my work. Best of thankfulness and love to my former local supervisor, Professor Kingsley Twum-Danso who fell ill after first year of supervision and later taken over by Professor Eric Sampane-Donkor, both at the Department of Medical Microbiology, University of Ghana for their encouragement, patience and friendly attitude throughout this study. I am very much indebted to the following advisors, Dr Charles A. Brown, Dr Richard H. Asmah, the former and present Heads of Department, Medical Laboratory Sciences, University of Ghana, respectively and Dr Phillips Gardiner of BMRC, Sheffield Hallam University for their outstanding and professional competencies by way of advice and suggestions.

I am very thankful to the University of Ghana for granting the Study Leave and also allowed me to carry out this research study in both Departments; Medical Microbiology and Medical Laboratory Sciences (Microbiology Division) under the College of Health

Sciences. I feel privileged to express my deepest appreciations to Professor Edwin Kwame Wiredu and Reverend Professor Patrick F. Ayeh-Kumi, for their immeasurable contributions that earned my funding support from the College of Health Sciences Endowment Fund. In particular, my sincere thanks go to Professor Yao Tettey (former Provost), Mrs. Amma K. Kwaa (former College Secretary) and Mrs. Yvonne Lartey (Secretary to the College Endowment Fund) for the endorsement of my sponsorship submission that took effect from the 2nd year of my study.

Special thanks to all the Biomedical Scientists who helped in diverse ways during my studies, especially, Mr Thomas Dankwa, Mr Ebenezer Kofi Mensah, Mr Daniel Somuah, Mr Charles Yao Agebe, Mr Pascal Hodogbe, Mrs Esther Okine, Miss Nana Eghele Adade, Miss Marjorie Quarchie and those not mentioned. A lot of gratitude also goes to Mr Michael Olutaiwo, for singlehandedly collected part of the carbapenem-resistant isolates in my absence while in the UK. My thanks also go to Yasin Al-Luaibi, Peter Kanengoni and other colleague PhD students who in diverse ways shared ideas and thoughts, during my studies.

A special note of thanks to Professor Kwadwo Ansah Koram and Professor Dorothy Yeboah Manu for the good cooperation they had with me during my studies and also granting the permission to perform my plasmid experimental studies. My sincere thanks to Astra-Zeneca, Ghana branch for substantially supplementing the supply of the meropenem discs that made screening easier for the detection of carbapenem resistance. Finally, to my wife, Sarah and daughters, Magdalene and Helen for their understanding and care throughout this research study. I sincerely thank them for their patience and encouragement given to me.

Declaration

I hereby declare that this thesis submitted for the degree of PhD is the result of my own research and that this thesis has not been submitted for a higher degree to any other university or institution. All sources of information have been properly referenced.

Part of this research work in Chapter 3, 4 and 5 would be prepared for publications in agreement with my principal supervisor, Dr Keith Miller.

Francis Samuel Codjoe

September, 2016

Dedication

Firstly, to the Almighty God from whom all good things come for granting me strength, wisdom and knowledge to pursue the PhD study. Secondly, to my immediate family: my beloved wife, Mrs Sarah Codjoe; dear daughters, Mrs Magdalene Yeduwah Addo and Helen Awurama Codjoe, and lastly, to all my brothers, sisters, friends and everyone who helped in diverse ways throughout my studies.

Abbreviations

AGAMH	AngloGold Ashanti Mines hospital
BADST	Boronic acid-disc synergy test
BLAST	Basic local alignment search tool
bp	Nucleotide base pair
CDC	Center for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CRE	Carbapenem-resistant Enterobacteriaceae
DHP-1	Inhibitor dehydropeptidase-1
DNA	Deoxyhyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ENRH	Effia-Nkwanta Regional hospital
ESBL	Extended-spectrum β -lactamase
EUCAST Testing	European Committee on Antimicrobial Susceptibility
g	gram
GNB	Gram-negative bacteria
GES	Guiana extended spectrum
GIM	German imipenemase
ICARE	Intensive Care Antimicrobial Resistance Epidemiology

ICUs	Intensive care units
IMI-1	Imipenem-hydrolyzing β -lactamase
IMP-1	Imipenem-resistant <i>Pseudomonas</i> -1
IDSA	Infectious Diseases Society of America
kDa	Kilo Daltons
KPC	<i>Klebsiella pneumoniae</i> carbapenemase
KATH	Komfo Anokye Teaching hospital
KBTH	Korle Bu Teaching hospital
LPS	Lipopolysaccharide
LTACHs	Long-term acute care hospitals
LTCFs	Long-term care facilities
MALDI-TOF MS	Matrix-assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry
MBLs	Metallo- β -lactamases
MDR	Multi-drug resistant
mg	milligram
MHT	Modified Hodge test
MICs	Minimum inhibitory concentrations
ml	millilitre
MLST	Multilocus sequence typing
mM	millimolar

MOH	Ministry of Health
NCBI	National Centre for Biotechnology Information
NDM-1	New Delhi metallo- β -lactamase-1
ng	nanogram
NHSN	National Healthcare Safety Network
NMCA	Not metalloenzyme carbapenemase A
NMIMR	Noguchi Memorial Institute for Medical Research
nmol	nanomole
OXA	Oxacillinase
PAGE	Polyacrylamide gel electrophoresis
PBP	Penicillin-binding proteins
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulphate
SFC-1	<i>Serratia fonticola</i> carbapenemase-1
SHV-1	Sulfhydryl variable-1
SIM	Seoul imipenemase
SME	<i>Serratia marcescens</i> enzyme
SPM	Sao Paulo metallo- β -lactamase-1
TAE	Tri acetate EDTA
TEMED	N, N, N, N-tetramethyl-ethane-1, 2-diamine

TEM-1	Temoneira-1
VIM-1	Verona integron-encoded metallo- β -lactamase-1
v/v	volume to volume
WHO	World Health Organization
w/v	weight to volume

Abstract

In Ghana, little is known about the nature and spread of carbapenemases in carbapenem-resistant (CR) pathogens. The aims of the present study were to detect carbapenemase activity by using simple phenotypic tests, molecular typing to characterise the resistance genes and to determine the relatedness of the CR isolates collected from different hospitals in the country.

A total of 111 CR isolates were identified by disc diffusion susceptibility testing and the MIC E-test method. Phenotype-based methods including the modified Hodge test, boronic acid-disc synergy test, nitrocefin assays, plasmid analysis and sodium dodecyl sulphate polyacrylamide gel electrophoresis for the expression of the outer membrane protein were performed for each of the CR isolates. Amplified DNA products were examined for common ESBL encoding genes (*bla*TEM-1 and *bla*SHV-1) and carbapenemase resistance genes (*bla*KPC-1, *bla*IMP-1, *bla*NDM-1, *bla*VIM-1 and *bla*OXA-48). Enterobacterial repetitive intergenic consensus (ERIC) by PCR technique was used to establish the relatedness of isolates.

Overall, a carbapenem-resistant prevalence of 2.9% (111 of 3840) was detected from the total of Gram-negative bacterial pathogens. In MIC E-test assays, 56.8% of CR isolates showed complete resistance to imipenem, meropenem and ertapenem at ≥ 32 $\mu\text{g/ml}$, of which 24.3% were found in *Pseudomonas aeruginosa* isolates and 18.9% in *Acinetobacter baumannii* isolates. In all, no KPC-1 and IMP-1 genes were detected. Carbapenemase genes identified were *bla*NDM-1 in *Acinetobacter baumannii* isolates, *bla*VIM-1 in *Pseudomonas* species and *bla*OXA-48 was only present in *Klebsiella pneumoniae* isolates. None of the carbapenemase-positive gene carriers harboured two

or more of carbapenemase resistance genes. Transfer experiments revealed the possible spread of the resistance genes from pathogens to commensal organisms by conjugation. Close relatedness with co-occurrence of *oprD* loss was detected among a small number of carbapenemase resistance gene carrying isolates of *Acinetobacter baumannii*. This is the first report of the detection and characterisation of carbapenemase resistance genes in Ghana.

Chapter one

1. Introduction

1.1 Background

Over seventy years the antimicrobial era has been marked by successive discoveries of a wide range of antibiotics and the subsequent emergence of antibiotic resistance. Bacterial resistance continues to increase and, drug researchers and manufacturing industries are not producing new drugs to replace the existing antimicrobials against which resistance has developed. The effects on current infection rates cannot be simply estimated (Carlet *et al.* 2012, WHO 2014, Freeman *et al.* 2015).

Howard and Scott (2005) reported that the economic impact related to antimicrobial resistance was expected to cost over \$105 billion annually worldwide. In recent times, development of antimicrobial resistance is rapidly changing, and the impending public health challenges these may cause in many health sectors need worldwide coordinated interventions. Many deaths have occurred as a result of this in Europe and the European Centre for Disease Prevention and Control (ECDC) had estimated that 25,000 people may die each year from infections related to antimicrobial resistance (Carlet *et al.* 2012, Moyane, Jideani and Aiyegoro 2013). Global reports documenting the infection burden of common and diverse bacterial pathogens that have developed resistance to available antimicrobial agents is alarming. Spellberg *et al.* (2011) reported the annual costs of combating resistant bacterial infections to be between \$21 billion and \$34 billion in the USA alone. At the World Health Day held in the year 2011, two bodies; the World Health Organization (WHO) and the Infectious Diseases Society of America (IDSA)

predicted that antimicrobial resistance worldwide may have major consequences for human health unless concrete solutions are found (Spellberg *et al.* 2011, WHO 2015). The cost burden of bacterial resistance worldwide may be difficult to quantify currently according to the World Health Organization (WHO) due to limited information on drug resistance investigations carried out between 2013 and 2014 among member states. Only 17% of countries participated in Africa while in the other continents 62% to 100% responded (WHO 2015). This has prompted the WHO to adopt new strategies through education and to create awareness for its member states to know the extent of the problem of antimicrobial resistance at each country level. Indeed, the current emergence of carbapenem-resistant Enterobacteriaceae (CRE) is a source of worry to healthcare providers due to its limited treatment options, and has become a major public health challenge globally (Schwaber and Carmeli 2008, Public Health England 2015, WHO 2015).

1.2 Gram-negative bacteria and infections

Infections acquired from clinically relevant Gram-negative bacilli include a wide variety of species. Urinary tract infections (*Escherichia coli*, *Proteus* species, *Enterobacter* species, *Serratia marcescens*), respiratory infections (*Pseudomonas aeruginosa*, *Haemophilus* species, *Klebsiella* species, *Legionella pneumophila*), and gastrointestinal infections (*Salmonella typhi*, *Salmonella enteritidis*, *Shigella* species, *Helicobacter pylori*) are the primary Gram-negative infections in most hospital settings (Peleg and Hooper 2010, Richet 2012). In recent times, a non-fermentative Gram-negative organism, *Acinetobacter baumannii* has been associated with hospital-acquired infections. Similarly, other less encountered Gram-negative bacteria such as *Stenotrophomonas maltophilia* and *Aeromonas* species have been increasingly reported

in many episodes of bacteraemia, meningitis, pneumonia, wound or surgical site problems in hospital intensive-care settings (Datta and Wattal 2010). Most of these Gram-negative bacteria are resistant to multiple drug agents and are increasingly becoming resistant to almost all available antimicrobial drugs worldwide (Xu, Flavin and Flavin 2014).

In Ghana, there are very few nationwide studies conducted on infections associated with antibiotic resistance. Most studies were carried out in a few hospitals and focussed on common pathogens such as *Staphylococcus aureus* and *Salmonella typhi*, rather than multi-resistant Gram-negative pathogens such as *Pseudomonas* and *Acinetobacter* species, commonly encountered in the developed countries (Newman *et al.* 2011, Donkor, Foster-Nyarko and Enweronu-Laryea 2013). In the United States, a survey based on National Healthcare Safety Network (NHSN) data showed that, 13% of *Escherichia coli* and *Klebsiella*, 17% of *Pseudomonas aeruginosa* and 74% of *Acinetobacter baumannii* in intensive-care units were multidrug-resistant (CDC 2008). Similarly, studies conducted in non-African countries in East Asia by Kyungwon *et al.* (2011) on these same organisms further indicated significant increase in the Korean Nationwide Surveillance of Antimicrobial Resistance program (Kyungwon *et al.* 2011).

1.2.1 Common antimicrobials for Gram-negative bacterial infections

Development of several new and modified compounds active against drug-resistant Gram-positive organisms has been conducted in the last few years. In the case of resistant Gram-negative bacteria, few new antibiotics and no new classes of antimicrobials against resistant Gram-negative bacilli can be anticipated in the near future (WHO 2014). The use of suitable empirical antimicrobial treatment significantly improves patient outcomes and reduces their hospital stay in many instances by clinicians (Fraser *et al.* 2006). Most of the first line and low-cost drugs with broad-spectrum activity such as co-trimoxazole, gentamicin, tetracycline, chloramphenicol, ampicillin, fluoroquinolones and some cephalosporins have been in use as effective therapeutic agents for the management of Gram negative bacterial infections since the 1990s (Khadri and Alzohairy 2009, Habte-Gabr 2010). Carbapenems became the antimicrobial agents of choice for many serious bacterial infections, especially in hospitals, but most drugs, both β -lactams and other non β -lactam antimicrobials, have been inactive against multi-resistant Gram-negative bacteria (Moyane, Jideani and Aiyegoro 2013).

1.3 Antimicrobial resistance in Gram-negative bacteria

Imprudent use of antimicrobials has led to the development of resistance which has now reached a grave situation. Bacterial reactions to antimicrobials, in particular, may depend significantly on the concentration of antimicrobial drug present at any one time. Different types of mechanisms, such as acquisition of genes encoding enzymes (β -lactamase types) that destroy the antibiotic, efflux pumps to remove the agents from the bacterial cell before reaching the target site, and alteration of binding sites that may take place before sufficient concentrations are reached to eliminate the entire poly-microbial

population. Gram-negative bacteria are no exception to these interactions (Walsh 2013). Gram-negative bacteria are often more resistant to antimicrobials, when compared with Gram-positive pathogens due to their outer membrane structure. The same outer membrane protects the bacteria from most dyes, antimicrobials and detergents that may damage either the internal membrane or the peptidoglycan in the cell wall. The outer membrane of these bacteria renders some protective resistance to lysozyme and penicillin attacks. However, in combination, lysozyme with ethylenediamine tetra-acetic acid (EDTA) and ampicillin antimicrobial, which serve as an alternative drug treatment for some pathogenic Gram-negative organisms, have been shown to breach the defensive outer membrane. There are other antimicrobials which can be used, including streptomycin, chloramphenicol and nalidixic acid (Xu, Flavin and Flavin 2014).

1.3.1 Clinical significance and risk of ESBLs

The discovery of Gram-negative resistance became apparent soon after the first semisynthetic penicillin, ampicillin, an agent found to be active against Gram-negative bacteria, was clinically introduced. Multidrug-resistant Enterobacteriaceae have been a growing concern worldwide (Pereira *et al.* 2011). In 1963, Temoneira-1 (TEM-1) was named after Temoneira, the affected patient, the first plasmid-encoded β -lactamase carrying *Escherichia coli* strain to have conferred resistance against the drug ampicillin (Datta and Kontomichalou 1965, Altayb *et al.* 2014). Over the years, diverse resistance mechanisms have changed the distribution of plasmids and new mobile genetic features have been contributory in the horizontal transmission of resistance genes, with these multiple genes conferring resistance to many antimicrobials. Among the Enterobacteriaceae, TEM-1 and sulfhydryl variable-1 (SHV-1) β -lactamases were the most prevalent plasmid-mediated enzymes frequently found spreading in countries

worldwide (Medeiros 1997). In the 1970s, resistant Gram-negative bacteria had become more common in most hospital-acquired pathogens with TEM-1 and SHV-1 enzymes. Most of these bacteria carried multiple β -lactamases as well as other multidrug-resistant genes. In the early part of the 1980s, a number of new antimicrobials were clinically introduced in the health-care delivery systems, including the third-generation cephalosporins. Due to misuse of these agents, Germany in 1983 experienced the first extended-spectrum β -lactamase (ESBL) in a species of *Klebsiella*. ESBLs are resistance enzymes usually confer resistance in most Gram-negative bacterial pathogens as a result of more-selective pressure from the use of β -lactams: oxyimino-cephalosporins (such as cefotaxime, ceftriaxone, ceftazidime, or cefepime) and monobactams (aztreonam) but not carbapenems, which had undergone hydrolysis and further mutations (Bush 2013, Blair *et al.* 2015). The ESBL enzymes result from point mutation in the parent β -lactamases, TEM-1 and SHV-1 by one to four amino acid changes which form the basis of resistance presumably due to evolutionary selective pressure from the use of β -lactams, such as oxyimino-cephalosporins and aztreonam. To date, the number of known β -lactamases has increased, and there are now over 1000 that have been identified. The most recognizable among the mutants of SHV-1, named SHV-2, deactivated the extended-spectrum cephalosporin drugs and often carried many other resistance genes on its parent plasmid that conferred reduced susceptibility to other unrelated classes of antimicrobials (Knothe *et al.* 1983, Altayb *et al.* 2014).

1.3.2 Other types of ESBLs

Equally important ESBLs of clinical significance are the CTX-M and AmpC β -lactamases families as indicated in table 1.1. The CTX-M family is classified under classes of β -lactamases as class A ESBLs. For the past decade, CTX-M-type ESBL enzymes have become most prevalent in clinical isolates, mostly in *Escherichia coli* isolates in Asia, Europe and South America (Wang *et al.* 2013).

Earlier, there were confusions as to where MEN-1 and Tolo-1 enzymes belong. CTX-M-1 was subsequently found to be similar to the MEN-1 enzyme; while CTX-M-44 and CTX-M-45 were known to be the same as Toho-1 and Toho-2 respectively (Ma *et al.* 1998, Bush 2013). Since CTX-M-1 recognition in clinical circles in the 1980s, over 130 variants have been identified and genetically classified based on amino acid differences into 5 major divisions, CTX-M-1, -2, -8, -9, or -25 mostly identified in *Escherichia coli* and *Klebsiella pneumoniae* isolates from varying geographical locations (Rossolini, D'Andrea and Mugnaioli 2008, Rogers, Sidjabat and Paterson 2011). In 1999, a CTX-M-15 variant was recovered from India; belonging to the CTX-M-1 group, it was shown to have dominance in the clinical setting and also shown to have worldwide distribution. Thus, more allelic variants were subsequently recovered from different Gram-negative bacterial isolates in both clinical and community settings and those yet to arrive are a threat to patients' conditions in the clinical environment (Cantón, González-Alba and Galán 2012, Wang *et al.* 2013).

Table 1.1 Beta lactamases classifications, ESBL activity and representative enzymes

Molecular class	Functional group	ESBL activity ^c	Representative enzymes
A	2a	N	PC1
	2b	N	TEM-1, SHV-1
	2be	Y	CTX-M-14, -15
	2br	N	TEM-30, SHV-10
	2ber	Y	TEM-50, TEM-121
	2c	N	PSE-4, CARB-3
	2ce	N ^e	RTG-4
	2e	Y	SFO-1, FEC-1, L2
	2f	Y	KPC-2, SME-1 ^f
B	3a ^g	Y	IMP, VIM, NDM, L1
	3b	N	CphA
C	1	N	AmpC, ACT-1
	1e	Y	GC1, CMY-37
D	2d	N	OXA-1, OXA-10
	2de	V	OXA-11, OXA-15
	2df	Y	OXA-23, OXA-48

Adapted and modified from (Bush 2013).

c= Based on hydrolysis of cefotaxime, ceftazidime, or cefepime

e= In spite of k_{cat} values generally $>1 \text{ s}^{-1}$, resistance to cefepime and ceftazidime is seen in producing organisms

g= Includes subclasses B1 and B3

Y= $k_{cat} >5 \text{ s}^{-1}$, N= $k_{cat} <5 \text{ s}^{-1}$, V= variable within the functional group.

AmpC β -lactamases are also of importance and the enzymes can be chromosome or plasmid-mediated. After sequencing of *ampC* gene from *Escherichia coli* K-12 strain, it was designated as class C according to Ambler's structural classification of β -lactamases (Table 1.1). Thus, differences in molecular structures between β -lactamases classes A and B actually determined *ampC* gene classification (Jaurin and Grunstrom 1981). AmpC-like β -lactamases mainly from *Enterobacter* and *Pseudomonas* with ESBLs hydrolyse both penicillins and cephalosporins. Of clinical importance, plasmid-mediated AmpC enzymes occurring in Gram-negative bacteria have detection problems with the phenotypic methods, therefore, dissemination associated with ESBLs pose serious risk of treatment failures (Rand *et al.* 2011). AmpC enzymes are inducible, unaffected by EDTA and clavulanic acid inhibitors, usually produced in low quantities and often suppress detection ability. The main mechanisms that initiate acquisition of plasmid-mediated *ampC* genes and over expression in bacterial strains are largely due to mutation at the AmpC attenuator and promotor regions (Getzlaff *et al.* 2011, Lakshmi *et al.* 2014).

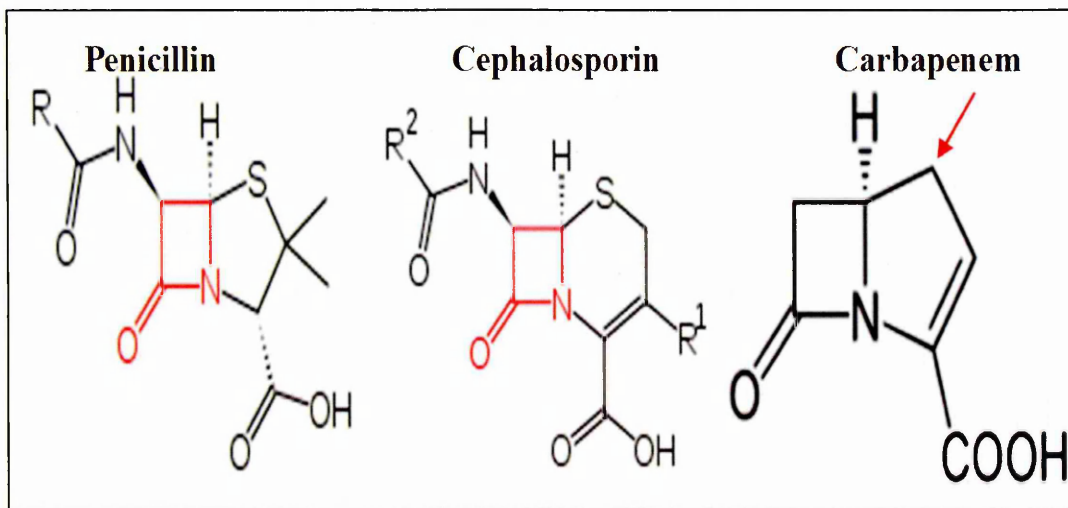
A study by Cottell, Webber and Piddock (2012) earlier reported selective pressure of broad-spectrum cephalosporins such as cefotaxime and ceftazidime as the main cause of production for AmpC types of β -lactamases (Cottell, Webber and Piddock 2012). This suggestion has been entirely modified following a careful re-evaluation on a number of plasmid-mediated bacteria by the same group of investigators recently, that selective pressure of antimicrobials only increase the numbers of antimicrobial resistance isolates, and that production of AmpC β -lactamases largely depend on; suitability of plasmids, stability of bacterial strain interactions, complexity of the plasmids, ability to conjugate freely and survival of plasmids at different conditions (Cottell *et al.* 2014).

1.4 Carbapenem antimicrobials

Carbapenems are bactericidal β -lactam antimicrobials with proven efficacy in severe infections caused by extended spectrum β -lactamase (ESBL) producing bacteria (Hawkey and Livermore 2012). There are a few examples, namely imipenem, meropenem, doripenem, ertapenem, panipenem and biapenem, in use worldwide as a result of the rising resistance to cephalosporin antimicrobials in the Enterobacteriaceae group. Recent emerging mechanisms of resistance accumulate through the spread of carbapenem-destroying β -lactamases leaving narrow therapeutic options (Patel and Bonomo 2013). The search for carbapenem agents was initially from diverse sources. Among these carbapenem agents, selection for treatment depends on the pathogen present.

1.4.1 Carbapenem mode of activity and structure-function relationship

Carbapenems are potent members of the β -lactam family of antimicrobials that are structurally related to the penicillins. The term “carbapenem” explains the changes in that family from penicillin and cephalosporin antimicrobials: “carba-” signifies the replacement of a carbon atom for the sulfur at position 1, and “-penem” stands for the presence of a double bond between positions 2 and 3 (Knapp and English 2001) as shown in Figure 1.1 where carbapenems have a carbon atom while the other β -lactams have a sulphur atom (red arrow).



Adapted from Knapp and English (2001)

Figure 1.1 Three β-lactam agents depicting the functional groups

Mode of action of carbapenems is initiated first by penetrating the bacterial cell wall and binding to enzymes known as penicillin-binding proteins (PBPs) (Mouton *et al.* 2000, Zhanel *et al.* 2007). Earlier study reported that inhibition of PBPs 2 and 3 generally occurs in Gram-negative bacillus-shaped bacteria to form spherical cells and filamentous organisms, respectively (Hayes and Orr 1983). The main inhibiting series of PBPs are 1a, 1b, 2, and 3; and the resultant lethal effect is the inactivation of an inhibitor of autolytic enzymes within the cell wall which leads to the killing of the bacteria (Sumita and Fukasawa 1995, Bonfiglio, Russo and Nicoletti 2002).

Current understanding describes transpeptidase inhibition as the main enzyme target of carbapenems during bacterial cell wall synthesis. Through transpeptidation, a co-valent bond is formed by PBPs comprising of carboxypeptidase and transpeptidase enzymes which in effect prevent their peptide cross-linking activities during peptidoglycan biosynthesis. The lethal effects are thought to result in cell death by autolytic action within the bacterial cell (van Dam, Olrichs and Breukink 2009). According to Papp-

Wallace *et al.* (2011), the mode of action of carbapenems remains uncertain. Much emphasis is placed on the rigid nature of the glycan backbone. Vitality of the cell wall is affected when PBPs are repressed, the glycan backbone weakens due to autolysis and eventually the cell is destroyed by osmotic pressure in Gram-negative bacteria (Papp-Wallace *et al.* 2011, Meletis 2016).

Generally, carbapenems are preferred over other types of antimicrobials in treating invasive or life-threatening infections because of their concentration-independent killing effect on the infecting bacteria (Abbot *et al.* 2013, Watkins and Bonomo 2013). They are broad-spectrum and act against Gram-positive, Gram-negative bacteria and including anaerobes. Notably, a wider spectrum of activity is found among the cyclic amine types of carbapenems with pyrrolidine derivatives namely meropenem, doripenem, panipenem and ertapenem. Exceptionally, biapenem also in the cyclic amine group, panipenem has a marginal effectiveness against Gram-negative bacterial strains.

Comparatively, among the carbapenems, doripenem is highly stable against hydrolysis of most β -lactamases such as ESBL and AmpC-producing β -lactamases in the Enterobacteriaceae group (Marti *et al.* 2009, Chahine, Ferrill and Poulakos 2010), but at the same time is described to have lower MICs with respect to meropenem and imipenem for *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Mandell 2009, Bassetti, Ginocchio and Mikulska 2011). Evidence has shown that doripenem is least susceptible and much slower in carbapenemase hydrolysis (from 2 to 150 fold) than imipenem (Queenan *et al.* 2010). Ertapenem effectiveness against *Pseudomonas aeruginosa* is relatively limited when compared with meropenem or imipenem. Studies also confirmed that *Acinetobacter baumannii* has increased susceptibility to imipenem

or doripenem compared with that of meropenem (Oliver *et al.* 2004, Papp-Wallace *et al.* 2011).

Several reviews on carbapenems and its carbapenemase-producing genes in Gram-negative bacteria have been reported. According to Papp-Wallace *et al.* (2011) and Bedenić *et al.* (2014), it has been established that prudent use of carbapenems, early recognition of infections and careful improvement in new treatment options for different classes of β -lactamases can significantly reduce the threat of spread in hospitals (Papp-Wallace *et al.* 2011, Bedenić *et al.* 2014).

1.4.2 Carbapenem usage and side effects

Carbapenemases as a mechanism of resistance have been reviewed in the context of the effective use of carbapenems in combination therapy to enhance successes in patient outcomes (Lakshmi *et al.* (2014). Many enzymes are produced by bacteria that degrade β -lactam antimicrobials. Such enzymes are known as β -lactamases. However, bacteria find it difficult to effectively degrade carbapenem agents incorporated with β -lactamase inhibitors for *in-vivo* use. The first compound to be used was imipenem, given with cilastatin, which inhibits the renal metabolism of imipenem and prolongs its half-life (Birnbaum *et al.* 1985). Imipenem is noted for its dose-dependent gastrointestinal side effects as compared with the other carbapenems. Ertapenem is the carbapenem that has the lowest activity against *Pseudomonas* species and other non-fermentative Gram negative bacteria. Currently, different kinds of carbapenems are used in clinical practice as antipseudomonal agents; doripenem, imipenem and meropenem. The agents; ertapenem, imipenem and meropenem are poorly absorbed orally and require parenteral administration to be effective. Recently approved doripenem is of value among the

current available carbapenems for treating serious infections (Kattan, Villegas and Quinn 2008, Watkins and Bonomo 2013).

Carbapenems have trivial hepatic metabolism effects leading to hepatotoxicity with jaundice, although this is an uncommon medical condition for these agents (Thyrum *et al.* 1997, Zhanel *et al.* 2007). Most carbapenems are subject to degradation by the enzyme dehydropeptidase-1 (DHP-1) located in renal tubules and require co-administration with a DHP-1 inhibitor such as cilastatin. The later types of carbapenem agents including doripenem and ertapenem require no β -lactamase inhibitor as they are made stable in their mode of activity against Gram-negative bacterial infections. These compounds vary in their binding to PBP, thereby giving unique differences of activity towards different types of organisms. This can be seen in those that inhibit PBP3 and the opportunistic pathogen, *Pseudomonas aeruginosa*, as a specific example.

1.5 Development of carbapenem resistance

Infection with CRE is emerging as an important challenge in health-care settings and a growing concern worldwide (Schwaber and Carmeli 2008, Nordmann, Naas and Poirel 2011). Carbapenem agents are very effective antimicrobials and administered intravenously with little or no allergic cross reactions in hospitals (Cunha *et al.* 2008). Each carbapenem agent has its own function, making its selection specific and delicate in clinical practice for serious infections (Boeser 2008). The importance of the use of the carbapenems against Gram-negative pathogens cannot be overemphasized.

1.5.1 Intrinsic resistance of Gram-negative bacilli

Development of resistance to carbapenems may be due to intrinsic or acquired resistance mechanisms or both. Large numbers of bacteria, both commensals and pathogens, naturally tend to be resistant to certain classes of antimicrobial agents. This insensitivity is termed intrinsic resistance, the occurrence of which limits and complicates drug selections for treatment. This can increase the risk for development of acquired resistance. For instance, Gram-negative organisms reduce the uptake of β -lactam drugs, by selectively altering their cell membrane porin channels. Reduction of outer membrane permeability in this manner prevents the β -lactams from reaching their targets.

Forsberg *et al.* (2012) have shown transmission of different antimicrobial resistance genes from soil bacteria to clinical pathogens. Carriage of such genes was laterally exchanged to clinical pathogens and multiple mobilisation sequences including non-coding regions were identified in short-read sequence data from many soil bacteria (Forsberg *et al.* 2012). For example, early metallo- β -lactamases (MBLs) studied and detected were chromosomal in nature from mainly opportunistic and environmental pathogenic bacteria including *Stenotrophomonas maltophilia*, *Bacillus cereus* and *Aeromonas* species (Franco *et al.* 2010). These bacteria are only opportunistically pathogenic, and *Stenotrophomonas maltophilia* is the only organism commonly associated with frequent hospital acquired infections. Others in the same category, often considered commensals, typically carry chromosomal metallo- β -lactamase enzymes, which are not transferrable, from the groups which had a serine-based hydrolytic mechanism of action (Queenan and Bush 2007, Poirel, Potron and Nordmann 2012). In

the mid-1990s, chromosomal MBLs were detected in most carbapenem-resistant *Pseudomonas aeruginosa* and subsequently *Acinetobacter* species in clinical specimens. In recent times, MBL genetic materials are mobile in the Enterobacteriaceae family (Patel and Bonomo 2013, Walsh 2013).

1.5.2 Acquired resistance of Gram-negative bacilli

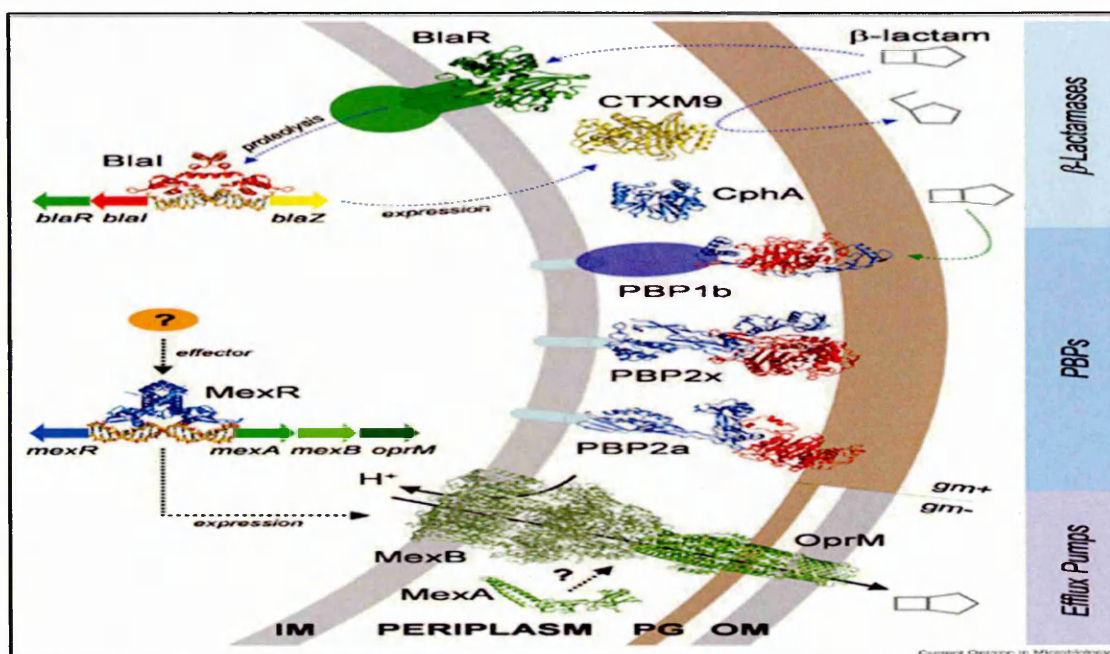
Bacteria have acquired multiple mechanisms of resistance including enzymatic inactivation, target site mutation and efflux pumps. Of these, the development and emergence of inactivating enzymes were established early following the discovery and clinical introduction of the β -lactam class of antibiotics. Over time, the β -lactam-hydrolysing enzymes extended their spectra of activity beginning with penicillinases, followed by cephalosporinases, then to extended-spectrum β -lactamases (ESBLs) and most recently, to the metallo- β -lactamases (MBLs) and other carbapenemases. The MBLs have hugely impacted the utility of carbapenems (often considered as last resort drugs) which are used for the management of multi-resistant Gram-negative bacilli (Garcia 2013).

Many of the acquired carbapenemases found in Enterobacteriaceae are plasmid-mediated and have several ways of spreading amongst bacterial isolates. Apart from these enzymes, there are other important mechanisms conferring carbapenem-resistance that have been observed in recent times. First, the carbapenems least susceptible to hydrolysis (especially, imipenem and meropenem) can be destroyed in the presence of plasmid AmpCs in combination with ESBL enzymes associated with porin loss making Gram-negative bacteria insusceptible to carbapenem agents (Bedenić *et al.* 2014). A significant number of ESBL genes have the potential to transfer between organisms. In

contrast, those strains having their porins mutated or their expression modulated typically do not have potential for mobilisation but may proliferate locally within hospitals. This type of mechanism is recognisable in *Klebsiella*, *Enterobacter* species and *Escherichia coli* as well as other genera. Among the carbapenems, ertapenem is the worst affected; isolates may continue to be susceptible to other carbapenems. In most cases, reduced susceptibility to ertapenem depends largely on the degree of AmpC/ESBL presence and exact changes of the porins. The overexpression of efflux pumps and loss of *OprD* porin are the most common mechanism of carbapenem resistance in *Pseudomonas aeruginosa*, notably to imipenem. Other β -lactams may be affected by this mechanism. *Pseudomonas aeruginosa* efflux-pump overexpression occurs more regularly when meropenem is used when compared with imipenem. Both commensals and pathogenic bacteria have different mechanisms of using their efflux pumps to remove amphipathic or lipophilic substances in and out of the cells. These mechanisms have been recognised in other organisms such as *Enterobacter aerogenes* and *Klebsiella* species against imipenem agent (Walsh 2000).

Generally, Gram-negative bacteria are more resistant to a large number of antimicrobials and other chemotherapeutic agents than Gram positive bacteria due to cell wall differences, external decreased membrane permeability, efflux pumps and the presence of various broad-spectrum- β -lactamases (e.g. ESBL and/or AmpC cephalosporinase). The resistance may be attributed to the presence of broad-specificity drug-efflux pumps (Wilke, Lovering and Strynadka 2005, Armand-Lefèvre *et al.* 2013) as illustrated in Figure 1.2, taking the opportunistic pathogen occurring in clinical strains of *Pseudomonas aeruginosa*, as an example. The structural proteins involved in β -lactam resistance are sub-divided; including β -lactamases, PBPs and efflux pump

systems. Those with solid arrows are the two repressors, BlaI and MexR representing the resistance operons, while BlaZ expressing β -lactamases. There are other structures, glycosyltransferase in class A PBPs and BlaR in the cytoplasmic protease domain which remain less well understood and are represented by blue and green solid outlines, respectively. The diagram also depicts the collaboration of β -lactams and its subgroupings; BlaR as the β -lactam-dependent signalling agent, acylation of the PBPs (poor in PBP2x and 2a), ring hydrolysis by the β -lactamases, and proton antiport of the antimicrobials by the efflux pump groups. The recently determined structure of *Streptococcus pneumoniae* PBP1b is representing the closely-related resistance element PBP1a, and the MexB and OprM structures are used at this point as models. The structures are manually put together to co-ordinate AcrB and TolC as their respective homologues. The effector accountable for terminating MexR repression and the details of the exact interaction of MexA with MexB-OprM are not yet known.



Adapted from (Wilke, Lovering and Strynadke 2005)

Figure 1.2 Resistance due to β -lactamase, PBP and efflux pump mechanisms in *Pseudomonas aeruginosa*

Note: gm +/-, Gram-positive/negative; Mex, multidrug efflux; IM, inner membrane; OM, outer membrane; PG, peptidoglycan. CphA and CTXM9 are shown as representatives of the >40 available metallo- β -lactamase and serine structures.

One of the structures of drug-efflux pump systems extensively reviewed by Poole (2005) was MexAB-OprM. This is a three-component pump; inner resistance-nodulation-division (RND) transporter 'pump' MexB, the outer membrane porin OprM, and the soluble periplasmic MexA which acts strongly on a large number of antimicrobials, including β -lactams and its β -lactamase inhibitors (clavulanic acid as an example). Understanding of this three-way efflux pump and its structural features were reviewed by Eswaran *et al.* (2004) and each component was investigated using *Escherichia coli* as an example; the orthologous inner membrane pump and outer membrane porin constituents AcrB and TolC have been resolved to 3.5 and 2.1Å

resolution respectively, while the structure of periplasmic component MexA from *Pseudomonas aeruginosa* has been solved to 3.5Å resolution. The structural basis of the inner membrane pump, AcrB, has been determined in the presence of numerous hydrophobic small molecular compounds, which indicates a varied binding mode for each ligand, at least in this efflux pump element (Yu *et al.* 2003). The three proteins; β -lactamase, PBP and efflux pump mechanisms interact to create a path to eliminate antimicrobial ligands. According to Kaatz (2005) this has provided new ideas and understanding of target sites to drug development initiatives in future (Kaatz 2005).

Thirdly, non-carbapenemase specific mechanisms such as mixture of porin loss in addition with specific efflux pump systems have been reported in *Acinetobacter* isolates. Weak detection levels have been found for OXA carbapenemases, more than their absence (Doumith *et al.* 2009, Public Health England 2014). Carbapenemase inhibition of carbapenems are of enormous clinical concern, however, resistance may be low level, compromising detection and reading of results for most hospital laboratories potentially having the consequence of increased patient mortality (Akova *et al.* 2012). According to a report issued by Public Health England (2014) carbapenems are known substrates for all carbapenemases, and undetected cases in the health care setting may cause complications when dealing with serious infections involving these enzymes (Public Health England 2014).

1.5.3 Risk factors for acquisition of CRE infection

There are a number of factors that predispose persons to infections by CRE and other multi-drug resistant (MDR) Gram-negatives including ESBL producers. Exposure to these resistant organisms can cause serious infections in patients with the following

reported risk factors: immune-suppression, advanced age, admission to ICU, mechanical ventilation, previous exposure to antimicrobials, organ or stem-cell transplantation, and prolonged hospital stay (Gasink *et al.* 2009, Arnold *et al.* 2011). Health-care associated infections caused by CRE, mainly *Klebsiella pneumoniae*, have been encountered most commonly in ventilator-associated pneumonia, bacteremia, urinary tract and surgical site infections. Generally, KPC-producing carbapenemases in the β -lactamase molecular class A are rare, however, the variants are rapidly spreading, with KPC-2 and KPC-3 frequently reported in the UK (Woodford *et al.* 2008). The gene is carried on a transposon which increases the risk of spreading in many hospital settings extensively worldwide (Yigit *et al.* 2001, Srinivasan and Patel 2008). In clinical situations, where KPC-producing bacteria are a major concern, early intervention has to be taken to prevent death by administering effective empiric antimicrobials when the patient is immunocompromised, undergoing organ transplants or during cancer treatment (Patel *et al.* 2008, Hara *et al.* 2013).

There are reports which suggest that long-term care facilities play a crucial role in the spread of CRE. Neuner *et al.* (2011) reported readmission of 72% patients who were discharged with carbapenem-resistant *Klebsiella pneumoniae* bloodstream infections. While in another study, Ben-David *et al.* (2011) reported 42% in their initial hospital stay with records from post-acute care facilities in Israel. Growing evidence suggests early detection of CRE-colonized patients on admission to long-term acute care hospitals (LTACHs) may help to prevent institutional outbreaks and limit regional spread of this emerging public health threat. A prospective study in Central Virginia by Lewis *et al.* (2013) concluded that CRE is more prevalent in regional acute care institutions than LTACHs (Lewis *et al.* 2013).

1.6 Gram-negative bacteria and carbapenemase resistance

The carbapenem class of antimicrobials is able to kill most bacteria that produce β -lactamase inhibitors, as their functional structure is an improvement over the other classes of β -lactam agents. Improper management of multidrug-resistant infections caused by Enterobacteriaceae producing extended spectrum β -lactamases (ESBLs) against carbapenems further leads to resistance in patients (Pitout and Laupland 2008).

1.6.1 The significance of carbapenemases

Over the years, resistance to carbapenems has emerged and continues to proliferate among bacterial pathogens, particularly *Klebsiella pneumoniae*. A new class of enzymes that affects many Gram-negative bacteria, known as *Klebsiella pneumoniae* carbapenemases (KPCs) is capable of rendering carbapenems ineffective. A KPC-producing *Klebsiella pneumoniae* isolate and its importance have been increasingly recognised in a wide range of *Klebsiella pneumoniae* ST258 due to the spread the organism exhibits worldwide (Arnold *et al.* 2011). The KPC enzyme inactivates carbapenam agents, last-resort treatments for serious infections which do not respond to other antimicrobial drugs (Yigit *et al.* 2001, Weisenberg *et al.* 2009). Most KPCs carry similar resistance profiles to most ESBLs, and reduced susceptibility to carbapenems; they do not, however, confer resistance but require an additional mechanism to be effective (Nicasio, Kuti and Nicolau 2008). The enzyme type produced is no longer limited to *Klebsiella pneumoniae* isolates but also has the ability to disseminate to and transfer between various bacterial species on plasmids. The genetic element is often found in association with resistance determinants for other antimicrobials which give rise to multidrug- and pandrug-resistant isolates (Arnold *et al.* 2011, Walsh and Toleman 2012).

The KPC enzyme was first described in North Carolina and found in a *Klebsiella pneumoniae* isolate in 2001 (prevalent also in Israel, and Greece; outbreaks elsewhere in Europe. Some UK cases imported via patient transfers) and remained the most important mechanism of resistance for the production of *blaKPC*, a carbapenemase gene. The gene is carried on a transposon (a piece of genetic material) which increases the risk of spreading in many hospital settings extensively worldwide (Yigit *et al.* 2001, Srinivasan and Patel 2008). The most recent data reported by the Centre for Disease Control and Prevention (CDC) revealed that CRE caused by the KPC enzyme have been described in 36 states of the USA. This is due to global spread of *Klebsiella pneumoniae* carbapenemase and has been accountable for a number of recounted outbreaks of carbapenem-resistant Enterobacteriaceae (CDC 2011).

Klebsiella pneumoniae carbapenemase (KPC) and New Delhi metallo- β -lactamase (NDM) are among the common types of carbapenemase found in intensive care units (ICUs) which have high rates of transmission and excessive mortality when not detected. With the advent of New Delhi metallo- β -lactamase-1 (NDM-1) and other NDM variants from Gram-negative bacilli the effective use of carbapenems is diminishing. MBLs are different from other β -lactamases as they use zinc instead of the amino acid serine as the nucleophile at the active site. These MBLs are not inactivated by monobactam antimicrobials such as aztreonam. However, organisms identified in United States health-care facilities harbouring the NDM-1 enzyme were found to have expressed another resistance mechanism to monobactams (CDC 2010).

Other Gram-negative bacteria such as *Escherichia coli*, non-fermenting *Pseudomonas* and *Acinetobacter* species in recent times have acquired the resistant gene NDM-1 which hydrolyses many carbapenem agents (Chen *et al.* 2011, Joucic *et al.* 2011). NDM-1 has a metallic ion as a cofactor and was first isolated from a Swedish patient of Indian origin after several hospitalisations in New Delhi, India (Livermore and Woodford 2000, Yong *et al.* 2009). Currently, there are few alternatives to carbapenems for the treatment of hospital infections involving Gram-negative bacteria. In the United Kingdom, thirty-seven NDM-1 producers were investigated harbouring plasmids, clonally diverse from those found in South India, however, the NDM-1 types were readily transferrable across different types of bacteria which end up in strains which become resistant to multiple-antimicrobials (Kumarasamy *et al.* 2010). Expression of carbapenemases including the NDM-1 in enteric bacteria has caused increased morbidity and mortality particularly in those who have travelled to the Indian subcontinent. Infections have occurred in many parts of the world including the USA, Canada, Europe, China, Israel, and are currently an emerging concern in South America (Woodford *et al.* 2008, Toye *et al.* 2009, Hara *et al.* 2013).

There are other carbapenemase enzymes in existence, some are geographically associated and spread among the Enterobacteriaceae family including metallo enzymes IMP, VIM (scattered globally), non-metallo enzyme OXA-48 (widespread in *Klebsiella pneumoniae* in Turkey, the Middle-East and North Africa). Rarely encountered types are SME, IMI and SPM which also occur as acquired carbapenemase enzymes across the globe (Carrër *et al.* 2010, Kumarasamy *et al.* 2010).

1.7 Molecular classification of carbapenemase enzymes

A large variety of carbapenemases have been identified in *Enterobacteriaceae* belonging to 3 classes of β -lactamases: the Ambler classes A, B and D β -lactamases (Table 1.2) adapted from Overturf (2010). These classes are of greatest clinical importance among nosocomial pathogens. Class A, C, and D β -lactamases all share a serine residue in the active site. The clinical role of a fourth class (Ambler class C) is unknown but possesses some extended activity towards carbapenems (Jacoby and Munoz-Price 2005, Queenan and Bush 2007).

1.7.1 Class A carbapenemases

A number of this class of enzymes have been identified; some are chromosomally encoded - NmcA (not metalloenzyme carbapenemase A), SME (*Serratia marcescens* enzyme), IMI-1 (Imipenem-hydrolysing β -lactamase), SFC-1 (*Serratia fonticola* carbapenemase-1), with the others being plasmid encoded - KPC (KPC-2 to KPC-13), IMI (IMI-1 to IMI-3), derivatives (GES-1 to GES-20) of GES (Guiana extended spectrum), but all actively hydrolyse carbapenems and are partially inhibited by clavulanic acid (Nordmann, Naas and Poirel 2011, Bedenić *et al.* 2014). Of these, the KPCs are the most prevalent and after a few years of its discovery, had spread worldwide and caused outbreaks in many Asian, North American and European countries as well as in Africa. KPC producers have evolved to be multidrug resistant to β -lactams thereby limiting therapeutic options for treating KPC-related infections in patients (Nordmann, Cuzon and Naas 2009). A review of KPC genes by Perez and van Duin (2013) stated that there are currently 12 additional variants of *bla*KPC existing globally (Perez and van Duin 2013). A study has shown that due to limited therapeutic

options, KPC resistant clones are disseminating internationally in several areas that have different multi-locus locations with additional β -lactamase content which differ by size, number, and structure of plasmids to compare producer isolates from different countries (Woodford *et al.* 2008). However, a single genetic element (transposon Tn4401) has been found in *blaKPC* genes (Cuzon *et al.* 2010, Nordmann, Naas and Poirel 2011). For instance, between 2009 and 2010, Canadian Nosocomial Infection Surveillance Program investigated 7 cases of KPC-3-positives involving *Serratia marcescens* (1), *Escherichia coli* (1), *Klebsiella oxytoca* (1) and *Klebsiella pneumoniae* (4) from a hospital and used MLST to identify these organisms. This study revealed ST512 as a single-locus variant from the original known ST258 *Klebsiella pneumoniae* isolates widespread in Greece. The indication was that there was interspecies and intra-species were plasmid-mediated transfer belonging to the repFIIA replicon type. In another incidence, an elderly patient who visited Greece in 2012 contracted community acquired KPC-2-positive *Klebsiella pneumoniae*. This was isolated from the patient's urine confirming it is widespread in Greece (Mataseje *et al.* 2012, Chan *et al.* 2013, Jamal *et al.* 2013, Munoz-Price *et al.* 2013).

1.7.2 Class B carbapenemases

These enzymes are mainly in the class of β -lactamases having the ability to hydrolyse carbapenems but are susceptible to inhibition by EDTA, a chelator of Zn^{2+} and other divalent cations. The mechanism of hydrolysis depends on interaction of the β -lactam drugs with zinc ions in the active site of the enzyme. The most common metallo- β -lactamase families include the NDM-1, IMP-type carbapenemases, VIM (Verona integron-encoded metallo- β -lactamase) GIM (German imipenemase), and SIM (Seoul imipenemase). The genes encoding these enzymes are often located within a variety of integron structures and incorporated in to the gene cassettes (Giakkoupi *et al.* 2003, Queenan and Bush 2007).

By mid-2010, the NDM-1 gene may have been acquired by bacteria from the community and introduced to other countries including Europe and the United States through tourists travelling around the globe, while the same strains have been found in environmental samples in India (Walsh *et al.* 2011, Khan and Nordmann 2012). At least, 8 variants have been described and identified in this group. NDM-genes are dominant in *Klebsiella pneumoniae* and *Escherichia coli* isolates but have also been found in association with *Acinetobacter baumannii* and *Pseudomonas aeruginosa* organisms (Pitout *et al.* 2008, Poirel *et al.* 2010, Bedenić *et al.* 2014).

Currently, up to 18 varieties of IMP-type carbapenemases have been identified. This type was first recognized in the 1990s in Japan. The majority of these enzymes were investigated in *Acinetobacter* and *Pseudomonas* species as well as those in the Enterobacteriaceae family. The IMP genes have also been found in Brazil and Canada

after first reports from Europe in 1997. These gene types have spread slowly to other countries in the Far East and subsequently to the United States and Australia (Queenan and Bush 2007, Bush *et al.* 2011).

VIM genes are rarely encountered in Enterobacteriaceae but occur most commonly in *Pseudomonas aeruginosa* and *Pseudomonas putida*. Another growing family of carbapenemases first isolated in Verona, Italy, in 1997, and exhibited predominantly integron-associated metallo- β -lactamases. The family consists of 14 members. The VIM and IMP families have some similarities in terms of which plasmids they are carried on and that they are integron-associated. The VIM family has amino acid sequence diversity of up to 10%, 15% for IMP, and 70% between the two families. Both hydrolyse all β -lactams except monobactams, and are susceptible to all β -lactam inhibitors (Marsik and Nambiar 2011).

1.7.3 Class D carbapenemases

These enzymes are serine- β -lactamases poorly inhibited by EDTA or clavulanic acid. These carbapenemases are of the oxacillinase (OXA) enzyme type, and have a weak activity against carbapenems. The enzymes are found primarily in non-fermenter organisms such, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and rarely in isolates of Enterobacteriaceae family in most countries including the United Kingdom and in the United States (Moquet *et al.* 2011, Poirel *et al.* 2011c). The OXA β -lactamase with carbapenemase activity was first described by Paton *et al.* (1993), and the enzyme purified from a multidrug-resistant *Acinetobacter baumannii* isolated in 1985 from a patient in Edinburgh, Scotland. Activity of OXA carbapenemases is

increased by upstream elements which control gene expression (Carrër *et al.* 2010). The major concern with OXA carbapenemases is their ability to rapidly mutate and expand their spectrum of activity. Studies by Mathers *et al.* (2013) reported frequent detection of class D among the Enterobacteriaceae family making this a threat and a major public health problem worldwide (Mathers *et al.* (2013). Currently, OXA-48 is the most common while non-nosocomial OXA-24 type has been found in environmental *Acinetobacter* species and the spread of OXA-23 type which occurs globally is more encountered in USA and Europe. The OXA-58 group had been described significantly over the globe in several outbreaks (Evan and Amyes 2014). OXA-48-type producers are among the most difficult carbapenemase producers to be identified due to their point mutant analogues with ESBLs; thus their true prevalence rates are difficult to estimate. Over the years, there have been 102 unique OXA sequences identified of which 9 are extended spectrum β -lactamases and at least 37 are considered to be carbapenemases (Queenan and Bush 2007, Nordmann, Naas and Poirel 2011).

Table 1.2 Carbapenemases by classification, activity and organisms

Gene Type	Classification by Ambler Class	Activity Spectrum	Organism(s)
KPC	A	All β -lactams	Enterobacteriaceae, <i>P. aeruginosa</i>
SME	A	Carbapenems and aztreonam, but not 3rd/4th G cephalosporins	<i>S. marcescens</i>
NMC-A, IMI	A	Same as for SME	<i>Enterobacter</i> spp.
GES	A	Imipenem and 3rd/4th cephalosporins	<i>P. aeruginosa</i> and Enterobacteriaceae
IMP, VIM, NDM	B (metallo- β -lactamases)	All β -lactams; can test susceptible to aztreonam	<i>Pseudomonas</i> spp. <i>Acinetobacter</i> spp. Enterobacteriaceae
OXA	D	Weakly active against carbapenems	<i>A. baumannii</i> , <i>P. aeruginosa</i> , and rare Enterobacteriaceae

Note: KPC= *Klebsiella pneumoniae* carbapenemase, SME= *Serratia marcescens* enzyme, NMC-A= Non-metallo-enzyme carbapenemase, IMI= Imipenem-hydrolysing β -lactamase, GES= Guiana extended-spectrum β -lactamase, IMP= Imipenem-resistant *Pseudomonas*, VIM= Verona integron-encoded metallo- β -lactamase, NDM= New Delhi metallo- β -lactamase, OXA= Oxacillinase.

1.8 Laboratory detection of carbapenem-resistant organisms

The presence of a carbapenemase can be detected by a number of methods in clinical laboratories. These include automated systems or disc diffusion, minimum inhibitory concentrations (MICs), selective agar, modified Hodge test, synergy tests (e.g., E-tests or double disc tests) and molecular methods. The majority of the genes controlling carbapenemase production are transferrable by plasmids. The presence of carbapenemases signifies some relevance to clinicians; however, care has to be taken in the management of patients to carbapenem therapy which may change depending on the

mechanism of resistance existing at that time. Currently, detection of the enzymes is difficult because of the different mechanisms involved and unreliable techniques practised in some clinical laboratories (Perez and van Duin 2013). On the other hand, extended-spectrum β -lactamase-inhibitors, such as clavulanic acid, tazobactam, and sulbactam when used in combination with a carbapenem agent are also unreliable for phenotypically detecting carbapenemase production in bacterial isolates.

1.8.1 Phenotype based methods

The baseline test that first predicts carbapenemase enzyme production is disc diffusion or the use of automated systems. For disc diffusion, impregnated discs containing a standard amount of an antibiotic agent are placed on agar plate seeded with a bacterium to be tested. As the organism grows during overnight incubation, the antibiotic agent diffuses into the agar medium. Susceptibility of the test organism is proportional to the zone of inhibition produced by the antibiotic used. With the automated systems, instruments are used to analyse antibiotic susceptibility testing with standardised inoculum for the test strain and diluted in a specialised broth with a drop of antimicrobial susceptibility testing indicator added. The final inoculum turbidity is adjusted to 0.5 McFarland Standard. The inoculum is poured into a panel, closed in a secured place and the inoculated panel is then placed into, for example, instrument for VITEK or VITEK 2 automated system. The panel is then read automatically by the instrument, the data generated is analysed with preliminary algorithms and compared to the controlled results as appropriate. Either one or both methods have shown variability in their ability relating to the underlying mechanism of carbapenem resistance. Several reports of carbapenemase detection using phenotypic based methods in *Acinetobacter* species, *Pseudomonas* species and Enterobacteriaceae have been documented (Liu *et al.*

2006, Miriagou *et al.* 2010). Reference MIC levels are more sensitive in determining resistant breakpoints for carbapenem susceptibility by broth microdilution and agar dilution, according to Patel, Rasheed and Kitchel (2009), than E-test, disc diffusion, and many automated systems. For instance, a number of automated systems studied by Queenan and Bush (2007) identified up to 87% of KPC-producing *Klebsiella pneumoniae* study isolates as being susceptible to imipenem or meropenem.

The determination of ertapenem MICs by automated testing tends to identify cases which are ertapenem-resistant, which in effect is recommended as a sensitive laboratory test of KPC production irrespective of the method employed (CDC 2010). Carbapenem susceptibility breakpoints against meropenem and imipenem keep on changing in recent times, due to lack of global consensus. For instance, previous MIC breakpoints EUCAST established were ≤ 2 $\mu\text{g/ml}$ and > 8 $\mu\text{g/ml}$ as susceptible and resistant respectively (Abbott *et al.* 2013), compared with the CLSI breakpoints of susceptible at ≤ 4 $\mu\text{g/ml}$ and resistant at ≥ 16 $\mu\text{g/ml}$ which may compromise results when testing on non-fermenting *Acinetobacter* species (CLSI 2013). Perez and van Duin (2013) established that detection with the new MIC lower breakpoints without using a phenotypic test such as the carbapenem-EDTA combination tests or modified Hodge test may result in lack of differentiation between various mechanisms of carbapenem resistance. For reliable results, European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) revised MIC breakpoints (Table 1.3) according to Hara *et al.* (2013) have been accepted and recommended for the detection of carbapenemase-producers.

Table 1.3 Breakpoint values (MIC, mg/L) for carbapenems according to guidelines in Europe (EUCAST) and the United States (CLSI)

	EUCAST		CLSI	
Carbapenem	S	R	S	R
Doripenem	≤ 1	≥ 4	≤ 1	≥ 4
Ertapenem	≤ 0.5	≥ 1	≤ 0.5	≥ 2
Imipenem	≤ 2	≥ 8	≤ 1	≥ 4
Meropenem	≤ 2	≥ 8	≤ 1	≥ 4

Note: EUCAST, European Committee on Antimicrobial Susceptibility Testing (www.eucast.org/clinical_breakpoints), version 2.0, 2012; CLSI, Clinical and Laboratory Standards Institute, M100-S22, 2012; S, sensitive; R, resistant.

The use of chromogenic agar preparations have emerged for identification of MDR organisms from surveillance cultures such as CHROMagar KPC and CHROMagar™ *Acinetobacter* (CHROMagar; Paris, France). Culture media are made selective by adding chromogenic substrates and agents that inhibit growth of other Gram-positive, Gram-negative and yeast isolates. The CHROMagar™ culture media were originally formulated for the screening of patients in the ICU and later redesigned so that *Acinetobacter* species appear as bright salmon red colonies. A new formulation, with the addition of '*Klebsiella pneumoniae* carbapenemase supplement' was able to pick out carbapenem-resistant *Acinetobacter baumannii*. Recent modifications to CHROMagar *Acinetobacter* have improved selective growth for organisms resistant to carbapenems (Gordon and Wareham 2009, Wareham and Gordon 2011). According to Arnold *et al.*

(2011), CHROMagar KPC has a sensitivity of 100% and specificity of 98.4% compared with polymerase chain reaction (PCR). This technique is used for confirmatory identification of KPC production though is expensive in terms of cost and is mostly performed in research laboratories (Arnold *et al.* 2011).

The modified Hodge test has been used extensively and is a phenotypic technique for detecting carbapenemase activity routinely used in clinical pathology laboratories. This test was recommended by the CLSI in 2009 (CLSI 2009), however, it is not specific for the detection of all carbapenemase enzymes, most significantly with isolates showing weak positive results and AmpC producers. Bonnin *et al.* (2012) performed modified Hodge tests on 19 carbapenemase-producing *Acinetobacter baumannii* isolates and found negative results for all tested NDM-producing isolates and only weak positive results for VIM-, IMP- and OXA-type producers (Bonnin *et al.* 2012). There are reports related to susceptibility testing in which the tests showed reduced susceptibility but give consistently negative results for the presence of carbapenemases. These may be due to greater genetic exchange among NDM-1 bacterial strains especially and using non-zinc-supplemented Mueller-Hinton agar medium (Castanheira *et al.* 2011, Girlich, Poirel and Nordmann 2012).

Double-disc synergy testing has several versions; carbapenem with -clavulanate, -cloxacillin -EDTA or 2-mercaptoproionic acid and one which utilizes a double sided E-test (BioMérieux, Solna, Sweden), imipenem versus imipenem with EDTA, and are used as a screening test for MBL producers. This method is efficient for detection of MBL carbapenemases with high resistance, but may be deficient for detecting MBL

producers with low resistance to imipenem according to Walsh *et al.* (2005). Other results have shown sensitivity with the imipenem-EDTA disc method, 95.7% for *Acinetobacter* species and 100% for *Pseudomonas* species (Queenan and Bush 2007, Overturf 2010). A recent study by Nordmann, Naas and Poirel (2011) indicated there is no inhibition test available for detection of OXA-48/OXA-181 producers and observed that EDTA by itself has inhibitory action against some bacteria, due to permeability of the outer membrane, and can lead to false-positive results (Nordmann, Naas and Poirel 2011). According to a recent report from Public Health England (2014), resistance to any of the carbapenem drugs may be confirmed using inhibitor based tests (Table 1.4). For the detection of carbapenemases in a community may largely depend on the availability of existing β -lactamase inhibitors (Public Health England 2014).

Table 1.4 Interpretation of inhibitor-based phenotypic synergy tests

^a Carb resistance mechanism	Synergy between				Resistance to	
	Carb + clavulanate *	Carb + cloxacillin*	Carb + boronic acid	Carb + EDTA / dipicolinic acid	^b Aztre	^c Temo (MIC $\geq 64\text{mg/L}$ or no zone around $30\mu\text{g}$ disc)
ESBL or AmpC + porin loss	+/-	+/-	+/-	-	R	-
MBL (IMP, NDM, VIM)	-	-	-	+++	S	++
KPC	+/-	-	+++	-	R	+/-
OXA-48	-	-	-	-	S	+++

Adapted from Public Health England (2014)

Note: ^a Carba = carbapenem; ^b Aztre = aztreonam; ^c Temo = temocillin; R = resistant; S = susceptible; +/- = positive or negative; - = negative; + = positive.

The newly developed Carba NP test, derived from the name “Carbapenemase Nordmann-Poirel”, is in use to detect carbapenemase producers in Enterobacteriaceae. The technique requires no expertise, is reproducible, inexpensive and may easily be adapted as an additional important test in any clinical laboratory. The test may help healthcare facilities to curb and implement rapid containment measures to limit the spread of carbapenemase producers in hospitals (Nordmann, Naas and Poirel 2011). Nordmann, Poirel and Dortet (2012) described the test as most efficient as compared with molecular-based methods. The Carba NP test is performed in wells and colour change from red to orange or yellow indicates the tested strains are producing carbapenemases. The Carba NP test rapidly and reliably identifies carbapenemase producers by changes in pH values using phenol red as the indicator. Colour develops

within 2 hours detecting strains that are imipenem resistant due to non-carbapenemase-mediated mechanisms, such as combined mechanisms of resistance or from strains that are carbapenem susceptible but express a broad-spectrum β -lactamase without carbapenemase activity. Reports have shown that the test is 100% sensitive and specific for Enterobacteriaceae (Nordmann, Poirel and Dortet 2012) and 100% specific and 94.4% sensitive for *Pseudomonas* species harbouring carbapenemases (Dortet, Poirel and Nordmann 2012a). In contrast, a study by Tijet *et al.* (2013) observed low performance of Carba NP test and false-negative results in OXA-48-like *Pseudomonas aeruginosa* and Enterobacteriaceae isolates with only 80% specificity (Tijet *et al.* 2013). Another study reports that Carba NP test, however, cannot differentiate among carbapenemase classes, particularly, weak carbapenemase activity in strains harbouring GES-type carbapenemases in high prevalence areas, Brazil and South Africa. An updated version designated “CarbAcinet NP test” for identifies all the types of carbapenemase in *Acinetobacter* species with high-level of sensitivity and specificity (Dortet *et al.*, 2014, Hammoud, Moubareck and Sarkis 2014).

1.8.2 Problems in phenotype based detection

The recent changes in the breakpoints for carbapenems (CDC 2011) have further added to the complexity of dealing with multidrug-resistance in the Enterobacteriaceae family. Many laboratories in the USA conform to CLSI standards, approval must come first from this body before clinical laboratories can use the recommendations to detect carbapenemase producing isolates. On the other hand, most European, Asian and African countries use EUCAST or CLSI as their standards for their detection of carbapenemase producing isolates. Delays in breakpoint recommendations on automated kits and devices for use resulted in some laboratories not following strictly laid down rules, hence, undetected CRE with lower breakpoints silently disseminated

KPC-producing *Klebsiella pneumoniae* in a long-term facility for children and young adults (Viau *et al.* 2012).

During the period of transition, there may be inconsistencies between laboratories regarding identification of ESBLs and CREs. Many different laboratory interpretations of MIC levels may have led to confusion among clinicians about when it is suitable to use an extended spectrum cephalosporin drug, a carbapenem, or neither of them. These determinants are also important for infection prevention and control practices. Pharmacists need to be responsive to these issues so that they can offer the best advice and other method in recommending appropriate antimicrobial choice. Pereira *et al.* (2011) offered suggestions on amended interdepartmental communication within hospital-care services during such transition periods to improve laboratory detection of ESBLs and CREs (Pereira *et al.* 2011).

1.8.3 Genotype based techniques

Molecular techniques have become an efficient tool for carbapenemase detection. Polymerase chain reaction (PCR) performed on colonies may give results within 4 to 6 hours with excellent sensitivity and specificity and this may reduce the chance of spreading organisms in hospitals. Wang, Gu and Lu (2012) reported a real-time PCR assay with specificity and sensitivity at 100% as compared with 90% phenotypic KPC activity when assessed by modified Hodge test (MHT) and sequencing. More recently, multiplex PCR and microarray techniques for detection of several carbapenemase genes in one test have been produced. These are mostly focused on the detection of genes in Enterobacteriaceae including their subgroups of carbapenemases. The limit of these

modern tools lacks sequence similarity to genes already described (Queenan and Bush 2007, Woodford *et al.* 2011, Abbott *et al.* 2013). Earlier studies largely focused their energies on KPC detection. With the emergence of equally important genes also spreading widely on plasmids, NDM, VIM and IMP in Class B group and recent underestimated Class D serine carbapenemase OXA-48 which is weakly detected by phenotypic methods have revolutionised for different molecular techniques. These are to improve the detection of unknown, dominant resistance genes and their variants to control sporadic dissemination into health-care facilities and community settings (Nordmann, Dortet and Poirel 2012). Notwithstanding the enormous contributions of molecular-based assays, they have their own drawbacks and frustrations to end-users mainly due to lack of trained personnel to properly man the equipment, labour-intensive, time-consuming and cost of all consumables and reagents. As reported in two previous studies by Naas *et al.* (2011) and Nordmann, Naas and Poirel (2011) molecular assays that are employed should be made as inexpensive, specific, sensitive as possible for the detection of the desired gene and rapid to detect β -lactamase resistance genes to avoid ambiguities of results to improve care-receivers outcome (Naas *et al.* 2011, Nordmann, Naas and Poirel 2011).

In relation to clinical laboratory practice, innovations of molecular techniques have been evaluated and are currently in use for the detection of carbapenemase producers. Check-MDR CT₁₀₂ DNA microarray, Check direct CPE assay and Hyplex-Super-Bug ID kit are on the market for diagnostic testing purposes. Excellent detection ability and reproducibility with these assays have been reported (Naas *et al.* 2011, Kaase *et al.* 2012, Nijhuis *et al.* 2013). Recent study report an intriguing finding in which a self-generating plasmid harbours the OXA-48 gene in multiple transposons Tn1999 rapidly

and silently disseminating as one clone have been detected using molecular techniques (Bakthavatchalam, Anandan and Veeraraghavan 2016).

Another important study by Sparbier *et al.* (2012) described the use of matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) as a new tool detecting resistance patterns in bacteria from fresh positive blood cultures. The laser-based ionisation technique can detect changes in mass to charge ratios. Carbapenam resistant bacteria use β -lactamases to physically disrupt the structure of β -lactam antimicrobials. The changes in the mass of the drugs make the resistant bacteria detectable by the MALDI-TOF MS technique, and return results within 4 to 5 hours. Secondary testing should be carried out to ascertain colonisation of resistant bacteria in the facility (Sparbier *et al.* 2012). MALDI-TOF MS is available in most research laboratories for confirmation of a host of bacterial resistance enzymes, recently including the OXA-48 carbapenemase activity with sensitivity and specificity of 98.9 and 97.1% respectively (Sauget *et al.* 2014). However, cost and requirement of trained expert to man the instrument limits its use in routine laboratories for diagnostic purposes (Bakthavatchalam, Anandan and Veeraraghavan 2016).

1.9 Epidemiology of carbapenemase-producing organisms

Across the globe, first reports of carbapenemases occurred in the 1980s. Its subsequent spread raised a number of concerns, and these have coincided with years in which no new antimicrobial agents against Gram-negatives have been developed. Japan reported the first carbapenemase from an *Aeromonas hydrophila* isolate in the 1980s. Sequentially, followed in London (1982) by Seoul imipenemase (SME-1) from *Serratia marcescens*, imipenemases (IMI-1) in California (1984) and NMC-A in France (1990), both from *Enterobacter cloacae* (Garcia 2013).

Carbapenem resistance involves one or more of several diverse mechanisms of actions in multi-resistant Gram-negative bacteria. The most frequently encountered β -lactamase enzymes are the acquired carbapenemases in the Ambler class A group of which *Klebsiella pneumoniae* carbapenemases (KPCs) predominate worldwide (Woodford, Turton and Livermore 2011, Public Health England 2015). An alarm call on the spread of carbapenem-resistance has been in existence since KPC was identified in 1996 in the United States. Clinical characteristics of KPC carrying organisms vary with local differences and conditions. Countries such as Israel, Greece and Colombia have outbreaks of KPC through their existing local situations and others mainly by importation for instance, in Canada, Australia and New Zealand (Munoz-Price *et al.* 2013, The *et al.* 2015). Following worldwide spread of *Klebsiella pneumoniae* ST258 type strain, and throughout the USA in particular, multilocus sequence typing (MLST), a specific molecular tool was used to find out the global epidemiology linkages and help understanding the dissemination of KPC genes. There are fresh fears and challenges ongoing and under discussion by researchers as to how to curb the spread of carbapenem resistance across the globe for epidemiological reasons. Detection has been difficult due

to differences in their genetic makeup within and across countries. Researchers have observed divergent resistant patterns from CREs in Europe, the United States and South America. The unusual resistance factors have been monitored through a surveillance programme, named the Intensive Care Antimicrobial Resistance Epidemiology (ICARE) by the CDC from 2000 onwards. Earlier CRE infections, particularly KPC, were confined to hospitals in New York. Between 2000 and 2010, the infection rate had increased from 1% to 12% in 42 States respectively (Kuehn 2013) with a 50% associated mortality rate (Freifeld *et al.* 2011, Nordmann, Naas and Poirel 2011). Thirty-nine states of the USA, including Puerto Rico, were hit by the threat of KPC positive organisms within the next few years. Strict control measures were instituted, such as short-stay in acute-care settings, made the CRE infections infrequent and contained the rate below 5% as compared with 8% in long-term acute-care hospitals monitored by the CDC Network (CDC 2013). The occurrence resulted in many other KPC-positive *Klebsiella pneumoniae* isolates to be categorised while the majority have been found in *Pseudomonas aeruginosa* (Robledo, Aquino and Vazquez 2011), *Pseudomonas putida* and others in the Enterobacteriaceae family (Bennett *et al.* 2009).

Studies have shown that the spread of KPC-positive organisms in some parts of the USA was not related to travel. Most infections seemed to occur in hospitals, with the majority of the outbreaks contracted in long-term care facilities (LTCFs) for care receivers with severe medical conditions who needed longer stays for their clinical management (Endimiani *et al.* 2009, CDC 2013). Patel *et al.* (2008) reported 38% infection-specific mortality and 48% in hospital mortality for patients infected with carbapenem-resistant *Klebsiella pneumoniae* isolates, contrasting with 12% and 20% for carbapenem-susceptible *Klebsiella pneumoniae* respectively (Patel *et al.* 2008). The clinical impact of KPC-positive bacteria occurs within 48 hours of admission, with

isolates identified from samples such as blood, wound swabs, urine and sputum. Patients with a history of frequent exposure in a health-care facility may easily trigger community-acquired infections (Gasink *et al.* 2009, CDC 2013). The guidance as to the detection and control of these isolates have been expanded with appropriate recommendations as a public health measure in 2012 by CDC on regional bases all over USA (Snitkin *et al.* 2012, CDC 2013).

Currently, 39 countries in the European Union including France and the United Kingdom have come together to fight the burden of CREs in their respective countries. The study is being coordinated by the European Centre for Disease Prevention and Control in Stockholm to keep epidemiological data of patients who test positive for such bacteria in their health-care institutions (Glasner *et al.* 2013, McKenna 2013). Between the years 2003 and 2007, the UK experienced the first recorded KPC gene with a KPC-4 variant in an *Enterobacter* species from a blood sample and the first case of a known *Klebsiella pneumoniae* producing KPC-positive isolate, all from Scotland (Livermore *et al.* 2008, Woodford, *et al.* 2008). There were scattered increases in numbers as the KPC-positive isolates continued to be identified by the UK national public reference laboratory in 2008 (4 hospitals 5 isolated) and in 2009 (12 hospitals 13 isolated) with *Klebsiella pneumoniae* ST258 the most frequent strain to be captured by MLST (10 out of 12). The source was traced to Greece, Cyprus or Israel, with patients having travelled to those countries in the time prior to admission. Between 2010 and the first six months of 2012, the epidemiological pattern rapidly changed in terms of numbers identified, 231 KPC-positive strains in 2010, 368 in 2011 and 293 for the first six months in 2012 were referred for further confirmation at the national reference laboratory (Livermore 2012, Public Health England 2015).

Rapid spread of CREs across Europe was observed in late 2005 when an identified KPC isolate in Israel was later found to be genetically related to a type strain in New York. France acquired its first KPC-2-positive *Klebsiella pneumoniae* in 2005 from a patient who had had 3 months admission in a New York City (NY, USA) hospital. The strain type was isolated from blood and urine cultures. From 2009 to 2012, there have been several CREs investigated. In a recent study, five cases out of 20 were confirmed and linked their sources to countries such as Kuwait, China, Italy and Israel. Community acquired strains were found to be rare and most of the documented cases in France were from persons colonisation with the strains (Naas *et al.* 2005, Munoz-Price *et al.* 2013).

Carbapenemases became a public health concern in 2007 when a total of 1,275 CRE infections were identified across their health care centres. KPC-positive organisms found its way in to other countries including Italy, Colombia and the United Kingdom (Schwaber *et al.* 2011, Cantón *et al.* 2012, Patel and Bonomo 2013). In early 2008, Sweden was the next country to identify multidrug-resistant KPC-positive isolates, this time with a different metallo- β -lactamase enzyme. More cases of this type of enzyme were seen within three years in the USA and in the UK. Other Enterobacteriaceae, such as *Escherichia coli* were harbouring NDM, exhibiting more resistant traits than KPC-positive *Klebsiella* isolates (Yong *et al.* 2009). There were no clear-cut patterns of dissemination for carbapenemase positive *Klebsiella* bacteria including those with NDM-1 genes into hospitals. For instance, following a Colorado episode, the CDC used whole-genome sequencing, a technology utilised for the first time to resolve outbreaks in the hospital. Researchers were in doubt as to exactly how NDM was escalating among the population. Studies later revealed that bacteria carrying the NDM enzyme found its way outside the boundary of hospital settings into community water and

sewage environments with some epidemiological link to parts of Southern Asia (Kumarasamy *et al.* 2010, Patel and Bonomo 2013).

In Italy, the enzyme type described in Israel ST258 isolates located in transposon Tn 4401 was detected in KPC-positive isolate (KPC-3 gene type) (Giani *et al.* 2009). Over the years, 7 different patients with wound infection in Verona hospital at the surgical ICU had their isolates closely related to KPC-3-positive *Klebsiella pneumoniae* ST258 (Mazzariol *et al.* 2012). The situation was more disturbing when in 2011, the same strain type carrying colistin-resistance was found in Palermo general hospital in different acute wards and elsewhere from isolates (*Acinetobacter baumannii* and *Pseudomonas aeruginosa*) in other countries (Mammina *et al.* 2012, Aboulmagd and Alsultan 2014). Spain also confirmed a KPC-3 variant among 8 suspected cases through colonisation in a hospital in Madrid from a Spanish man in his mid-sixties in 2009, and 7 of the isolates were ST384 while the other one was carrying ST388 type (Curiao *et al.* 2010). A later outbreak which occurred in late 2009, this time a variant closely related to the KPC-2 gene type had been found in three *Citrobacter freundii*. The earlier isolates, none were attributed to travels outside the country (Gomez-Gil *et al.* 2010). However, in close neighbour Portugal, a KPC-2-positive *Escherichia coli* isolate was recovered from river water although there were no reports of medical cases in connection of KPC-positive isolates in recent times from their hospitals (Poirel *et al.* 2012).

Poland has a history of high prevalence of CREs carrying KPC genes. A KPC-positive case was confirmed in Warsaw. Later, a number of cases were confirmed by the national reference laboratory in five Warsaw hospitals towards the end of 2008, with more identified from 2009 to 2012 (not published). Most common sources of infections were either through stool or urine and ST258 type of strains were high among the isolates countrywide (Baraniak *et al.* 2011, ECDPC 2011). In Greece KPC-positive *Klebsiella pneumoniae* isolates were confirmed in Sweden and France in 2007 (Tagmark *et al.* 2007, Cuzon *et al.* 2008). An outbreak of KPC-2-positive *Klebsiella pneumoniae* was reported afterwards in a tertiary hospital in Heraklion (Maltezou *et al.* 2009), and two years later the type strain had spread into all tertiary hospitals including most acute-care services countrywide (Zarkotou *et al.* 2011, Gaibani *et al.* 2014).

Currently, KPC-positive organisms are frequently encountered in Greece and are not only found in the ICU, patients on surgical and medical wards have been victims of such infections. Outbreaks in long-term care facilities have been described, *Escherichia coli* carrying KPC-2-positive gene contributed to 40% infections outside ICUs (Mavroidi *et al.* 2012). However, there has been no evidence of horizontal transmission into the community. On the other hand, several confirmed cases of colonisation of KPC-positive *Klebsiella pneumoniae* from Greece have been reported in other countries most of them following international travel (Van der Bij and Pitout 2012). There have been several other carbapenemase enzymes established in most KPC-positive isolates in Greece. A surveillance study involving 40 hospitals had 5% VIM-1 or VIM-4 genes and the whole study team all reported 378 KPC-positive isolates in their findings. Other studies have revealed much higher numbers in VIM isolates or the presence of both VIM and KPC in most isolates. Of all the epidemic cases, Greece had recorded most

KPC-positive isolates having other genes linkages, and ST258 type included with the genetic make-up closely related (Giakkoupi *et al.* 2011, Lascols *et al.* 2013).

Israel is one such country with a successful story of controlling CRE infections after strain ST258 was suspected to have been imported from the USA in 2005 and eventually spread over all Israeli health-care facilities (Navon-Venezia *et al.* 2009). Most contributing risk factors at that period included poor infection control set-up, previous use of antimicrobial and ICU stay. Until middle of 2008, outbreak of KPC-positive Enterobacteriaceae had been controlled considerably, with incidence reduced by 79% in the country and bacteraemia taking up 50% as the attributable mortality rate of all clinical infections (Schwaber *et al.* 2008, Borer *et al.* 2009). Acquiring CRE infections from the community was not encountered, however, in long-term care facilities (LTCFs) the carriage were well established in a study carried out by Cantón *et al.* (2012) due to frequent transfer of patients from acute-care facility to the latter for further management. One of the measures taken to control the spread was compulsory search of CREs from rectal samples in high risk patients (Ben-David *et al.* 2011, Cantón *et al.* 2012).

The most hit areas of the Asian continent by the CRE isolates were India, Hong Kong, Taiwan, South Korea, and central, north-west, eastern and south-west parts of China. In India in particular, KPC-positive isolates were observed during a phase-3 clinical trial of tigecycline drug between 2002 and 2006 from three organisms; *Klebsiella pneumoniae*, *Proteus mirabilis* and *Escherichia coli* (Jones *et al.* 2009). By the end of 2010, an active surveillance study identified 9 more organisms harbouring the *bla*KPC gene in which 8 *Klebsiella pneumoniae* isolates were with 7 KPC-2 and 1 KPC-3, and 1 *Escherichia coli* isolate with KPC-2. Interestingly, one of *Klebsiella pneumoniae*

isolates was co-harboured KPC-2 with OXA-1, NDM-1, TEM-1, CTX-M-15 and SHV-12 β -lactamases, and RmtB known to be transferring aminoglycoside resistance (Kumarasamy and Kalyanasundaram 2012). The SENTRY programme carried out (between 2006 and 2007), identified OXA-181 as the gene recognisable in Indian health-care facilities (Castanheira *et al.* 2011). Earlier KPC-positive organisms seemed to be rare until French researchers reported KPC-positive *Escherichia coli* suspected to have been imported from India by a patient who had been colonised (Patron *et al.* 2012).

The most common carbapenemase gene in China is KPC-2 type found in a number of *Klebsiella* species. Zhejiang province recorded KPC-positive *Klebsiella pneumoniae* in China in the year 2004 from a mid-seventy year old ICU patient (Wei *et al.* 2007), and then significant numbers of KPC-positive Enterobacteriaceae were later identified in the eastern part of China. As many as 95 isolates of *Klebsiella pneumoniae* that showed susceptibility to carbapenem agents were later found to be carrying KPC-2 genes, closely related to ST258 type (Qi *et al.* 2012). The Shanghai region detected KPC-3 genes, one each in *Citrobacter freundii* and *Escherichia coli* respectively and infections were mainly from urine and sputum specimens. In many instances, the KPC-3 gene is more common in *Enterobacter cloacae* and *Klebsiella pneumoniae* isolates found abroad (Li *et al.* 2011). China had no effective infection control strategy in place and most patients received broad-spectrum antimicrobials including carbapenems in ICU wards where many patients had indwelling medical procedures complicating management. The most alarming situation was the detection of KPC-positive *Enterobacter cloacae* and *Citrobacter freundii* in hospital sewage, raising awareness of possible pollution of water bodies and reservoirs (Zhang, Lu and Zong 2012) while Ge

et al. (2011) studied and reported eminent community acquired infections with KPC-2-positive isolates in China.

Endemicity of KPC genes had been identified in these regions of Latin America, specifically, across Colombia among the Enterobacteriaceae family. Reported cases of KPC-positive isolates of KPC-2 type were found in *Klebsiella pneumoniae* isolates from two patients in 2005 (Villegas *et al.* 2006, Mojica *et al.* 2012), and both had no travels outside the country. Later in 2006, the country became the first to experience a KPC gene in *Pseudomonas aeruginosa* (Villegas *et al.* 2007). After two years, a KPC-3-producing *Klebsiella pneumoniae* outbreak occurred in which 20 died out of 32 patients. Fourteen of the death tolls were due to infections, with one patient travelling from Israel, a country with several outbreaks of KPC-3 genes (Lopez *et al.* 2011). A number of KPC genetic lineages were identified in Colombia; namely *Pseudomonas aeruginosa* ST235, ST308, ST1006 and 1060, while in *Klebsiella pneumoniae*, ST258 and ST51233 were identified (Cuzon *et al.* 2013, Munoz-Price *et al.* 2013). Subsequent reports on molecular investigations revealed extensive dissemination of KPC genes spreading across 3 regions out of 7 metropolitan areas studied in 2009 (Mojica *et al.* 2012, Maya *et al.* 2013). Argentina also had its KPC-2-positive *Klebsiella pneumoniae* identified in 2006.

By the end of 2010 following SENTRY studies carried out for 2 years, substantial increases in KPC-positive isolates in Argentina and Brazil were recorded (Gales *et al.* 2012). In another surveillance study conducted in 7 cities, 65 out of 514 *Pseudomonas aeruginosa* isolates were carrying KPC genes, and most had genetic relatedness to ST654, different from the ST258 *Klebsiella pneumoniae* type which suddenly spread up to mid-2009 in Argentina (Pasteran *et al.* 2012, Ramirez *et al.* 2013). A KPC-positive

enzyme in Brazil was recorded in 2006, a variant KPC-2-positive from 4 *Klebsiella pneumoniae* isolates in an intensive care unit with no trace of overseas travels (Monteiro *et al.* 2009). Subsequent expanded studies carried out across the country including waste water from hospital environment, revealed clone type-ST437 was common among KPC-2-harboursing *Klebsiella pneumoniae* isolates (Chagas *et al.* 2011). Brazil and other parts of the region had most species of *Pseudomonas* harbouring KPC-2 genes from hospital sittings. Venezuela and Chile, in particular, had no evidence of endemicity, however, several outbreaks of KPC-positive carriers from the Enterobacteriaceae group were latterly recorded (Maya *et al.* 2013).

Many countries have not instituted active surveillance studies specifically monitoring the KPC threat since its inception around the globe, the majority of which are in the African continent. Countries in Africa, including Morocco, Kenya and South Africa have reported NDM-1 as the most dominant carbapenemase gene (Poirel *et al.* 2011a, Poirel *et al.* 2011b). However, South Africa was the first to have reported a KPC-2-positive organism in 2012 (Brink *et al.* 2012). Strict infection control measures and regular surveillance programmes instituted by countries such as Australia and New Zealand have recorded prevalences as low as 1% of KPC harbouring genes in hospital acquired Enterobacteriaceae infections. The control measures were implemented after increased numbers of care-servicers were accepted to be treated from the Bali bombing episode in 2002 and the 2004 tsunami disaster which occurred in the Indian Ocean (CDC 2013, Munoz-Price *et al.* 2013).

1.10 Treatment options for CRE infections

Antimicrobial treatment of CRE infections has been challenged by the emergence of more complex resistance phenotypes as well as economic and regulatory pressures. Agents such as polymyxins and tigecycline have recently seen resurgence in their clinical usage (particularly colistin) in the management of multidrug-resistant Gram-negative infections, particularly CRE including carbapenem-resistant *Acinetobacter baumannii* in most hospitals (Hagihara *et al.* 2014).

The polymyxins are active agents and attain sufficient serum levels in the treatment of serious bloodstream and CRE infections. The agents produce additive or synergistic effects on humans against multidrug-resistant organisms including *Acinetobacter baumannii* isolates when combined with another antimicrobial agents such as tigecycline. In a study conducted by Lee *et al.* (2009), 16 patients with recurrent infections caused by KPC-producing *Klebsiella pneumoniae* evaluated that three out of twelve managed with polymyxin monotherapy experienced polymyxin resistance in their treatment. None of the four cases which were treated with polymyxin in combined therapy with tigecycline detected resistance to either antimicrobial agent (Lee *et al.* 2009, Marchaim *et al.* 2011). Out of several outcomes of monotherapies, polymyxin B monotherapy exhibited inferiority as against combination therapy in the management of patients (Petrosillo, Ioannidou and Falagas 2008). In an outbreak of KPC-2 infections in Greece, 22% resulted in polymyxin failure after treatment (Maltezou *et al.* 2009).

Tigecycline, a glycylcycline, is active *in vitro* against most carbapenem-resistant *Escherichia coli*. The drug is licensed for most complicated intra-abdominal, skin and soft tissue infections. Interestingly, a study reports success in various infections caused

by carbapenemase producers (Arnold *et al.* 2011). A later review of 10 studies including 33 patients with serious infections caused by multidrug resistant-Enterobacteriaceae, Kelesidis *et al.* (2008) reported 70% favourable outcomes with tigecycline treatment of cases, while 49% were only for intra-abdominal infections. Recurrence of infections was detected, prompting the management of the drug for a longer period in order to attain favourable results, due to delayed clearance of the CRE strain from the body which several studies had reported (Kelesidis *et al.* 2008). However, tigecycline use alone remains a concern in blood, urine, respiratory or other serious infections, and there have been several reports of development of resistance due to low concentration level (Falagas, Karageorgopoulos and Nordmann 2011, Patel and Bonomo 2013).

Combination therapy with tigecycline proved successful in many instances with positive clinical outcomes in the majority of infections involving KPC bacteria (Kelesidis *et al.* 2008). During an outbreak of KPC-2 infections in Greece, 88% of the patients were effectively treated with combination therapy involving tigecycline (Maltezou *et al.* 2009, Arnold *et al.* 2011). In a study involving over 1,000 patients with severe illnesses and a high prevalence of multidrug-resistant organisms, tigecycline showed a significant success and a positive tolerability profile when the drug was used (Bodmann *et al.* 2012, Hara *et al.* 2013).

Aminoglycosides, notably gentamicin, amikacin and tobramycin have different *in vitro* activities. Treatments of CRE infections may depend on the susceptible organism as studies have shown gentamicin activity against gentamicin-susceptible strains in urinary tract infections have positive outcomes. Of the aminoglycosides, amikacin appears to be the more active against CREs when compared with gentamicin or tobramycin (Abbott *et al.* 2013). While in another study, amikacin and tobramycin showed remarkably low

susceptibility to infections caused by MDR Gram-negative bacteria. This may be due to gentamicin modifying enzymes which have been carried by these MDR organisms. The use of aminoglycosides as monotherapy against carbapenemase-producing *Klebsiella pneumoniae* infections are considered ineffective, and therefore not recommended for clinical management of patients (Satlin *et al.* 2011, Hara *et al.* 2013).

Fosfomycin, a bactericidal antibiotic that inhibits bacterial cell wall biogenesis has seen its use renewed globally in response to the recent threat of antimicrobial resistance including carbapenem-resistant *Klebsiella pneumoniae* isolates (Falagas *et al.* 2008, Neuner *et al.* 2012). The drug is effectively used to treat urinary tract infections and has low rates of resistance. However, poor outcomes may occur when treating complicated *Pseudomonas aeruginosa* as a urinary pathogen. Many patients that developed treatment failure were immunosuppressed or had urethral stents due to the use of fosfomycin as monotherapy in kidney transplant cases.

Combination therapies have shown remarkable outcomes in dealing with MDR and CRE infections. The most commonly used combinations are colistin, polymyxin B or tigecycline combined with a carbapenem. In a retrospective study by Qureshi *et al.* (2012) on patients with bacteremia, the monotherapy, either tigecycline or colistin-polymyxin B alone had 58% mortality rate as compared with 13% for the tigecycline or colistin-polymyxin B combined with a carbapenem on a 28-day assessment, and this was observed in infections caused by KPC-producing *Klebsiella pneumoniae* isolates (Qureshi *et al.* 2012). Lee and Burgess (2012) also studied several antimicrobials for treatment of patients and recommended combination therapy (polymyxin plus tigecycline, polymyxin plus carbapenem, polymyxin plus aminoglycoside) as the best option compared with monotherapy (tigecycline or colistin-polymyxin B) in

complicated infections involving MDR and CRE (Lee and Burgess 2012). In future, treatments for infections caused by carbapenemase producers may involve new β -lactamase inhibitors such as methylidene penems; avibactam, MK-7655; the maleic acid derivative ME1071; ('neoglycoside') plazomicin, a novel aminoglycoside; the polymyxin derivatives NAB739 and NAB7061; and the siderophore monosulfactam, BAL30072 combined with cephalosporins and novel classes of antimicrobial agents effective against these pathogens (Hawkey and Livermore 2012, Patel and Bonomo 2013). Continual studies as regards to combination therapy trials need to be intensified until more efficient and acceptable combined therapy is accepted and recommended for the management of CRE infections.

1.11 Prevention of CRE infections

The non-availability of antimicrobials for the management of CRE infections should be given first line importance and newer drugs effective against carbapenemase producers should be developed at the earliest opportunity (Nordmann, Naas and Poirel 2011). Worldwide, the detection of carbapenemase producers in a clinical laboratory is based first on susceptibility testing results obtained by disc diffusion or by automated systems (Miriagou *et al.* 2010). The difficulty of the clinical laboratory to detect these enzyme producers early is the fact that some organisms that harbour *bla*KPC, have MICs that are elevated but still within the susceptible range for carbapenems due to complex mechanisms exhibited by carbapenem-resistant bacteria.

To curtail the spread, CDC (2013) proffers guidelines for areas where CRE are not endemic (acute care facilities) and these should firstly review microbiology records for the preceding 6-12 months to determine whether CRE have been recovered at the facility. Secondly, if the review finds previously unrecognized CRE, perform a point prevalence culture survey in high-risk units to look for other cases of CRE, and thirdly perform active surveillance cultures of patients with epidemiologic links to persons from whom CRE have been recovered. In areas where CRE are endemic, an increased likelihood exists for importation of CRE, and facilities should consider additional strategies to reduce rates of CRE.

In areas of low to middle income status such as in most African countries, including Ghana, active surveillance had not be given primary importance because of scarce resources in terms of competent staffing to carry out screening, poorly equipped laboratories and material shortages. Therefore, strict compliance with hand hygiene has to be made mandatory, contact precautions, screening patients with no link to known CRE patients in high-risk areas, and rectal swab taken to the laboratory for identification of CRE may go a long way to minimise the spread of outbreak of CRE infections. According to Hara *et al.* (2013) several studies have shown remarkable success in many health care settings implementing portions of these guidelines. Usage of drugs such carbapenems, fluoroquinolones, aminoglycosides and others inappropriately is associated with increased emergence of resistance, CRE included (ECDC 2011). Therefore, careful use in the ICUs of such antimicrobials may require tailored guidance and be supported by laboratory evidence.

1.12 Problems and clinical relevance of carbapenemase production

Globally, antimicrobial resistance in the family Enterobacteriaceae is classified as the leading cause of nosocomial infections in patients with multiple invasive devices or in the immunocompromised (Marchaim *et al.* 2008). Movement of carbapenemase encoding genes through mobile genetic elements has emerged and become a second step of resistance in horizontal transfer of plasmids between different species of bacteria (Diene and Rolain 2014). High levels of CRE in ICUs were reported in a study carried out in Europe, *Klebsiella pneumoniae* and *Escherichia coli* organisms were found to be endemic and multi-resistant with carbapenemase genes. Serious cases involving transplantations, long stay in the ICUs are at a high risk of acquiring these organisms (van Duijn, Dautzenberg and Oostdijk 2011, van-Duijn *et al.* 2013).

Currently, the main reason for the spread of CRE infections and antimicrobial-resistance genes is the dissemination of plasmids into community populations through stool or wound contacts by injuries as one of the mode of transmissions (Schwaber and Carmeli 2014). There is the need, therefore, for aggressive detection, surveillance and control strategies to capture information to help researchers examine the multifactorial nature of antimicrobial resistance among Gram-negative bacteria (Spellberg *et al.* 2008). As in the UK today, other countries in Europe, Asia, the Far East and the Americas, CRE has become a serious threat, and the control of healthcare associated infections has become a priority for healthcare managers (Watkins and Bonomo 2013). Carbapenem resistance is uncommon in the community, however, reports have indicated the existence of spread of CRE into the environment in India (Walsh *et al.* 2011), and such an alarming effect cannot be overemphasised. Recent figures from the CDC (2013) indicated that the prevalence of CRE has initially increased from 1% to 4% and subsequently to 18% in

long-term acute-care hospitals according to the database of national surveillance. Electronic systems for sharing information between hospitals and nursing homes regarding patients with CRE are needed in order to curtail their spread. The threat calls for rapid detection methods, high-calibre personnel and well-equipped laboratory setups to strengthen surveillance studies since the practice of microbiology is still a complex field that requires experience and logic. Thus, highly trained microbiologists are needed to interpret results using systems of electronic laboratory reporting to correlate with instant updates of epidemiological data from investigating centres (CDC 2013).

The routine practice of “culture and susceptibility” as a matter of rule should be augmented with quicker methodologies. Typical culture and sensitivity testing take several days to produce results, allowing transmission of undetected CRE and delaying effective antimicrobial therapy. Frequent changes of MIC breakpoints of resistance for carbapenems though relevant to patient care without performing phenotype based test may cause improper differentiation between various mechanisms of carbapenem resistance (Endimiani *et al.* 2010). Ability to monitor the emergence of resistance, using both phenotypic and genotypic methods depending on the availability of resources, and expertise to perform the tests and interpret results for clinicians is crucial. There is the need for governments to equip clinical laboratories to carry out genetic finger printing of bacteria so that they are used subsequently to detect, trace, or track cases of CRE outbreaks, their sources and how to control their spread.

With the exception of South Africa (Brink *et al.* 2012), a number of studies have also been carried out mostly in north African countries Egypt (Kaase *et al.* 2011, Abdelaziz *et al.* 2012), Morocco (Hays *et al.* 2012) and Libya (El Salabi *et al.* 2012) which have revealed significant number of threats of carbapenemase resistance genes in their

hospitals. Other countries have extended the search of spread into the community; recently Senegal (Moquet *et al.* 2011), Kenya (Poirel *et al.* 2011b) and Nigeria (Yusuf *et al.* 2013) have recorded significant number of emerging CRE isolates from hospitals to the community settings. Most countries in Africa are not carrying out extensive work to ascertain the presence of CREs. This is due to a number of factors including economic problems, political instability, and inconsistent or poor availability of resources to carry out rapid clinical laboratory identifications and perform cheaper surveillance studies. The imminent threat of untreatable infections from CREs have been highlighted by researchers worldwide, as evidenced by the emerging inter-hospital spread that occurred in Israel (Samra *et al.* 2007), and later many countries in Europe, Australia, China, India, the Middle East, and South America (Queenan and Bush 2007, Overturf 2010, Nordmann, Naas and Poirel 2011, Patel and Bonomo 2013, Walsh 2013).

1.13 Future solutions to curb global CRE threat

The growing threat to public health includes the rapid spread of CRE into the community. Organisms in the Enterobacteriaceae family are the common cause of community infections, and in recent times the occurrence of CRE also have been evidenced to spread from their main niche among hospital exposed patients into the community among otherwise healthy persons through faecal carriage (Jacob *et al.* 2013). Multidrug-resistant Gram-negative bacteria such as *Acinetobacter* and *Pseudomonas* species, less encountered in hospitals are the main organisms. However, these organisms have the potential to spread resistance to other bacterial isolates and into the community (Nicolas-Chanoine *et al.* 2013, Sievert *et al.* 2013). The threat must be answered by strict adherence to infection control strategies and policy guidelines

designed for individual countries (Walsh *et al.* 2011, Nordmann, Poirel and Dortet 2012). Performing more efficient identification of carbapenemase producers in the clinical microbiology laboratory is an important first step and provides key evidence for the control of CRE infections. The early identification of carbapenemase-producing isolates both in clinical infections and at the carrier-state should be mandatory to prevent the development of untreatable infections. The right to obtain a prescription at the first sign of a trivial infection propels the threats of resistance globally. Therefore, international campaigns to educate health-care providers, patients and lay persons may be warranted to limit the over-use and abuse of antibiotics in humans and agriculture.

Development of novel antimicrobials remains the prime objective for CRE control, a look at critical interventions aimed at preventing the transmission and infections with these organisms are of great significance. In waiting for more effective treatment options, healthcare settings can use prudent antimicrobial stewardship and effective infection control methods to decrease the impact of these organisms where this emergence of carbapenem resistance is known to be uncommon. Heightening the awareness of the public health threat by governments, policy makers, and or taking political decisions based on active surveillance testing for CRE may have some impact and support health caregivers to curb the resistance.

1.14 Justification of the study in Ghana

Multidrug resistance globally has reached an alarming stage and WHO has described it as the one of the three most serious problems confronting human health. Bacterial resistance has been a major public health concern, therefore, surveillance studies of antibacterial resistance should be in place and coordinated to minimise gaps in information gathering to combat the phenomenon. Notably, lack of focus by member countries instituting early implementation and the development of a global programme for surveillance of antimicrobial resistance may greatly affect patient outcomes in the future and cause universal suffering in human health (WHO 2014).

Currently, there are limited and less effective antimicrobials circulating against carbapenemase-producing bacteria which are more difficult to treat. In a recent infection report released in the United Kingdom a surveillance study clearly revealed that, colistin was the only drug which remained effective against over 90% of carbapenem-resistant Gram-negative bacteria associated with diverse mechanisms of resistance (Patel and Bonomo 2011, Public Health England 2015). With the most sophisticated monitoring systems, frequent surveillance studies and proper documentation systems at the doorsteps of developed countries such as in the United Kingdom, France, Germany and other resourced countries, South Africa and Egypt are not comparable to the least endowed country like Ghana to monitor and tackle enhanced surveillance of carbapenem-resistant Gram-negative bacteria in its regional hospitals. Ghana already has a problem conducting large scale surveillance studies and in carrying out efficient laboratory management systems as regards to data collection, use of laboratory based equipment and availability of detection reagents to identify any antimicrobial resistance including CR isolates. Study parameters used in many developed countries to evaluate

bacterial resistance are not the same in Ghana, therefore, may pose serious challenges in the early detection of CR organisms for clinical management of patients. For instance, OXA-48 producing strains require more efficient techniques for detection in clinical laboratories. To date, no variant-types of carbapenemase-producing genes have been investigated or studied for epidemiological records in Ghana.

The demand for effective anti-microbial agents remains high due to escalating microbial resistance. High incidence of treatment failures, morbidity and mortality in many infectious diseases are strongly correlated with antimicrobial resistance. For instance, the overuse of β -lactam drugs has caused a considerable increase in the incidence of infections caused by ESBL-producing organisms with subsequent adverse effects on clinical outcomes (Kim *et al.* 2002). Ghana is no exception to threats of global antimicrobial resistance. The two most important Teaching Hospitals, Komfo Anokye (KATH) and Korle-Bu (KBTH) have carried out extensive studies on antimicrobial resistance in the country. Studies carried out by Newman *et al.* (2011) evaluated the extent of multi-drug bacterial resistance for a number of pathogens but did not evaluate the use of carbapenem agents for the detection of carbapenem-resistance in multi-drug resistant Gram-negative bacteria. The results were alarming due to improper monitoring and misuse of antimicrobials by many people without prescriptions (Newman *et al.* 2011).

Most hospitals in Ghana have limited facilities and resources to properly confirm the presence of ESBLs and the recently occurring KPC-type genes in clinical isolates. Standard testing regimens used elsewhere cannot be performed routinely because of the difficulty in detecting the underlying mechanisms of resistance based on phenotypic

identification of the organisms. Harmony and Kenneth (2003) reported that extremely broad antimicrobial use of carbapenem drugs pose substantial risks. Patients treated with carbapenem antimicrobials may no longer be subsequently treated for the same infection using cephalosporin antimicrobials (Harmony and Kenneth 2003). However, in most countries, including Ghana, carbapenems remain one of the few therapeutic options for treatment of serious infections including ESBL-producing organisms after they have been detected. In a study by Queenan and Bush (2007) the current epidemiology of metallo- β -lactamase production generally follows certain patterns of increasing occurrences that are country specific unless this is carefully monitored (Queenan and Bush 2007). The epidemiology of carbapenem-resistant organisms remains largely unknown in Ghana. Cases of CR organisms have been observed routinely by most microbiological laboratories in Ghana but no serious documentation has been kept. People, therefore, stand the chance of acquiring and bringing this emerging resistance into the country as many travel worldwide for various reasons including medical interventions. The spread of CR organisms was attributed to the risk of travel to the most affected countries in the Indian sub-continent for some form of surgical and medical investigations. At present, most hospital laboratories in Ghana lack the necessary diagnostic tools for CR detections; therefore, ensuring effective treatment of patients cannot be guaranteed.

In many parts of the world, studies have acknowledged that the location of KPC genes is on highly mobile genetic elements thereby contributing to their rapid spread with frequent co-transfer of multiple antimicrobial resistance factors. The infection strata may happen in acute care facilities where critically ill patients are exposed to invasive devices (e.g., ventilators or central venous catheters). Carbapenem-resistant organisms in these settings may become an important challenge and increase the likelihood of

importation of such infections into ICUs. In effect, KPC dissemination in hospitals causes serious outbreaks in ICUs (Brink *et al.* 2012). The carbapenemase enzymes produced render the carbapenems ineffective, contributing to mortality rates of 40%-50% in hospital settings (Queenan and Bush 2007, Schwaber *et al.* 2008, van Duijn *et al.* 2011, CDC 2013, Watkins and Bonomo 2013). According to Thaden *et al.* (2014) the current rate of mortality and poor outcome of patients in parts of United States community hospitals may fall between 48% and 71% for CRE infections. A number of CR studies have been investigated in African countries, namely South Africa (Brink *et al.* 2012), Kenya (Poirel *et al.* 2011b), Senegal (Kempf *et al.* 2012), Morocco (Poirel *et al.* 2011a), Nigeria (Yusuf *et al.* 2013, Ogbolu, and Webber 2014) and Egypt (Abdallah *et al.* 2015) with common carbapenemase variant types detected. However, there have been screenings but not extensive studies in hospitals to establish high levels of CRE detections in Ghana.

As a matter of policy, general housekeeping and infection control guidelines have been instituted all over hospitals in Ghana. However, curbing the spread of carbapenem resistance in Gram-negative bacterial infections needs serious interventions. Strategies have to be adopted by stakeholders to enhance surveillance studies among the regional and tertiary hospitals. The Ministry of Health (MOH) of Ghana is expected to spearhead this study with necessary logistical supplies, training of laboratory personnel, data collectors for regular analysis and feedback of results to develop a greater understanding of the epidemiology of carbapenem resistance in the country. Regional and national analysis of results may allow identification of patients prone to acquiring CR organisms, evaluation of changes in the epidemiology of carbapenemase-producing bacteria and monitoring of any interventions adopted to curb the spread of carbapenem resistance. District hospitals in the country should be monitored. Those with facilities to perform

reliable microbiological laboratory tests should be allowed to send suspected CR isolates to designated regional laboratory centres to access and manage the local data as well as providing the prospects for district-level results analysis. As part of the projected WHO programme report, enhanced surveillance studies need to be carried out in many countries (WHO 2014). Ghana's MOH should engage experts in the field of antimicrobial resistance in collaboration with the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, who have the mandate and responsibility to monitor the insurgence of carbapenem resistance. The situation of CR bacteria across the globe is disturbing. Ghana needs the collection and analysis of these CR data to be informed and fine-tuned its strategies year by year for the laboratory detection and management of patients. Epidemiological data of CR organisms may finally depend on the outcome of this study for the country.

1.15 Hypothesis

Carbapenem-resistant organisms are widely distributed among Gram-negative bacilli and the nature of the resistance loci vary significantly among these clinical isolates from selected hospitals in Ghana.

1.16 Main objective

The overall objective of the study is to investigate carbapenem resistance in Gram negative bacteria in Ghana using phenotype based detection and genotypic characterisation methods.

1.16.1 Specific objectives

- To identify the spectrum of Gram-negative bacteria resistant to carbapenems from clinical specimens.
- To determine the incidence and distribution of carbapenem resistance by phenotype based techniques.
- To characterise the presence of plasmid-mediated carbapenemase resistance genes.
- To determine the genetic diversity of the carbapenem-resistant isolates using ERIC-PCR technique.

Chapter two

2. Materials and methods

2.1 Materials

All the kits and reagents used for this study were prepared according to the manufacturer's recommendations and guidelines except where stated otherwise, and where some materials demanded re-constitution before use, this done according to the manufacturer's recommendations and guidelines.

2.2 Ethical Approval

Ethical approval for this study was obtained from the ethical committee of the School Biomedical & Allied Health Sciences, College of Health Sciences, University of Ghana (Ethics Identification Number: SAHS-ET/SAHS/PSM/ML/05/AA/26A/2012-2013). Sheffield Hallam University, Research Degree Sub-Committee confirmed the PhD registration (SHU: GT/RDSC 27th June 2014) on the research topic while Risk Assessment Unit of the Biomolecular science Research Centre, Sheffield Hallam University approved the use of Hazard group 2 organisms for this study.

2.3 Sample sites

A prospective study was carried out in four selected hospital laboratories in Ghana. Carbapenem-resistant (CR) isolates were recovered from September 2012 to September 2014 from patients who were diagnosed with various infections in four tertiary care hospitals located in four distinct regions in Ghana (Korle Bu Teaching hospital in the Greater Accra Region, Effia-Nkwanta hospital in the Western Region, AngloGold

Mines hospital in the Ashanti Region and Ho Regional hospital in the Volta Region) as indicated in Figure 2.1.



Figure 2.1 Map of Ghana depicting sample sites

Note: "The Star sign" indicating four regional hospitals namely; KBTH, GA/R= Korle Bu Teaching Hospital, Greater Accra Region, ENRH, W/R= Effia-Nkwanta Regional Hospital, Western Region, AGAMH, A/R= AngloGold Ashanti Mines Hospital, Ashanti Region, HRH, V/R= Ho Regional Hospital, Volta Region

2.3.1 Korle Bu Teaching Hospital

Korle Bu Teaching hospital (KBTH) is a referral hospital with a 2,000 bed capacity. The facility receives 1,500 daily visits of people and admits almost 250 patients each day (Annual Report of KBTH 2012). The Teaching Hospital, ranked third in Africa in terms of competent medical staff and resources continues to introduce specialised services. Within Africa, this referral hospital is among the few that perform DNA investigations and was the first to carry out kidney transplantation in the West African sub-region (Annual Report of KBTH 2012). Their clinical laboratories are manned by qualified and competent Biomedical Scientists.

2.3.2 Effia-Nkwanta hospital

The main regional hospital of the Western region of Ghana is situated at the capital, twin city, Sekondi-Takoradi. The municipality has a population of over 440,000 inhabitants which the hospital serves including the surrounding villages. According to the Ghana Health Service annual report (2005) the hospital has a greater than 400 bed capacity and serves as a referral point for many clinical conditions which need specialist interventions. The refurbished laboratory named Public Health Reference Laboratory (PHRL) is currently in use to cover public health issues in the region and other regional hospitals in the country. Currently, the Ghana Health Service has finalised the use of the hospital as an associate Teaching Hospital to the School of Medical Sciences (SMS), University of Cape Coast (UCC). The affiliation may allow more intakes of medical students to be enrolled due to the low bed capacity at the Cape Coast Regional Hospital (UCC 2015).

2.3.3 AngloGold Mines hospital

The AngloGold Mines hospital has a bed capacity of over 100 and is the referral facility in the municipality. However, other referral patients are admitted from the West and Central Districts, Adansi North and South Districts, and Amansie East. In the Ashanti region, the hospital is the second largest facility catering for over 40% of non-workers in the AngloGold Mining Company in and surrounding the Obuasi municipalities. On average, 15,000 patients are managed monthly by the hospital care providers (AngloGold 2007, <http://www.spyghana.com/anglogold-medical-facility-obuasi-formally-commissioned-obuasi/>).

2.3.4 Ho Regional hospital

The Ho Regional hospital has 150 beds serving an estimated population of over 260,000 inhabitants according to the 2000 census estimation. Ho municipal is the administrative capital of the Volta region surrounded by 24 other districts. The municipality shares borders in the west with Ho West District, to the south with Agotime-Ziope, to the north with Hohoe Metropolis and the Republic of Togo in the east. In 2012, Regional Hospital status was upgraded to a Teaching Hospital due to the establishment of the University of Health and Allied Sciences (UHAS) in the region (Ghana Health Service 2006, Health News 2012).

2.4 Bacterial isolates

A total collection of three thousand eight hundred and forty (3,840) Gram negative bacilli isolates were screened among the Enterobacteriaceae family and non-fermenting organisms belonging to different species; *Pseudomonas*, *Acinetobacter*, *Klebsiella*, *Enterobacter*, *Providencia*, *Shigella*, *Cronobacter*, *Sphingomonas* species and

Escherichia coli. Clinical specimens from different infection sites such as wound, urine, sputum, ear, aspirate, stool, cerebrospinal fluid, catheter tip, eye and high vaginal swab were obtained from the four selected hospitals in Ghana. These CR isolates were multidrug resistant to different classes of antimicrobials tested including carbapenems. A collection totalling one hundred and eleven (111) isolates which showed multidrug resistant (MDR) including intermediate or complete resistance to carbapenems (imipenem, meropenem and doripenem), identified by antimicrobial susceptibility testing were identified for this study (see Appendices 1 and 2).

2.5 Microbiological assays

2.5.1 Identification of bacterial isolates

All the 111 CR pathogens from various clinical specimens were identified to the genus levels by conventional methods. Quality control strain *Escherichia coli* ATCC 25922, a known susceptible strain to carbapenems, and *Klebsiella pneumoniae* carbapenemase positive NCTC 13438 were included in the identification. Stock cultures of the isolates were cultured onto MacConkey agar (Oxoid Ltd, UK) plates for 18-24 hours prior to identification and confirmation to species levels was determined by the use of Vitek 2 automated Compact system (BioMérieux, France). Inoculum of the isolate was adjusted to 0.5 McFarland standard, and then poured into a panel covered in a secured place. The inoculated panel was then read automatically by the device. The results generated were analysed with preliminary algorithms and compared with the control data as appropriate giving either excellent or good identification.

2.5.2 Antimicrobial resistance testing

All the MDR isolates were re-tested using three carbapenem discs (Oxoid Ltd, Basingstoke, UK); imipenem, meropenem and doripenem by the British Society for Antimicrobial Chemotherapy (BSAC) disc diffusion method (Andrews 2004) on Mueller-Hinton agar (MHA), (Biotec Ltd, UK). Impregnated discs containing a standard amount (10 µg) of each of the carbapenems were placed on agar plates seeded with the bacteria to be tested. As the organism grows during overnight incubation, the carbapenem agent diffuses into the agar medium. Susceptibility of the test organism is proportional to the zone of inhibition produced by the antibiotic used. According to Clinical and Laboratory Standards Institute (CLSI) breakpoints (CLSI 2012), an isolate is considered resistant or susceptible to the three carbapenems used if the zone sizes ranged from ≤ 15 to ≥ 23 mm (imipenem or meropenem) and ≤ 19 to ≥ 24 mm (doripenem) respectively. A reference strain of *Escherichia coli* ATCC 25922 was included as a negative control organism. For isolates with diameters ≤ 15 mm, minimum inhibitory concentrations (MICs) were determined using the E-test method (CLSI 2012, EUCAST 2012), and resistance was defined as isolates with MIC against the same carbapenems of ≥ 4 µg/ml according to CLSI breakpoints. Additionally, E-test strips (BioMérieux, France) for imipenem, meropenem and ertapenem showing MIC values greater or within 2 to 4, 2 to 4, and 2 µg/ml levels respectively were considered carbapenemase producers which were confirmed by the modified Hodge test.

2.5.3 Determination of minimum inhibitory concentration by the E-test method

For isolates with diameters ≤ 15 mm inhibition to any of the carbapenems, MICs were determined using E-test strips of 3 carbapenems (imipenem, meropenem, ertapenem) each gradient ranging from 0.002 to 32 µg/ml, and a cefpodoxime gradient from 0.016

to 256 µg/l for detection of ESBLs. Diffusion method on agar plates designed by the British society for antimicrobial chemotherapy (BSAC) followed (Andrews 2004). Prepared inoculum suspension in 0.85% NaCl of the test organism adjusted to 0.5 McFarland turbidity standard was swabbed onto the MHA plate. Impregnated E-test strips of the 3 carbapenems and cefpodoxime were placed on agar plates already inoculated with the bacterium to be tested and incubated at 35°C in air for 16-18 hours. The MIC results were read following the incubation. Resistance was defined as isolates with MICs against the same carbapenems of ≥ 4 µg/l (except ertapenem of 2 µg/ml) according to CLSI revised breakpoint recommendations (Table 1.3). Based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST 2012), organisms that were found to show resistance to imipenem and meropenem (MIC ≥ 4 µg/ml respectively), and ertapenem (MIC ≥ 2 µg/ml); according to the Clinical and Laboratory Standards Institute (CLSI 2012) were to be confirmed using the modified Hodge test. Cefpodoxime (BioMérieux, France) MICs by E-test method were carried out of which ≥ 1 µg/ml was considered as ESBL positive.

2.6. Phenotypic based methods for carbapenem-resistant organisms

The following techniques, modified Hodge test (MHT) and boronic-acid disc synergy test (BADST) were carried out as additional phenotype based techniques in this study.

2.6.1 Modified Hodge test

This method was performed for the detection of Class A carbapenemase previously described by Anderson *et al.* (2007). Briefly, the indicator organism *Escherichia coli* ATCC 25922 was prepared by obtaining an overnight culture which was adjusted to 0.5 McFarland turbidity standard followed by a 10 fold dilution in saline. This broth was

then used to inoculate the surface of plates of Mueller-Hinton agar (Biotec Ltd, UK) by swabbing. Meropenem (10µg) was placed at the centre. Three to five colonies of the test organisms were used to inoculate the plate in a line straight from the edge of disc to the end of the plate and incubated overnight at 35-37°C for 16-24 hours. The plate was read and interpreted as positive test when a clover-leaf like indentation of the *Escherichia coli* ATCC 25922 grew along the test organism growth streak within the disc diffusion zone.

2.6.2 Boronic acid-disc synergy test

Boronate derivatives have structural resemblance of β -lactams have long been used in searching the function of β -lactamase enzymes, particularly class C enzymes. However, in 2008, inhibitory effects of boronic acid and its derivatives such as 3-aminophenylboronic acid (APBA) and phenylboronic acid (PBA) have been confirmed to detect KPC production and other members of the class A carbapenemases with better sensitivity and specificity when meropenem disc supplemented with PBA. Cut-off value of ≥ 5 mm zone diameter difference with and without PBA was accepted (Doi *et al.* 2009). A cut-off value of ≥ 7 mm when meropenem disc is used, with and without 400 µg of PBA, was effective for detecting KPC-producing *Klebsiella pneumoniae* isolates and differentiating them from plasmid-borne AmpC-producing *Escherichia coli* and *Klebsiella pneumoniae* (Tsakris *et al.* 2011, Hrabák, Chudáčkova and Papagiannitsis 2014).

A modified boronic acid-disc synergy test (BADST) described by Doi *et al.* (2009) was performed on Mueller-Hinton agar (Biotec Ltd, UK). Carbapenem discs containing 10µg each of imipenem, meropenem and ertapenem were supplemented with 20 µl of 20 mg/ml 3-aminophenyl-boronic acid (APB) and dissolved in dimethyl sulphoxide (DMSO; Sigma-Aldrich Company Ltd Dorset, UK) to yield a 400 µg/ml final concentration. A suspension of the test organism was prepared according the same protocol described above then used to swab the surface of a MHA plate. The supplemented discs of imipenem, meropenem and ertapenem containing 400 µg/ml each and un-supplemented discs of each antimicrobial respectively were placed far apart on the plate and incubated at 35-37°C in air for 16-18 hours. A positive test for KPC enzyme production was recorded when the measured diameter of the growth-inhibitory zone around each carbapenem disc with boronic acid was ≥ 5 mm more than that around the disc containing the carbapenem alone.

2.6.3 Sensitivity and specificity calculation for modified Hodge test and boronic acid-disc synergy test

Determination of sensitivity (SE) and specificity (SP) were calculated based on the formulas described by Pasteran *et al.* (2010) to evaluate the reliability of MHT and BADST compared with PCR-based results for carbapenemase resistance genes. SE formula = $a/(a + c) \times 100$, where a represents the number of carbapenemase-producing isolates correctly identified and c represents the number of true carbapenemase-producing isolates incorrectly identified as non-producers. SP formula = $d/(b + d) \times 100$, where d represents the number of CR isolates correctly identified by MHT screen as non-carbapenemase producers and b being the number of CR isolates incorrectly identified as carbapenemase-producing isolates. The formulas $a/(a + b)$ and $d/(c + d)$

may be used to calculate positive predictive figure (PPF) and negative predictive figure (NPF), respectively.

2.6.4 Nitrocefin assay for β -lactamase enzyme identification

Beta-lactamase enzymes are the main components of resistance to β -lactam antimicrobials for most Gram-negative organisms. In recent times, nitrocefin interactions with ESBLs offer valuable evidence in assessing β -lactam compounds (Papanicolaou and Medeiros 1990, Dai *et al.* 2012).

2.6.4.1 Principle of nitrocefin assay

Nitrocefin in solution at neutral pH is expected to produce two absorption levels. One absorption peak is spectrophotometrically read at 217 nm when linked to 7-acyl group and another peak level at 386 nm. Subsequent to exposure to β -lactamase, the level considerably reduces at 386 nm, and a new level formed appears at 482 nm which relates to degraded nitrocefin. This difference in shift can be measured over time and allows estimation of activity of a given β -lactamase enzyme based on the rate at which it degrades nitrocefin, a coloured cephalosporin whose visible absorption spectrum (and hence colour) changes upon hydrolysis of the β -lactam ring.

2.6.4.2 Preparation of β -lactamase extracts

All 111 CR organisms were grown in 10 ml Mueller-Hinton broth at 37°C in a shaking incubator for 16-18 hours. The technique described by Dai *et al.* (2012) was used and modified. The overnight broth culture was centrifuged at 4,000 rpm for 10 minutes to recover the cell deposit. The pellet was washed once with sodium phosphate buffer (100

mM with pH of 7.0). The supernatant was removed and the wet weight of the pellet was determined. The pellet was re-suspended in sodium phosphate buffer using a minimum of 5 μ l of buffer per microgram of sample. The cell suspension was sonicated (Sonic & Materials, Inc., USA, model: VCX 750) for 5 minutes on ice (4 \times 15 s at 30-s intervals) and centrifuged at 6,400 rpm at 4°C for 20 minutes to obtain cell-free extract for nitrocefin assay. Disruption by sonication was to release β -lactamase enzyme into the cell-free suspension from periplasmic region of the cells. The β -lactamase extracts were stored at -20°C or colder until assay testing. Frozen samples were thawed at room temp (20-25°C) or in a refrigerator before use.

2.6.4.3 Microplate assay for determination of β -lactamase concentration

In each well, 10 μ l of collected extract was added to a 96-well plate. Total volume was adjusted to 50 μ l per well with β L Assay Buffer (100 mM Sodium phosphate, pH 7.0). Reaction mixture was prepared according to the manufacturer's instructions. In each well, a total reaction mix of 50 μ l was prepared comprising β L Assay Buffer 48 μ l and 2 μ l of nitrocefin solution. Reaction mix was added to the extract in each well and was then thoroughly mixed and the absorbance of the solution was measured at 390 and 486 nm every minute for a 15 minute period using a plate reader (Tecan Infinite® 200 PRO Männedorf, Switzerland). Positive controls were prepared in β L Assay Buffer and 10 μ l of diluted positive control pipetted into the desired well. Double distilled water was included as a negative control. The volume was adjusted to 50 μ l with β L Assay Buffer. For the blank, 10 μ L sodium phosphate buffer was used and adjusted in the same way as the positive control. The mixture was then incubated at 30°C for 1 hour.

2.7 Genotypic characterisation of carbapenemase-producers

All the 111 CR isolates were investigated for the presence of carbapenemase-producing genes with primers from the five most common genes namely; New Delhi metallo-beta-lactamase-1 (NDM-1), *Klebsiella pneumoniae* carbapenemase-1 (KPC-1), Verona integron-encoded metallo- β -lactamase-1 (VIM-1), Oxacillinase-48 (OXA-48) and Imipenem-resistant *Pseudomonas*-1 (IMP-1) in this study.

2.7.1 Genomic DNA extraction of CR organisms

DNA isolation procedures were carried out in accordance with guidelines set by Millar *et al.* (2002) in order to minimise contamination for false-positive results. Bacterial suspension of 1 ml was grown overnight at 37°C. The extraction procedure was conducted using a QiaAmp mini Kit (Qiagen, Hilden, Germany) for Gram negative bacteria according to Qiagen guidelines. The eluted chromosomal DNA (in 2 x 2 ml sterilised Eppendorf tubes) was stored at -20°C.

2.7.2 Preparation of primer sets

Each of the five primers of carbapenemase-producing genes (IMP-1, VIM-1, OXA-48, NDM-1 and KPC-1) was diluted from 100 $\mu\text{g}/\mu\text{l}$ stock solution and stored at -20°C by adding the desired volume of nuclease-free PCR water. To make a working solution, the stock solution was diluted in ratio 1:10 with nuclease-free PCR water with each primer sets (Table 2.1) according to the manufacturer's instructions. Exclusion of CTX-M primers was due to unavailability of reference control strain during the study.

Table 2.1 Primer sets for amplification of carbapenemase and extended spectrum**β-lactamase genes**

Gene	Primer sequence (5'→3')	Amplicons size (bp)	Reference
<i>blaIMP</i>	Forward - GGAATAGAGTGGCTTAAYTCTC Reverse – GGTTTAAAYAAAACAACCACC	232	(Poirol <i>et al.</i> 2011d)
<i>blaVIM</i>	Forward - GATGGTGTGTTGGTCGCATA Reverse - CGAATGCGCAGCACCAG	390	(Poirol <i>et al.</i> 2011d)
<i>blaOXA-48</i>	Forward - GCGTGGTTAAGGATGAACAC Reverse – CATCAAGTTCAACCCAACCG	438	(Poirol <i>et al.</i> 2011d)
<i>blaNDM</i>	Forward - GGTTTGGCGATCTGGTTTTTC Reverse – CGGAATGGCTCATCACGATC	621	(Poirol <i>et al.</i> 2011d)
<i>blaKPC</i>	Forward - CGTCTAGTTCTGCTGTCTTG Reverse – CTTGTCATCCTTGTTAGGCG	798	(Poirol <i>et al.</i> 2011d)
<i>blaTEM</i>	Forward - TCAACATTTTGTCGTCG Reverse – CTGACAGTTACCAATGCTTA	860	(Schlesinger <i>et al.</i> 2005)
<i>blaSHV</i>	Forward - TTTATCGGCCYTCACTCAAGG Reverse – GCTGCGGGCCGGATAACG	930	(Schlesinger <i>et al.</i> 2005)

Note: IMP, imipenem-resistant *Pseudomonas*; VIM, Verona integron-encoded metallo-β-lactamase; OXA-48, oxacillinase-48; NDM, New Delhi metallo-β-lactamase; KPC, *Klebsiella pneumoniae* carbapenemase; TEM-1, Temoniera-1; SHV-1, sulphdry1 variable-1

2.7.3 PCR reaction mix preparation

The PCR reaction mix was prepared aseptically using PyroMark Master Mix Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with 2 µl of DNA extract in a final reaction volume of 25 µl as indicated in Table 2.2 was prepared.

Table 2.2 Composition of PCR reaction mix

Reagent	Volume per PCR reaction (µl)	Final concentration
PCR master mix (QIAGEN) buffer, 2x	12.5	1x
Forward primer (MWG) working solution, 10 pmol	0.5	0.2 pmol
Reverse primer (MWG) working solution, 10 pmol	0.5	0.2 pmol
CorralLoad (QIAGEN), 10x	2.5	1x
DNA template, 1-5ng/µl	2.0	0.4 ng
Nuclease free water (QIAGEN)	9.5	made up appropriately to the total volume
Total volume	25.0	

2.7.4 PCR amplification conditions

Primer sets used were obtained from Eurofins MWG Ebersberg, Germany (Table 2.2).

The cycling conditions carbapenemase genes were: initial denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 1 minute, annealing at 58°C for 30 seconds, and elongation at 72°C for 1 minute 30 seconds, followed by a final elongation step at 72°C for 10 minutes in a thermal cycler (MJ Research PTC-150 MiniCycler™, USA). The PCR cycles were run between 2 and 3 hours.

2.7.5 PCR products for electrophoresis

Gels were prepared using 2% agarose (Invitrogen, Carlsbad, USA) in 1 x Tris-Acetate-EDTA buffer. Before solidification of prepared gels, 2.5 µl of ethidium bromide was added. All PCR products were analysed by electrophoresis in 1 x TAE buffer prepared from 10 x TAE buffer per litre (48.8 g Tris base, 11.4 ml Glacial acetic acid, 3.7 g EDTA disodium salt).

2.7.6 Loading and electrophoresis of amplified PCR products

The presence of coralload dye required no other loading dye for the amplified PCR products. Therefore, each well was loaded with 6 µl of test samples. A molecular marker 1 kb DNA ladder (Invitrogen, Carlsbad, USA) was used as an external reference for size comparison. Each electrophoretic set was run at 100 volts per centimetre for 1 hour.

2.7.7 Visualisation of bands on agarose gel

The amplified bands in the gel were visualized by a trans-illuminator (UV light) to confirm the PCR products.

2.7.8 Purification of PCR products for sequencing

Reagents needing to be prepared from the QIAquick PCR purification kit (Qiagen, Hilden, Germany) were prepared according to the manufacturer's instructions. The purified PCR products were stored at -20°C prior for sequencing.

2.7.9 Sequencing of purified PCR products

Purified PCR products for sequencing by Eurofins Genomics, Ebersberg were followed according to the protocol instructions. Forward primer of each appropriate carbapenemase gene (2 µl) was added to 7.5 µl of purified PCR DNA product and nuclease-free water respectively to make up a total volume of 17 µl for sequencing. The purified 26 positive carbapenemase producers including positive control strains were prepared and sent to Eurofins Genomics, Ebersberg, Germany for sequencing.

2.7.10 Determination of extended spectrum β-lactamase genes in total DNA extracts

Primers were identified from Schlesinger *et al.* (2005) study to amplify regions of the extended spectrum β-lactamase genes (*bla*TEM and *bla*SHV) (Table 2.2). Amplification conditions in a thermal cycler (MJ Research PTC-150 MiniCycler™, USA) with initial denaturation 15 minutes at 95°C and 35 cycles of 1 minute at 94°C, 1 minute at an annealing temperature of 47°C and 50°C designed for each primer set for TEM and SHV respectively, and 1 min at 55°C, followed by 10 minutes at 72°C for the final extension. The amplification cycles were run for 2 to 3 hours. All other set ups were followed as indicated in the above sections; 2.7.1, 2.7.2, 2.7.3 and 2.7.5 to 2.7.7.

2.7.11 Outer membrane protein analysis of CR isolates

A recent study by Sugawara and Nikaido (2012) claimed that the major proteins in the outer membrane of *Acinetobacter baumannii*, non-fermenter isolate which belongs to the low-permeability trimeric porin group, but have been found to equate to *Escherichia coli* OmpF/OmpC, and also shares close homology to *Pseudomonas aeruginosa* OprF and *Escherichia coli* OmpA (Sugawara and Nikaido 2012). Therefore, in-house MBL-

positive (VIM positive) and wild-type *Pseudomonas aeruginosa* control strains from Sheffield Hallam University (SHU) collection were included and used for the study analysis of OMPs. Two methods, with some modifications, were employed separately for organisms in the Enterobacteriaceae family and *Acinetobacter* species (Mizuno and Kageyama 1978), and for *Pseudomonas* species (Sambrook and Russell 2001) due to structural differences.

2.7.11.1 Separation of outer membranes of *Escherichia coli*, *Acinetobacter* species and other organisms

Bacterial cultures were grown to stationary phase (6 hours) aerobically at 37°C in Mueller-Hinton broth (Biotec Ltd, UK). Aliquots (0.1 ml) of the overnight culture were added to 20 mls of Mueller-Hinton broth in a 50 ml conical centrifuge tube (to allow for appropriate aeration). The cultures were then grown to an optical density of 0.6 at 600 nm (late exponential phase growth). The bacterial culture was then harvested by centrifugation at 4000 g (1600 rpm) for 15 minutes using Eppendorf 5804R with the 6 x 125 g rotor (Thermo Scientific). The pellet was subsequently re-suspended in 9ml of phosphate-buffered saline (PBS), 100 µg lysozyme per ml was added, and the solution was left for 20 minutes, or until the culture had a more translucent appearance. The culture was then disrupted at 4°C by sonication through five cycles of 30 seconds, followed by 30 seconds without sonication. The remaining solution was then subjected to two successive centrifugation steps (10 minutes at 2,500 g or 1,000 rpm) to remove whole cells, or large cell debris, with the supernatant being kept in each case. The supernatant was then centrifuged at 27,500 g (11,000 rpm) for 90 minutes to pellet the cell membranes. The pellet was then re-suspended in 1.7% (w/v) sodium-lauryl-sarcosinate by repeated pipetting within the 20-30 minutes at room temperature. Outer

membrane vesicles were then harvested by centrifugation at 27,500 *g* for 90 minutes. The pellet was re-suspended in 1.5 ml double-distilled filter-sterilised water and was centrifuged at 27,500 *g* for 60 minutes to remove the sodium-lauryl-sarcosinate. The pellet was re-suspended in 50 µl of double-distilled filter-sterilised water and stored at -20°C until required (Sambrook and Russell 2001).

2.7.11.2 Outer membranes separation from *Pseudomonas* species

Cultures were grown in 20 ml of Mueller-Hinton broth (Biotec Ltd, UK) to attain optical density of 0.6 at 600 nm (late exponential phase growth) before use. The bacterial broth was centrifuged at 1600 rpm (Eppendorf 5804R centrifuge, Thermo Scientific) for 15 minutes to harvest the deposit. The pellet was re-suspended in the following order; 4.5 ml of 20% (2M) Sucrose, 5.0 ml (0.1M Tris HCL pH7.8 at 25°C, 0.4 ml 1% Na-EDTA (pH7.0) and 0.9 ml of 0.5% (0.07M) lysozyme. The content was left for 20 minutes, then warmed to 30°C and kept for 60 minutes. After 30 minutes within the 60 minutes, deoxyribonuclease 3 µg/ml was added. The suspension was centrifuged to remove the cell debris at 13,000 rpm for 15 minutes at 30°C. The supernatant collected was centrifuged in Sorvall Lynx 6000 centrifuge (Thermo Scientific, rotor T29-8 x 50) at 29,000 rpm for 60 minutes. To the pellet obtained, 4 ml of 5mM MgCl₂ was added then centrifuge at 15,000 rpm for 20 minutes. The pellet was re-suspended in 1.5 ml of double distilled (DD) and filtered-sterilized water to remove the MgCl₂. After another centrifugation, the pellet was suspended in 50 µl of DD filtered-sterilized water and stored at -20°C until required (Mizuno and Kageyama 1978).

2.7.11.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Resolving gels were made at a 12% concentration of acrylamide: bisacrylamide (37:5:1) (Bio-Rad, UK) by mixing 4.8 mL of 40% acrylamide, 4 mL of 2 M Tris-HCl (pH 8.8), 6.9 mL of distilled water, 160 μ L of 10% SDS, 160 μ L of 10% ammonium persulphate (APS). Polymerisation of acrylamide was prepared by adding 16 μ L N,N,N',N'-tetramethylethylenediamine (TEMED) (Bio-Rad), the gel solution was cast between two glass plates in the apparatus and then layered with isopropanol equilibrated with saturated aqueous sodium chloride. Once gel polymerisation was complete, the saturated isopropanol was removed and the area at the top of the gel was dried with filter paper. The stacking gels were made containing 6% acrylamide concentration by mixing 1.5 mL of 40% of acrylamide, 2.5 mL of 0.5 M Tris-HCl (pH 6.8), 5.8 mL of distilled water, 100 μ L of 10% SDS, 100 μ L of 10% APS. Acrylamide polymerisation was initiated by adding 10 μ L TEMED, the gel solution was overlaid on the resolving gel, and 15-well comb was pushed into the gel to create sample wells. After complete polymerisation, the gel apparatus was assembled in the appropriate tank and covered in 1 x SDS-PAGE running buffer made from 10 x Tris-glycine-SDS buffer in 500 mL of deionised water at pH 8.3; Tris base 15.0 g, Glycine 72.0 g, SDS 5.0 g. The crude outer membrane protein samples were mixed with SDS-PAGE loading buffer at a 6:1 ratio, boiled for 5-10 minutes, and loaded into the wells created in the gel. Electrophoresis was set at 130 volts per centimetre until the blue colour in the loading dye in the sample buffer had reached the end of the gel (approximately 45 to 60 minutes). Outer membrane proteins were visualized by staining the gel for 1 hour with Coomassie brilliant blue R solution, and then destained with destain solution overnight and 2 other changes of destain solution over 2 to 3 hours until the gel was clear. Outer membrane protein bands were compared with standard pre-stained protein ladder (Thermo-Scientific, Leicestershire, UK).

2.8 Plasmid analysis of carbapenem-resistant organisms

2.8.1 Plasmid DNA extractions

All CR isolates were grown in 5 ml each in MHB for overnight. For the overnight broth, 50 µl was used to inoculate fresh MHB and incubated at 37°C in a shaker incubator for 16-18 hours. The plasmid DNA was extracted from the bacterial suspension using QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The eluted plasmid DNA was stored at -20 °C until required.

2.8.2 Purified plasmid DNA and concentration analysis

A NanoDrop® ND-1000 spectrophotometer (NanoDrop, Wilmington, USA) was used to measure the concentration of purified nucleic acid at a spectrum range from 220 to 750 nm. The manufacturer's protocol instructions were followed and used to measure purified plasmid DNA extracted from CR isolates. The spectrophotometer instrument works on a patented sample-retention methodology which relies solely on surface tension to hold up the sample in place. A volume of 1 µl of a DNA extracted sample was aliquoted onto the end of a receiving fibre (fibre-optic cable). A source fibre (second cable) was to bring into contact with the liquid DNA sample in between the gap causing liquid to be read at the fibre optic ends. The controlled gap for both paths was between 0.2 mm and 1 mm. The presence of the sample resulted in the passage of light from a pulsed Xenon flash lamp through the sample, and subsequent analysis of the light intensity by a spectrometer. The absorbance ratio falling at 260 nm and 280 nm was used to assess the DNA purity in the sample, and a ratio ~1.8 was considered as pure in state.

2.8.3 Detection of common β -lactamases and carbapenemase genes in plasmid

DNA extracts

All CR isolates including the numbers which were positive by PCR for carbapenemase genes from total DNA extracts were amplified for the corresponding genes in their plasmid DNA extracts. The primers, reaction mix, PCR cycle conditions, gel preparation and visualisation of PCR bands were set as described in sections 2.7.2 to 2.7.7 and 2.7.10 from total DNA amplification procedures.

2.8.4 Plasmid transfer experiment

To explore the carbapenemase genes located on transferable plasmids, a method described by Khajuria *et al.* (2014) was performed. The resistance pattern was based on inhibition against specific cephalosporins (cefotaxime and cefpodoxime) and carbapenems (imipenem, meropenem and doripenem). Carbapenem-resistant isolates used in this study were used as donor cells while *Escherichia coli* Top10 competent cells (Life Technologies, Carlsbad, USA) as the recipient organism (susceptible to cephalosporins and carbapenems tested). Conjugations were performed by mixing 1 ml of the donor cells to 10 ml of the recipient cells at log-phase in Mueller-Hinton broth (Biotec Ltd, UK) and incubated in a mild shaking incubator at 35°C for 16-18 hours. The transconjugants were selected on Mueller-Hinton agar (Biotec Ltd, UK) with selection based on growth on agar plates in the presence of cefotaxime (2 μ g/ml) and sodium azide (100 μ g/ml) incubated for 48 hours. Donor and recipient cells were killed by sodium azide and cefotaxime agents respectively while the resultant survival colonies were considered as the transconjugants (resistant to both agents), sub-cultured and stored at -80°C. The identity of the transconjugants was confirmed by indole positive test and decreased susceptibility to a panel of β -lactams including carbapenems.

2.8.5 Plasmid curing and restriction enzyme digestion experiment

The plasmid curing technique was by physical method of incubation at 45°C described by Di Martino *et al.* (1997). The method was used to investigate the 26 PCR-positive carbapenemase isolates for curing. The isolates were inoculated in 5 ml of Mueller-Hinton broth (Biotec Ltd, UK) each in duplicate. One broth culture was incubated at 37°C while the other at a higher temperature of 45°C overnight for plasmid curing. The curing was confirmed by loss of plasmid and antimicrobial susceptibility testing using antibiotics to which isolates were resistant. Digestion reactions was carried out in 50 µl reaction volumes with 20 µl or 1 µg of each plasmid DNA extract by simple boiling a suspension of the cured isolates in double-distilled water for 15 minutes then mixed with 20 µl of *EcoRI* and *HindIII* restriction enzymes (Invitrogen) in digest buffer and incubated in a water bath at 37°C for digestion to take place for 4 hours according to the protocol of instructions. The content was analysed by electrophoresis on a 1% agarose gel prepared with 1 x TAE buffer (40mM 56 Tris base, 20mM acetic acid, 1mM EDTA) at 100 volts per centimetre for 2 hours.

2.9 Molecular typing of carbapenem-resistant isolates by ERIC-PCR

A total number of CR isolates (111) was subjected to enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) amplification using total DNA extractions with the following primers: ERIC₁R (5'-ATGTAAGCTCCTGGGGATTAC-3') and ERIC₂F (5'-AAGTAAGTGACTGGGGTGAGCG-3'). Preparation of primer set and PCR reaction mix composition (Table 2.2) were followed as indicated in sections 2.7.2 and 2.7.3 respectively. PyroMark Master Mix Kit (Qiagen, Hilden, Germany) was used with 2 µl of DNA extract each in 25 µl final reaction volume. Based on using the standard ERIC-PCR annealing temperature at 52°C for majority of microbial pathogens, no amplified band was visible on agarose gel electrophoresis, rather upon several trials at different temperatures, 45°C annealing temperature proved to be useful for band size estimates and genotype analysis in this study. Amplification conditions were as follows: denatured for 15 minutes at 95°C and then subjected to 45 cycles of denaturation for 30 seconds at 94°C, annealing for 45 seconds at 45°C, extension for 7 minutes at 72°C, and a final extension for 10 minutes at 72°C.

2.9.1 Electrophoresis and computer analysis of gene sequence and ERIC-PCR data

The amplified products were prepared and electrophoresed as described in sections 2.7.5 and 2.7.6. Gene Ruler 1 kb DNA Ladder Plus (Invitrogen, Carlsbad, USA) was used as a molecular marker standard. The presence of coralload dye in the reaction mixture required no loading dye again for the amplified PCR products. Each well was loaded with 10 µl of test samples including the molecular marker standard and electrophoretic set was run at 100 volts per centimetre for 45 minutes. Gel images were digitally photographed and documented.

2.9.2 Sequence data and analysis of phylogenetic trees

The phylogenetic data were evaluated using the Neighbor-Joining method (Saitou and Nei 1987). The evolutionary history and taxa analysis were taken from 500 replicates to prepare the bootstrap consensus tree (Felsenstein 1985). Less than 50% of the replicates collapse and conform to divisions of the corresponding branches. The evolutionary distances were measured using the maximum composite likelihood technique (Tamura, Nei and Kumar 2004) and these are calculated in units of the number of base substitutions per position. The analysis was based on 14 nucleotide sequenced data. Codon sites were included, first + second + third and noncoding areas. All ambiguous sites were removed for each sequence pair. There were 490 sites in the final analysis. Phylogenetic data were conducted in Molecular Evolutionary Genetics Analysis version 6.0 (MEGA6) (Tamura *et al.* 2013).

2.9.3 ERIC-PCR genetic relatedness analysis

With the complexity of diverse band sizes, the formation of clustering analysis could not have been assessed by extrapolation alone. For more discriminating interpretations of gel electrophoretic images by ERIC-PCR products, specifically written software designed by the GelCompar II evaluation software (Applied Maths, Kortrijk, Belgium) was sourced through the website (<http://www.applied-maths.com/download/software>) before a full featured license granted for a month (2/10/2015 until 1/11/2015) to generate computer-assisted images for the dendrogram data analysis for this study. Clustering information of different CR Gram-negative bacteria with control strains were determined by random enterobacterial repetitive intergenic consensus-PCR (ERICPCR) fingerprint patterns using Gel Compar II image analysis software and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster method.

3. Phenotypic and genotypic characterisation of carbapenem-resistant isolates

3.1 Background and Aims

Carbapenemase producing bacteria have limited treatment options and are difficult to manage by clinicians in health care facilities due to the presence of ESBLs, hydrolysis of β -lactams, including carbapenem antimicrobials, by β -lactamase enzymes, use of inactive transpeptidases, and expulsion of the antibiotic through efflux pumps (Walsh *et al.* 2005, Wilke, Lovering and Strynadka 2005, Temkin *et al.* 2014). In recent times, carbapenemase producing genes have gained worldwide recognition as they can spread across different strains and species. The enzymes have been characterised as various classes of carbapenemases including the most common types: the New Delhi metallo- β -lactamases (NDM), verona integron-encoded metallo- β -lactamases (VIM), oxacillinase-48 (OXA-48), imipenem-resistant *Pseudomonas*-1 (IMP-1) and *Klebsiella pneumoniae* carbapenemases (KPCs) with determinant encoding genes expressed as; *bla*NDM, *bla*VIM, *bla*OXA-48, *bla*IMP, and *bla*KPC, respectively (Nordmann, Naas, Poirel 2011).

Recently, a few African countries have extensively investigated carbapenemase producers among the Enterobacteriaceae group to find out the prevailing types and distribution in their locations such as in South Africa (Coetzee and Brink 2011), Kenya (Poirel *et al.* 2011b), Morocco (Hays *et al.* 2012), Tanzania (Mushi *et al.* 2014), Nigeria (Ogbolu and Webber 2014) and Uganda (Okoché *et al.* 2015). More importantly, in

terms of resistance to carbapenems, in sub-Saharan regions there is limited data available to ascertain the extent of the spread of carbapenemase gene carriers. Current studies show there is a lack of interest, poor implementation of surveillance studies and an unsystematic approach to carry out research studies on carbapenemase-producing bacteria in this part of the sub-region (Manenzhe *et al.* 2014). Recently, a nationwide laboratory based antimicrobial resistance study carried out in Ghanaian health care facilities by Opintan *et al.* (2015) reported no data on the prevalence and distribution of CRE, despite the fact that there are increased numbers of multidrug-resistant organisms in the country (Opintan *et al.* 2015). Previous studies attested to the high incidence of multidrug resistance among common pathogens attributed to the unregulated use of antimicrobials and improper diagnosis of patients' infections in most developing countries (Newman *et al.* 2011), Nigeria (Ogbolu *et al.* 2011) and Rwanda (Ntirenganya *et al.* 2015).

Outbreaks of CRE infections are considered a serious threat to patient care and tracing the source of transmission in hospitals from infected patients is a cumbersome task to undertake in any facility. A study by Sahin *et al.* (2015) recommended early detection of CRE organisms from colonised patients as an important step in curbing infections and its spread. Clinical manifestations of CRE organisms are similar to infections caused by susceptible pathogens, however, detection methods are more critical in reducing their dissemination within hospitals. The problem arises when interpreting the susceptibility testing results of an organism that has demonstrated resistance to a carbapenem via disc diffusion methodology while proving susceptible once its MIC is determined by alternative methods. This may complicate interpretation in a clinical laboratory with consequences for public health. Therefore, for meaningful detection, more comprehensive methods are needed to detect either carbapenemase gene producers

or carbapenemase non-PCR positives to avoid any ambiguities in reporting results to clinicians (Sahin *et al.* 2015). The aim of this study was to apply various techniques to identify carbapenem-resistant Gram-negative organisms and their resistance genes from the hospital environment.

The objectives were to:

- Identify carbapenem-resistant bacteria using various phenotype based techniques
- Determine the prevalence and distribution of carbapenemase producers from various clinical specimens
- Investigate the presence of ESBL by cefpodoxime E-test and nitrocefin assay
- Detect carbapenemase-producing isolates using PCR-based assay
- Determine the presence of outer membrane proteins of carbapenemase non-PCR positives and carbapenemase gene producers by SDS-PAGE
- Determine the phylogenetic relationships of isolates PCR-positive for carbapenemase genes

3.2 Method summary

Suspected CR isolates were collected between the months of September 2012 through September 2014 from four selected regional hospitals in Ghana. Identification of bacterial strains was by both conventional and automated systems (Vitek 2) to obtain a species level of identification of organisms. Disc diffusion assays and MIC determination using E-test strips for imipenem, meropenem and ertapenem were performed. The presence of ESBL enzyme was determined by combined disc assay using cefpodoxime with or without clavulanic acid and a nitrocefin assay. Outer

membrane protein analysis was performed using SDS-PAGE where extractions of *Pseudomonas* isolates were differently treated from those in the Enterobacteriaceae group and *Acinetobacter baumannii* isolates because of the specific efflux pump systems exhibited in the cell wall structure in order to visualise the different porin levels within each bacterial cell.

A modified Hodge test and boronic-acid disc synergy test were also carried out as additional phenotype-based methods on strains showing reduced susceptibility and complete inhibition to carbapenems. Total DNA was extracted directly from all CR isolates for molecular characterisation of carbapenemase genes with five common primers by PCR-based assay and sequenced purified positive carbapenemase gene producers to determine phylogenetic relationships with controlled strains compared to established strains from the GenBank database. Full description is in Chapter two of this study.

3.3 Results

3.3.1 CR isolates demographics and characteristics

The study identified 111 non-duplicated CR isolates from 55 male and 56 female patients with ages between 3 days and 91 years (Appendix 1). Of the 111 isolates, 51 were *Pseudomonas aeruginosa*, 31 *Acinetobacter baumannii*, 12 *Escherichia coli*, 7 *Pseudomonas putida*, 3 each for *Klebsiella pneumoniae* and *Enterobacter cloacae*, and one each for *Cronobacter sakazakii*, *Providencia stuartii*, *Shigella sonnei* and *Sphingomonas paucimobilis*, respectively from ten different sample collection sites (Tables 3.1 and 3.2).

3.3.2 Resistance pattern of CR isolates

The study revealed the lowest frequency of resistance to imipenem (58%), followed by meropenem (66.7%) and doripenem (75%) by the disc diffusion method with corresponding minimum inhibitory concentrations evaluated using the E-test method (BioMérieux, France). In the MIC investigations, doripenem was replaced by ertapenem, due to its good selectivity for community-acquired pathogens, notably *Pseudomonas aeruginosa* mutants, and also detect cross-resistance better with meropenem and imipenem than doripenem. *Providencia stuartii* was the only CR isolate which showed 100% susceptibility to meropenem while *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Cronobacter sakazakii*, *Shigella sonnei* and *Sphingomonas paucimobilis* isolates were 100% resistant to imipenem, meropenem and doripenem, respectively. The highest measurable MIC levels were observed in 56.8% of CR isolates at ≥ 32 $\mu\text{g/ml}$ by the E-test method of which 24.3% and 18.9% were detected in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates, respectively. Susceptibility testing results in table 3.3 were based on ≥ 23 mm as susceptible, 20-22 mm as intermediate and ≤ 19 mm as resistant for all carbapenems. However, figure 3.1 shows E-test thresholds of ≥ 32 $\mu\text{g/ml}$ for resistance to the 3 carbapenems (imipenem, meropenem, ertapenem), levels ranging from ≥ 16 to ≤ 4 $\mu\text{g/ml}$ were resistant to 1 or 2 of the carbapenems, and levels at < 4 $\mu\text{g/ml}$ showed intermediate resistance to either 1 or 2 of carbapenems tested. Details of these resistant isolates are shown in Table 3.3 and Figure 3.1.

Table 3.1 Distribution pattern of carbapenem-resistant organisms by age groups, sex and source of specimens

	<i>P. aeruginosa</i>	<i>A. baumannii</i>	<i>E. coli</i>	<i>P. putida</i>	<i>En. cloacae</i>	<i>K. pneumoniae</i>	<i>C. sakazakii</i>	<i>P. stuartii</i>	<i>S. sonnei</i>	<i>S. paucimobilis</i>
Age (years)										
0-9	15	9	3	2	1	0	0	0	0	0
10-19	4	4	1	0	1	0	0	0	0	0
20-29	4	5	2	3	0	0	1	0	0	1
30-39	7	5	2	0	1	1	0	0	0	0
40-49	2	0	2	1	0	1	0	1	0	0
50-59	7	1	2	0	0	0	0	0	0	0
60-69	3	2	0	1	0	1	0	0	1	0
70-79	7	3	0	0	0	0	0	0	0	0
80-89	2	1	0	0	0	0	0	0	0	0
90-99	0	1	0	0	0	0	0	0	0	0
Sex										
Female	17	21	9	3	2	2	1	1	0	0
Male	34	10	3	4	1	1	0	0	1	1
Specimen										
Wound (47)	24	13	5	1	1	1	1	1	0	0
Urine (31)	16	14	6	2	2	1	0	0	0	1
Sputum (7)	4	1	0	1	0	1	0	0	0	0
Ear (4)	1	0	0	3	0	0	0	0	0	0
Aspirate (5)	2	3	0	0	0	0	0	0	0	0
CSF (2)	2	0	0	0	0	0	0	0	0	0
Stool (1)	0	0	0	0	0	0	0	0	1	0
Catheter tip (1)	1	0	0	0	0	0	0	0	0	0
HVS (1)	0	0	1	0	0	0	0	0	0	0
Eye (1)	1	0	0	0	0	0	0	0	0	0

Numbers indicate the total number of CR isolates in each category

Table 3.2 Distribution pattern of carbapenem-resistant organisms from selected hospitals in Ghana

	<i>P. aeruginosa</i>	<i>A. baumannii</i>	<i>E. coli</i>	<i>P. putida</i>	<i>En. cloacae</i>	<i>K. pneumoniae</i>	<i>C. sakazakii</i>	<i>P. stuartii</i>	<i>S. sonnei</i>	<i>S. paucimobilis</i>
Hospital										
KBTH, GA/R (93) ^a	45 (48.4%)	28 (30.1%)	10 (10.8%)	4 (4.3%)	2 (2.2%)	1 (1.1%)	1 (1.1%)	1 (1.1%)	0 (0.0%)	1 (1.1%)
ENRH, W/R (6)	2 (33.3%)	3 (50.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (16.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
AGAMH, A/R (7)	4 (57.1%)	0 (0.0%)	1 (14.3%)	1 (14.3%)	0 (0.0%)	1 (14.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
HRH, V/R (5)	0 (0.0%)	0 (0.0%)	1 (20.0%)	2 (40.0%)	1 (20.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (20.0%)	0 (0.0%)
Total number 111(%)	51 (46)	31 (28)	12 (11)	7 (6.3)	3 (2.7)	3 (2.7)	1 (0.9)	1 (0.9)	1 (0.9)	1 (0.9)

^a Total number of CR isolates obtained from the hospital;

Note: KBTH, GA/R= Korle Bu Teaching Hospital, Greater Accra Region; ENRH, W/R= Effia-Nkwanta Regional Hospital, Western Region; AGAMH, A/R= AngloGold Ashanti Mines Hospital, Ashanti Region; HRH, V/R= Ho Regional Hospital, Volta Region

Table 3.3 Resistance pattern of carbapenem-resistant isolates against carbapenems

Bacterial species	Antimicrobial agents								
Number of CR isolates	Imipenem % for each group of CR isolates			Meropenem % for each group of CR isolates			Doripenem % for each group of CR isolates		
	S	I	R	S	I	R	S	I	R
51 <i>P. aeruginosa</i>	11.8	5.9	82.3	0.0	0.0	100.0	2.0	0.0	98.0
31 <i>A. baumannii</i>	00.0	9.7	90.3	0.0	0.0	100.0	0.0	0.0	100.0
12 <i>E. coli</i>	16.7	25.0	58.3	0.0	33.3	66.7	0.0	25.0	75.0
7 <i>P. putida</i>	14.3	0.0	85.7	0.0	0.0	100.0	0.0	0.0	100.0
3 <i>K. pneumoniae</i>	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0
3 <i>En. cloacae</i>	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0
1 <i>C. sakazakii</i>	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0
1 <i>P. stuartii</i>	0.0	0.0	100.0	100.0	0.0	0.0	0.0	0.0	100.0
1 <i>S. sonnei</i>	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0
1 <i>S. paucimobilis</i>	0.0	0.0	100.0	0.0	0.0	100.0	0.0	0.0	100.0

Note: CR= carbapenem-resistant, S= susceptible, I= intermediate, R= resistant

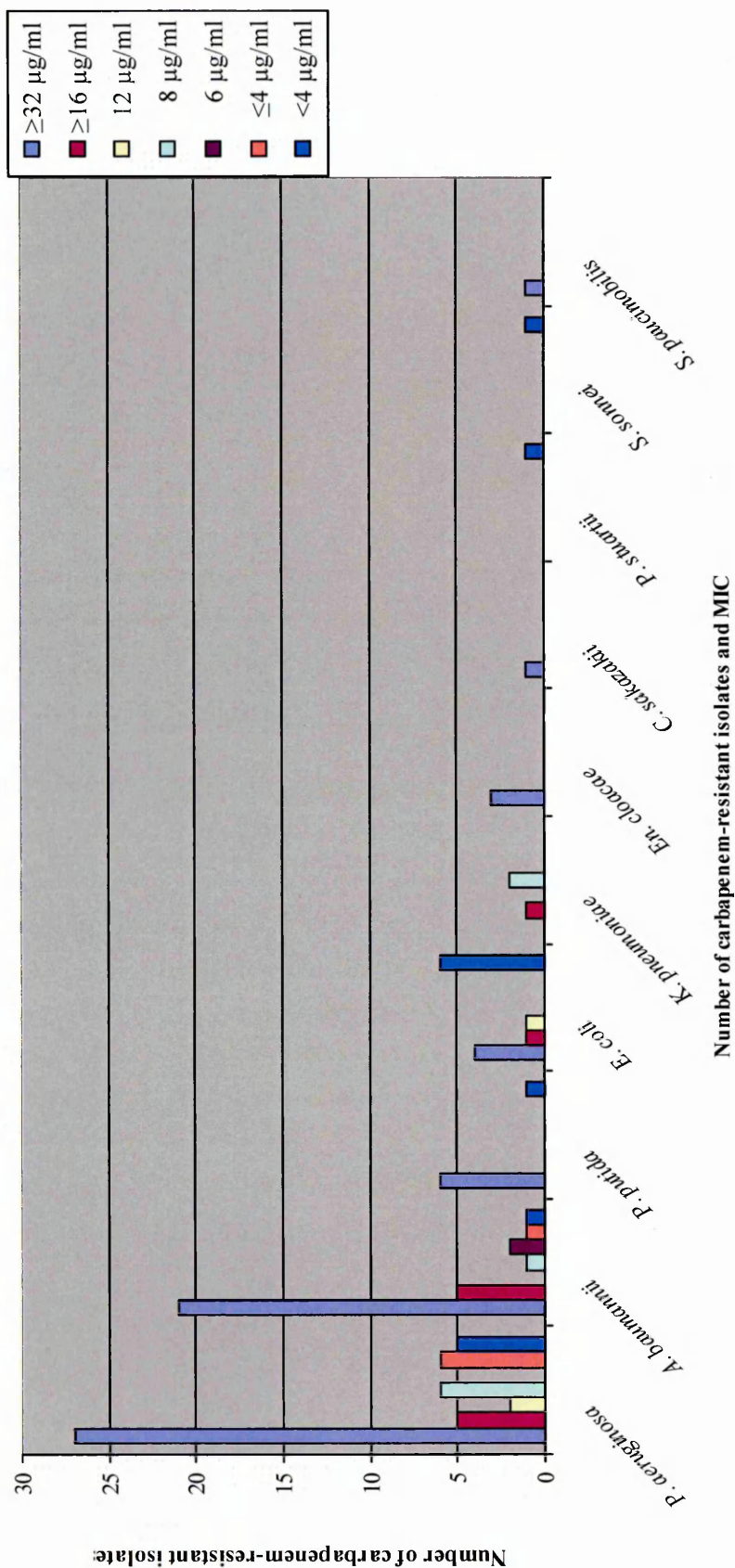


Figure 3.1 Carbapenem-resistant isolates and E-test MIC against carbapenems

3.3.3 ESBL determination by cefpodoxime and nitrocefin assay

Cefpodoxime with and without clavulanic acid was used to screen for ESBL as described in Chapter 2 (Section 2.5.2). Of all the CR isolates, none showed ESBL activity with the test when the results were interpreted according to Livermore and Woodford (2004) established guidelines for cefpodoxime ESBL determination. A difference of ≥ 5 mm in ratio for either cefpodoxime combination with clavulanic acid compared to the same disc when tested alone (Livermore and Woodford 2004). Readings measured in millimetres were between zero and three for all the CR isolates including *Klebsiella pneumoniae* (NCTC 13438) carbapenemase-positive (KPC-positive) and *Escherichia coli* (ATCC 25922) as positive and negative control strains, respectively (see Appendix 2). These results indicated that cefpodoxime disc and clavulanic acid were not good indicators for determining ESBL activity in CR isolates. In contrast, MIC determination using the E-test method was more discriminating ranging from 8 to ≥ 256 $\mu\text{g/ml}$ to cefpodoxime for ESBL detection (see Appendix 2).

The nitrocefin assay for detection of β -lactamase activity was conducted as described in Section 2.5.3. The assay was also considered non-specific since colour intensity of β -lactamase activity was determined from the sample within 2 to 22 minutes. All CR isolates were measured against a blank sample and negative and positive β -lactamase controls to determine a cut-off point based on the background β -lactamase activity. A sample that showed a measurable colour development at the same condition and time with the positive control was considered as positive β -lactamase test. The nitrocefin assay was controlled with KPC-positive (NCTC 13438) strain as a positive control which yielded measurable quantity of β -lactamase activity comparable to all the CR isolates studied. In all, β -lactamase activity for the 26 carbapenemase producers was

from 84% to 174% compared with the negative control. The presence of β -lactamase activity in the test extracts showing more than 46% is considered positive for nitrocefin assay. Details of these results are shown in Table 3.4.

3.3.4 Prevalence of carbapenemase detection based on phenotypic tests

From this study, phenotypic identification for carbapenemase activity was revealed in 21 of 111 CR isolates (18.9%) by the modified Hodge test (MHT) method and 3/111 (2.7%) by boronic acid-disc synergy test (BADST) screen. A total of 21.6% exhibited carbapenemase production by both methods. None of the CR isolates were positive for both MHT and BADST phenotypic test. The three *Enterobacter cloacae* isolates were the only isolates which showed positivity from the BADST screen. Details of the carbapenemase activity among the CR isolates are shown (Table 3.5, Figures 3.2 and 3.3).

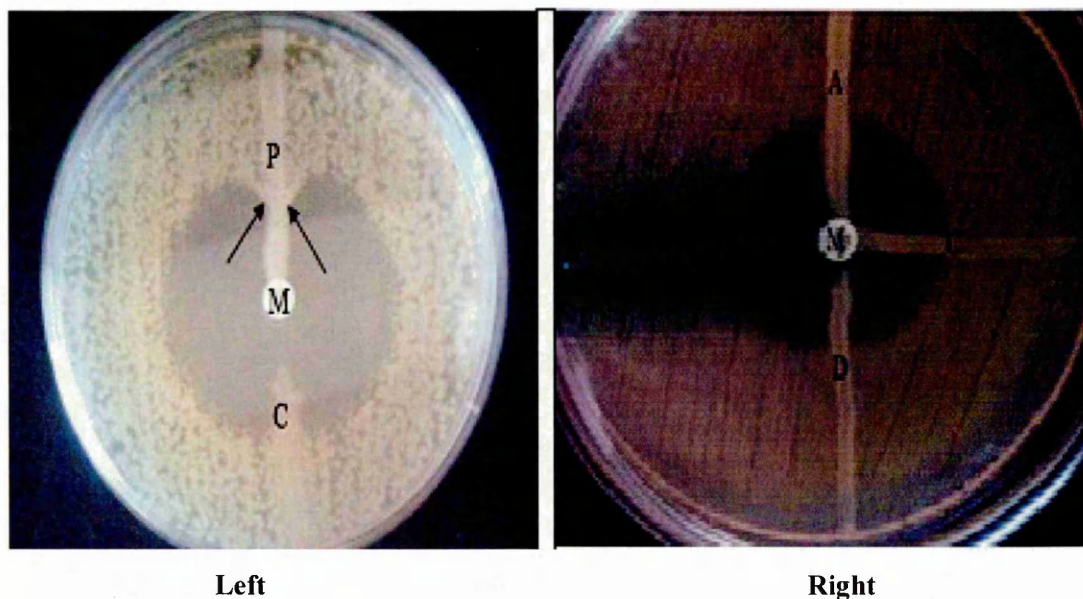


Figure 3.2 Positive and negative modified Hodge test on Mueller-Hinton agar plate

Left: M= meropenem disc, P= positive modified Hodge test (arrowed clover-leaf like indentations), C= negative *Escherichia coli* ATCC 25922 control strain

Right: M= meropenem disc; A, B, C & D= carbapenem-resistant organisms showing no clover-leaf like indentations

Table 3.4 Detection of β -lactamase activity using nitrocefin assay

Sample Number	Average A_{390/486} 2 - 22 min linear range	Ratio against Kpc-positive control	Relative % activity in comparison to Kpc-positive control
1	0.510499998	0.817834931	81.78349
2	0.575563631	0.922068648	92.20686*
3	0.539854537	0.864861705	86.48617
4	0.548899997	0.879352778	87.93528
5	0.555518177	0.889955284	88.99553*
6	0.570063629	0.913257494	91.32575*
7	0.567218174	0.908698997	90.8699*
8	0.553281817	0.886372572	88.63726
9	0.570809093	0.914451748	91.44517
10	0.591654539	0.947846721	94.78467
11	0.598890901	0.95943957	95.94396
12	0.527181815	0.844559658	84.45597*
13	0.625409083	1.001922422	100.1922
14	0.613027275	0.982086427	98.20864
15	0.555863635	0.890508716	89.05087
16	0.54130908	0.867191922	86.71919
17	0.564954536	0.905072587	90.50726
18	0.57553637	0.922024975	92.2025
19	0.562490908	0.901125788	90.11258*
20	0.579454547	0.928302	92.8302
21	0.611599998	0.979799891	97.97999
22	0.559299995	0.896013858	89.60139
23	0.613327265	0.982567019	98.2567
24	0.520481809	0.833826064	83.38261
25	0.620736355	0.994436584	99.44366
26	0.568572727	0.910869028	91.0869
27	0.572400001	0.917000426	91.70004*
28	0.584799994	0.936865553	93.68656
29	0.543463637	0.87064358	87.06436
30	0.592763635	0.949623524	94.96235
31	0.533481815	0.854652429	85.46524
32	0.585745459	0.938380214	93.83802
33	0.56287273	0.901737477	90.17375
34	0.617990911	0.990038308	99.00383
35	0.520345444	0.833607605	83.36076
36	0.574809096	0.920859862	92.08599
37	0.534554547	0.856370975	85.6371
38	0.601818177	0.964129146	96.41291

Sample Number	Average A ₄₉₀ 2 - 22 min linear range	Ratio against Kpc-positive control	Relative % activity in comparison to Kpc-positive control
39	0.562563631	0.901242292	90.12423*
40	0.609718182	0.97678517	97.67852
41	0.629872723	1.009073295	100.9073
42	0.597136367	0.95662876	95.66288*
43	0.594772724	0.952842138	95.28421*
44	0.571963635	0.916301356	91.63014
45	0.548518186	0.878741107	87.87411
46	0.572172723	0.91663632	91.66363*
47	0.571427275	0.915442092	91.54421*
48	0.573954544	0.919490846	91.94908
49	0.599745458	0.960808594	96.08086
50	0.750145452	1.201753489	120.1753*
51	0.844909083	1.353567413	135.3567
52	0.74403636	1.191966558	119.1967
53	0.775781821	1.242823653	124.2824
54	0.665109087	1.065522912	106.5523
55	0.55108182	0.882848118	88.28481
56	0.739799998	1.18517979	118.518
57	0.968872726	1.552160553	155.2161
58	0.736945445	1.180606718	118.0607
59	0.848936368	1.360019234	136.0019
60	0.726718176	1.164222355	116.4222*
61	0.727609093	1.165649627	116.565
62	0.744336361	1.192447168	119.2447*
63	0.766090908	1.227298545	122.7299
64	0.798309093	1.278912958	127.8913*
65	0.769827274	1.233284306	123.3284
66	0.77927273	1.248416184	124.8416
67	0.729054543	1.167965278	116.7965
68	0.68872727	1.103359887	110.336
69	0.694172724	1.112083651	111.2084
70	0.970218182	1.55431601	155.4316
71	0.612518175	0.981270836	98.12708
72	0.770909087	1.2350174	123.5017*
73	0.764745458	1.225143097	122.5143
74	0.768218187	1.230706504	123.0707*
75	0.734899998	1.177329856	117.733
76	0.652163641	1.044783954	104.4784*
77	0.826881826	1.324687256	132.4687
78	0.582818183	0.933690638	93.36906

Sample Number	Average A ₄₉₀ 2 - 22 min linear range	Ratio against Kpc-positive control	Relative % activity in comparison to Kpc-positive control
79	0.769536365	1.232818262	123.2818
80	0.683127273	1.094388538	109.4389
81	1.092072731	1.749530324	174.953
82	0.876345461	1.403929349	140.3929
83	1.004709081	1.609571372	160.9571
84	0.760381818	1.218152427	121.8152*
85	0.674281819	1.08021788	108.0218
86	0.760672737	1.218618488	121.8618
87	0.951972718	1.525086278	152.5086
88	0.889636365	1.42522174	142.5222
89	0.670027272	1.073401979	107.3402*
90	0.765445449	1.226264503	122.6265
91	0.768681824	1.231449264	123.1449
92	0.97350909	1.559588134	155.9588
93	0.940027269	1.505949344	150.5949
94	0.632163633	1.012743395	101.2743
95	0.88874546	1.423794485	142.3794
96	0.673409099	1.078819758	107.882
97	1.040681806	1.66720066	166.7201
98	0.778554542	1.247265628	124.7266*
99	0.802754543	1.28603469	128.6035
100	0.976545448	1.564452463	156.4452*
101	0.796218195	1.275563281	127.5563
102	0.771590916	1.236109708	123.611*
103	0.772954545	1.23829428	123.8294
104	0.919490923	1.473049556	147.305
105	0.738954549	1.183825359	118.3825*
106	0.809799991	1.29732169	129.7322
107	0.806545453	1.29210783	129.2108
108	1.086909094	1.741258036	174.1258*
109	0.896536361	1.436275722	143.6276
110	0.810845446	1.298996536	129.8997
111	0.752799993	1.206006137	120.6006
+ve control (Kpc)	0.62420909	0.999512656	99.9513*
-ve control (Ec)	0.00110000	0.461100000	46.1100

= carrier of carbapenemase genes by PCR technique*

Note: +ve control (Kpc)= *Klebsiella pneumoniae* carbapenemase (NCTC 13438) positive control strain, -ve control (Ec)= *Escherichia coli* (ATCC 25922) negative control strain. All CR isolates indicating more than 46% were considered nitrocefin positive as this was the value recorded for the negative control.

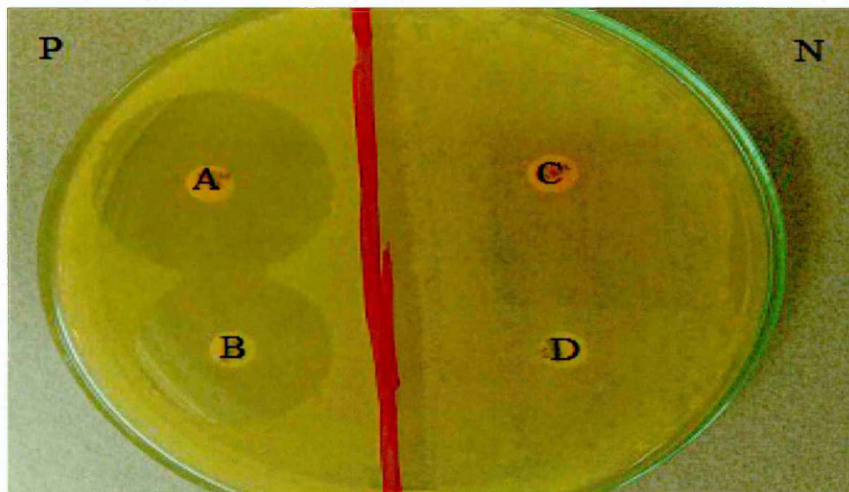


Figure 3.3 Two boronic acid-disc synergy tests on Mueller-Hinton agar plate

Note: P= positive boronic-acid disc synergy test {A= meropenem disc + boronic acid (31mm), B= meropenem disc alone (24mm)}; N= negative boronic-acid disc synergy test {C= meropenem disc + boronic acid (6mm), D= meropenem disc alone (6mm)}

3.3.5 Phenotypic and genotypic correlation of carbapenem-resistant isolates

Of the 21 MHT positive isolates none were detected by BADST screen as positive. Out of these, 11.7% (13/111) were *Acinetobacter baumannii* CR isolates of which 7.2% were carbapenemase positive, followed by 4.5% for *Pseudomonas aeruginosa* and 1.8% for *Klebsiella pneumoniae* isolates. Surprisingly, there were only two *Klebsiella pneumoniae* positive OXA-48 producers (1.8%) and one positive NDM-1 *Acinetobacter baumannii* isolate (0.9%) detected by PCR-based assay. Carbapenemase resistance genes, VIM-1, KPC-1 and IMP-1 were undetected by MHT as carbapenemase-producing isolates. A correlation was observed between MHT positivity and PCR assay for OXA-48 gene positivity. The only remaining CR *Klebsiella pneumoniae* isolate showed negative result for MHT, BADST as well as KPC resistance gene in this study. There was no correlation between BADST positivity and PCR-based results (Table 3.5).

3.3.6 Distribution and prevalence of carbapenemase-producing genes

Based on the PCR assay, 26/111 (23.4%) of genomic DNA extracts were carriers of PCR-positive carbapenemase genes of which 14.4% were *bla*NDM-1, 7.2% *bla*VIM-1 and 1.8% for *bla*OXA-48 genes. The highest prevalence was from *Acinetobacter baumannii* (9 NDM-1 positives) and *Pseudomonas aeruginosa* (2 NDM-1 and 7 VIM-1), followed by *Escherichia coli* (3 NDM-1), *Klebsiella pneumoniae* (2 OXA-48), one VIM-1 for *Pseudomonas putida* isolate, and one each NDM-1 for *Providencia stuartii* and *Shigella sonnei*, respectively. No carbapenemase encoding genes were found in *Enterobacter cloacae*, *Cronobacter sakazakii* and *Sphingomonas paucimobilis*.

In this study, females harboured more NDM-1 genes (*Acinetobacter baumannii* isolates) in ages between (3 days and 9 years) than the male counterparts in scattered age groups. However, more positive VIM-1 genes were detected in *Pseudomonas aeruginosa* isolates than any other strains from males between the ages of 50 and 59 years. None of the positive VIM-1 *Pseudomonas aeruginosa* isolates were detected from the female counterparts in all the year groups. Details of carbapenemase activity by phenotype-based assays, distribution and prevalence of carbapenemase resistance genes are shown in Tables 3.5, 3.6 while Figures 3.4 and 3.5 depict carbapenemase resistance gene distribution in the four hospitals, age groups, both sexes and source of specimens among the CR isolates. Clearly, phenotype-based tests were less efficient in detecting carbapenemase activity considering the percentage sensitivity and specificity values when compared with the PCR-based assay results obtained in this study (Figure 3.5). The percentage values were calculated using Pasteran *et al.* (2010) calculation formulas described in Chapter 2, section 2.6.3.

Table 3.5 Distribution of presumptive carbapenemase producers in phenotypic and genotypic assays among carbapenem-resistant isolates

Bacterial isolate		Phenotypic test		Type of gene				
Organism	CR total	MHT	BADST	KPC-1	NDM-1	VIM-1	OXA-48	IMP-1
<i>P. aeruginosa</i>	51	5	-	-	2	7	-	-
<i>A. baumannii</i>	31	13	-	-	9	-	-	-
<i>E. coli</i>	12	-	-	-	3	-	-	-
<i>P. putida</i>	7	-	-	-	-	1	-	-
<i>K. pneumoniae</i>	3	3	-	-	-	-	2	-
<i>En. cloacae</i>	3	-	3	-	-	-	-	-
<i>C. sakazakii</i>	1	-	-	-	-	-	-	-
<i>P. stuartii</i>	1	-	-	-	1	-	-	-
<i>S. sonnei</i>	1	-	-	-	1	-	-	-
<i>S. paucimobilis</i>	1	-	-	-	-	-	-	-
Total	111	21	3	-	16	8	2	-

Note: - = not found

Table 3.6 Prevalence of carbapenemase resistance genes and the tested assays

Carbapenem-resistant isolate (n) ^a	Number (%) by:				
	MHT (n=21)		^b BADST (n=3)		PCR amplification assay
Carbapenemase genes (26)	SE	SP	SE	SP	
NDM-1 (16)	(6.3) ^c	(78.9)	(0)	(0)	16 (100)
VIM-1 (8)	(0)	(79.6)	(0)	(0)	8 (100)
OXA-48 (2)	(100) ^d	(82.6)	(0)	(0)	2 (100)
IMP-1 (0)	(0)	(0)	(0)	(0)	0 (0)
KPC-1 (0)	(0)	(0)	(0)	(0)	0 (0)

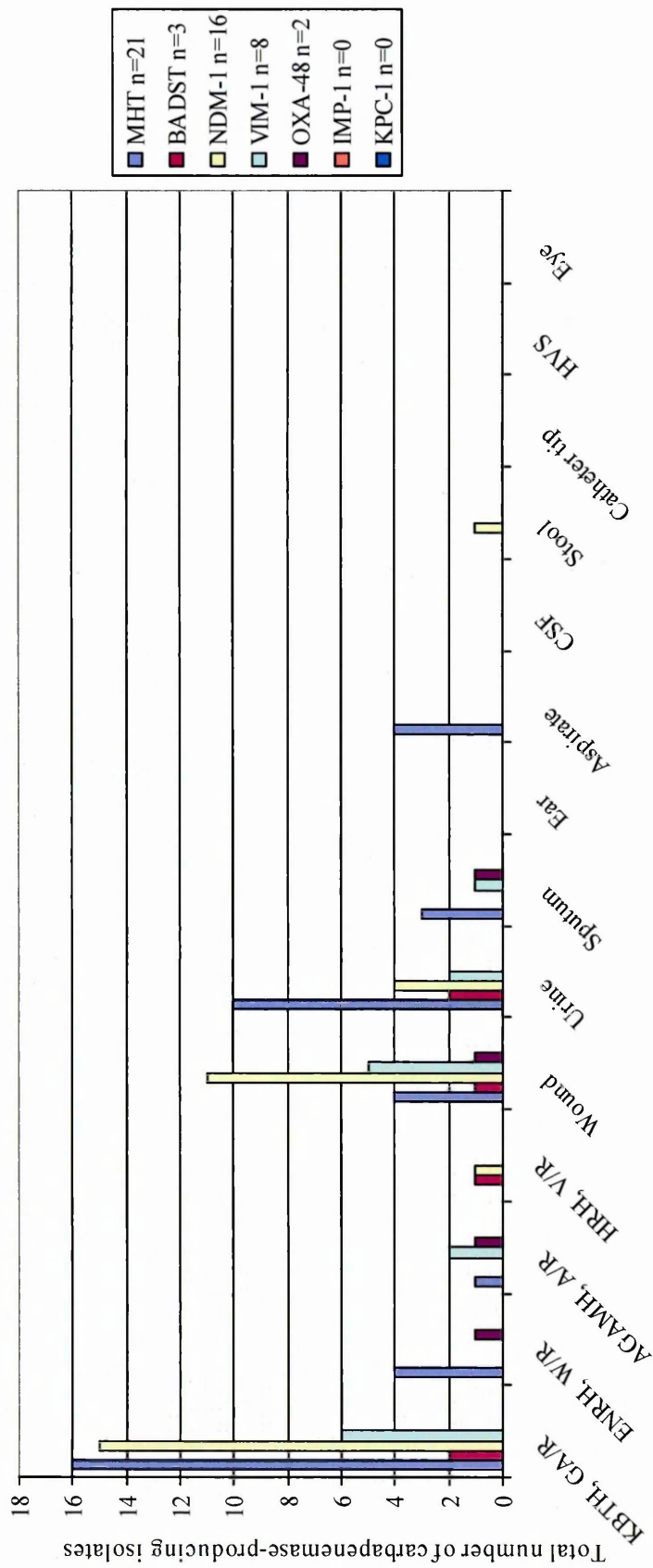
^a Total number of CR isolates (111)

^b None was detected by PCR assay

^c Only MHT positive (n=1) was detected by PCR assay

^d All MHT positives (n=2) were detected by PCR assay

Note: MHT= modified Hodge test, BADST= boronic acid-disc synergy test, n= number, SE= sensitivity, SP= specificity



Selected hospitals and type of specimens collected

Figure 3.4 Distribution of carbapenemase resistance genes among hospitals and type of specimens

Note: n= total number, KBTH, GA/R= Korle Bu Teaching Hospital, Greater Accra Region; ENRH, W/R= Effia-Nkwanta Regional Hospital, Western Region; AGAMH, A/R= AngloGold Ashanti Mines Hospital, Ashanti Region; HRH, V/R= Ho Regional Hospital, Volta Region

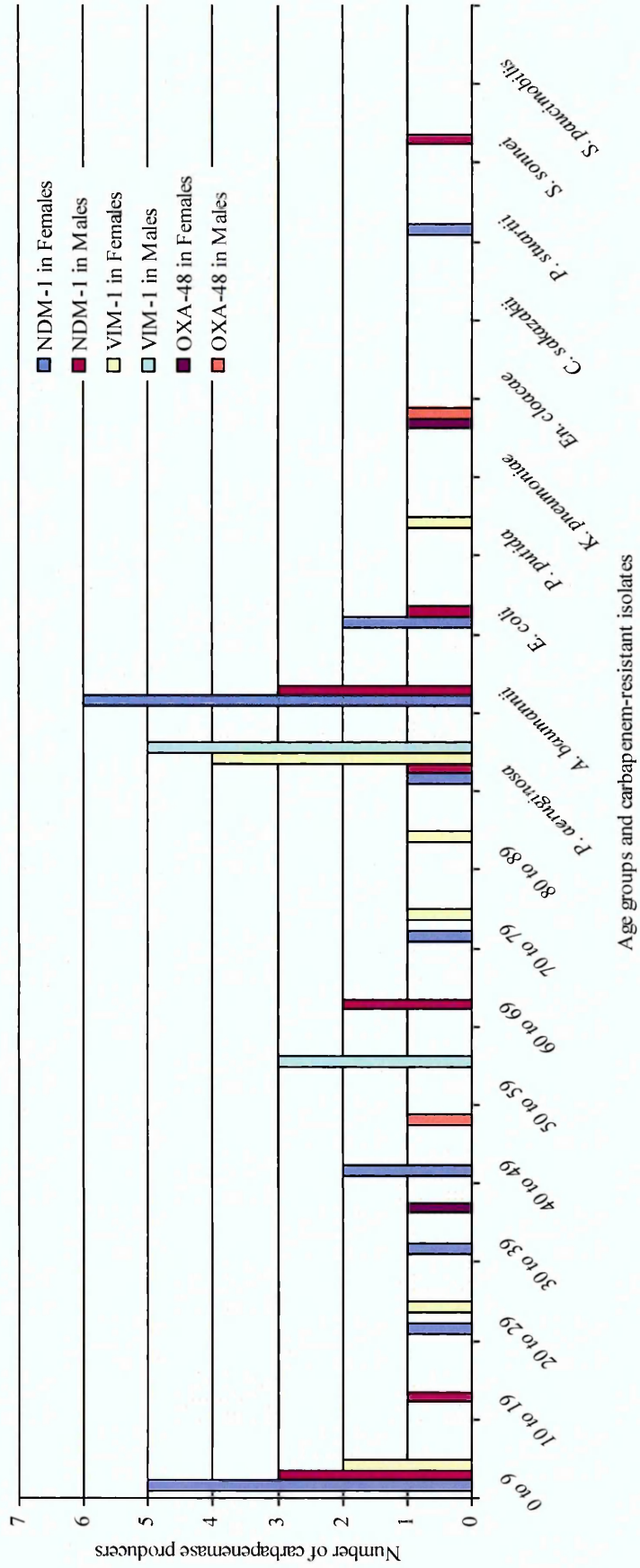


Figure 3.5 Distribution of carbapenemase-positive genes in both sexes, among age groups and carbapenem-resistant isolates

Note: Despite the differences in CR isolates, source of specimens and demographic information obtained from the four hospitals studied, one of the carbapenemase resistance genotypes was detected in each hospital in this study (Figures 3.4. and 3.5)

3.3.7 Analysis of outer membrane proteins by SDS-PAGE

All CR extracted samples were performed as described in Chapter 2, sections 2.7.11.1 and 2.7.11.2. The isolated fractions were subjected to SDS-PAGE investigations (Section 2.7.11.3) to observe the differences of protein profiles. In all, the levels of outer membrane proteins were varied because of diverse CR isolates studied. A study conducted on the OMPs of *Pseudomonas aeruginosa* strain by Hancock and Brinkman (2002) was adapted and used (Table 3.7) for the analysis of this study.

Table 3.7 Important outer membrane proteins of *Pseudomonas aeruginosa* strain

Gene	Protein function and name	Number of AAs	Known porin (class) ^a
<i>OprC</i>	Putative copper transport porin OprC	723	GP
<i>OprD</i>	Basic amino acid, basic peptide and imipenem porin OprD; also named Porin D, Protein D2	443	SP, P
<i>OprF</i>	Major porin and structural porin OprF; Porin F	350	P
<i>OprG</i>	Outer-membrane protein OprG	232	
<i>OprH</i>	PhoP/Q and low Mg ²⁺ -inducible outer-membrane protein H1	200	GP
<i>OprI</i>	Outer-membrane lipoprotein OprI	83	EP
<i>OprJ</i>	Multidrug efflux protein OprJ	479	EP
<i>OprM</i>	Major intrinsic multiple antibiotic resistance efflux protein OprM	485	EP

(Adapted from Hancock and Brinkman 2002)

^aP, general porin; SP, specific porin; GP, putative gated porin; EP, OprM family member of efflux and protein secretion porins

In support of Hancock and Brinkman (2002), a recent study by Sugawara and Nikaido (2012) explicitly described that the major proteins in the outer membrane of *Acinetobacter baumannii* (not in the high-permeability trimeric porin group) relates to *Escherichia coli* OmpF/OmpC and shares close homology with *Pseudomonas aeruginosa* OprF and *Escherichia coli* OmpA (Sugawara and Nikaido 2012). In this study, in-house *Pseudomonas aeruginosa* control strains from the Sheffield Hallam University (SHU) collection, MBL-positive (SHU: 2048, VIM positive) and wild-type MBL-negative (SHU: 2054, VIM negative) isolates were used alongside the 111 isolates identified to analyse various expression of porins, specifically OprD and OprF in all the CR isolates on SDS-PAGE (Tables 3.8, 3.9 and 3.10).

Of the control strains, the wild type showed absence of porin band (~44 kDa) on SDS-PAGE (Figure 3.6) and was compared with the CR isolates on SDS-PAGE which expressed different OMP levels (Figure 3.7 and Appendix 3). The important OMPs for *Pseudomonas aeruginosa* isolate including imipenem-resistance determinant porin OprD in addition to OprM/J, OprF and OprH/G are shown in Table 3.8. Of the 51 CR *Pseudomonas aeruginosa* isolates, 48 (94.1%) reduction or loss of OprD was observed, including the VIM-1 carbapenemase positive-producers while 3 with moderate to high-level of porin expression were observed. For the 31 *Acinetobacter baumannii* isolates, 30 (96.8%) showed significantly diminished OmpA or OprF porin expression (Table 3.9), which is similar to the OprF of *Pseudomonas aeruginosa* isolate. Similar analysis of OMPs in the Enterobacteriaceae group (Table 3.10) confirmed that the two OXA-48 positive *Klebsiella pneumoniae* isolates were deficient of a major 35-kDa OmpC, the 3 *Enterobacter aerogenes* and 10 *Escherichia coli* isolates lacked a major 37-kDa OmpF, and the 2 *Escherichia coli* isolates had decreased expression of a major 37-kDa OmpF, which is also highly similar to *Pseudomonas aeruginosa* OprF. For all the CR isolates,

a detailed analysis of major porins in relation to carbapenem MIC levels is shown in the Appendix 4.

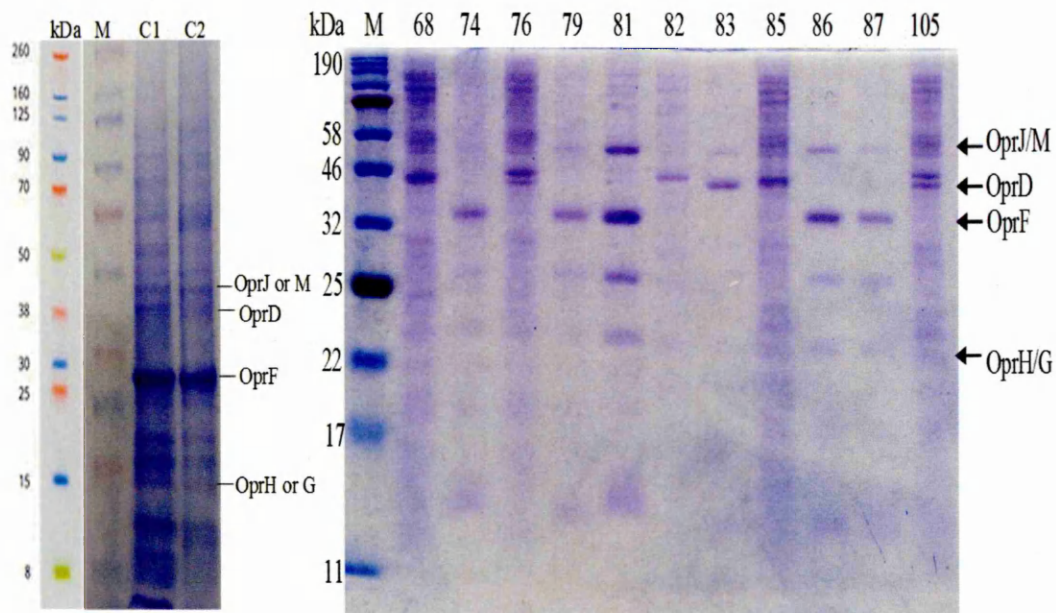


Figure 3.6 Outer membrane proteins of control strains and selected carbapenem-resistant isolates on SDS-PAGE

Note: M= protein marker in kDa; C1= Control SHU 2048 VIM positive *Pseudomonas aeruginosa* strain; C2= Control SHU 2054 MBL negative *Pseudomonas aeruginosa* strain; 68-87 & 105= selected samples; *OprM/J* (48/49kDa)= major intrinsic multidrug efflux proteins; *OprD* (44kDa)= specific for β -lactam imipenem resistance; *OprF* (35kDa)= major porin for structural stabilisation of peptidoglycan; *OprH/G* (22/23kDa)= polycationic antimicrobial uptake and putatively in iron uptake. Band corresponding to *OprD* in C2 strain is showing a reduced or loss of expression

Table 3.8 Analysis of outer membrane proteins among *Pseudomonas* isolates on

SDS-PAGE

Isolate code	Name of isolate	Common outer membrane proteins of importance			
		<i>OprM/J</i>	<i>OprD</i>	<i>OprF</i>	<i>OprG/H</i>
1	<i>P. aeruginosa</i>	+	+++	++	+
3	<i>P. aeruginosa</i>	-	+	-	-
6*	<i>P. aeruginosa</i>	-	+	-	-
7*	<i>P. aeruginosa</i>	-	-	-	-
10	<i>P. aeruginosa</i>	-	-	-	-
12*	<i>P. putida</i>	-	-	-	-
13	<i>P. aeruginosa</i>	-	-	-	-
14	<i>P. aeruginosa</i>	-	-	+	-
15	<i>P. aeruginosa</i>	-	+	+	+
16	<i>P. aeruginosa</i>	-	+	++	+
17	<i>P. aeruginosa</i>	-	-	-	-
21	<i>P. aeruginosa</i>	-	-	+	-
24	<i>P. aeruginosa</i>	-	-	+	-
25	<i>P. aeruginosa</i>	-	-	+	-
26	<i>P. aeruginosa</i>	-	-	-	-
28	<i>P. aeruginosa</i>	-	+	+++	+
31	<i>P. aeruginosa</i>	-	+	-	-
32	<i>P. aeruginosa</i>	-	-	+	-
37	<i>P. aeruginosa</i>	-	+	-	+
39*	<i>P. aeruginosa</i>	-	-	-	-
40	<i>P. aeruginosa</i>	-	+	+	-
41	<i>P. aeruginosa</i>	-	+	-	-
44	<i>P. putida</i>	-	-	-	-
47*	<i>P. aeruginosa</i>	-	-	-	-
48	<i>P. aeruginosa</i>	-	-	+	-
52	<i>P. aeruginosa</i>	-	-	-	-
54	<i>P. aeruginosa</i>	-	-	-	-
57	<i>P. aeruginosa</i>	-	-	-	-
58	<i>P. aeruginosa</i>	-	-	-	-
59	<i>P. aeruginosa</i>	+	++	++	-
60*	<i>P. aeruginosa</i>	-	+	-	-
61	<i>P. aeruginosa</i>	-	-	-	-
62*	<i>P. aeruginosa</i>	-	+	-	-
63	<i>P. aeruginosa</i>	-	-	-	-
65	<i>P. aeruginosa</i>	-	-	-	-
66	<i>P. aeruginosa</i>	-	-	-	-
70	<i>P. aeruginosa</i>	-	+	-	+
71	<i>P. aeruginosa</i>	-	+	-	+
74*	<i>P. aeruginosa</i>	-	-	++	+
78	<i>P. putida</i>	+	-	-	-
79	<i>P. aeruginosa</i>	+	-	++	-

Isolate code	Name of isolate	Common outer membrane proteins of importance			
		<i>OprM/J</i>	<i>OprD</i>	<i>OprF</i>	<i>OprG/H</i>
80	<i>P. aeruginosa</i>	-	-	-	-
81	<i>P. aeruginosa</i>	+++	-	+++	+
83	<i>P. putida</i>	+	++	-	-
84*	<i>P. aeruginosa</i>	-	-	-	-
86	<i>P. aeruginosa</i>	+	-	+++	+
87	<i>P. aeruginosa</i>	-	-	++	-
88	<i>P. putida</i>	+	-	-	-
90	<i>P. putida</i>	-	-	-	-
92	<i>P. putida</i>	-	-	-	-
96	<i>P. aeruginosa</i>	-	-	-	-
99	<i>P. aeruginosa</i>	++	++	+++	+++
100*	<i>P. aeruginosa</i>	-	-	+	-
101	<i>P. aeruginosa</i>	-	-	-	-
104	<i>P. aeruginosa</i>	-	+	-	-
109	<i>P. aeruginosa</i>	-	+	-	-
110	<i>P. aeruginosa</i>	+	+	+++	-
111	<i>P. aeruginosa</i>	+	+	+++	-

* Carrier of carbapenemase genes by PCR assay

Note: +++= high level of protein, ++= moderate level of protein, += low/reduced level of protein, -= no amount of protein

Table 3.9 Analysis of outer membrane proteins among isolates of *Acinetobacter****baumannii* on SDS-PAGE**

Isolate code	Name of isolate	Common outer membrane proteins of importance			
		<i>OprM/J</i>	<i>OprD</i>	<i>OprF</i> ^a	<i>OprG/H</i>
4	<i>A. baumannii</i>	-	-	-	-
8	<i>A. baumannii</i>	-	-	-	-
20	<i>A. baumannii</i>	-	-	-	-
22	<i>A. baumannii</i>	-	-	-	-
27*	<i>A. baumannii</i>	-	-	-	-
29	<i>A. baumannii</i>	-	-	-	-
30	<i>A. baumannii</i>	-	-	-	-
34	<i>A. baumannii</i>	-	-	-	-
36	<i>A. baumannii</i>	-	-	-	-
38	<i>A. baumannii</i>	-	-	-	-
42*	<i>A. baumannii</i>	-	+	-	-
43*	<i>A. baumannii</i>	-	-	-	-
45	<i>A. baumannii</i>	-	-	-	-
46*	<i>A. buamannii</i>	-	-	-	-
49	<i>A. baumannii</i>	-	-	-	-
50*	<i>A. baumannii</i>	-	-	-	-
51	<i>A. baumannii</i>	-	-	-	-
56	<i>A. baumannii</i>	+++	++	++	-
64*	<i>A. baumannii</i>	-	-	-	-
69	<i>A. baumannii</i>	+	-	-	-
72*	<i>A. baumannii</i>	-	-	-	-
73	<i>A. baumannii</i>	-	-	-	-
75	<i>A. baumannii</i>	-	-	-	-
77	<i>A. baumannii</i>	-	-	-	-
82	<i>A. baumannii</i>	-	+	-	-
94	<i>A. baumannii</i>	-	-	-	-
102*	<i>A. baumannii</i>	-	-	-	-
103	<i>A. baumannii</i>	-	+	-	-
106	<i>A. baumannii</i>	-	+	-	+
107	<i>A. baumannii</i>	-	-	-	-
108*	<i>A. baumannii</i>	-	+	-	+

*Carrier of carbapenemase genes by PCR assay

^a *OprF* in *Pseudomonas* isolate is synonymous to *OmpA* in *Acinetobacter baumannii*

Note: +++= high level of protein, ++= moderate level of protein, += low/reduced level of protein, -= no amount of protein

Table 3.10 Analysis of outer membrane proteins among the Enterobacteriaceae isolates on SDS-PAGE

Isolate code	Name of isolate	Common outer membrane proteins of importance			
		<i>OprM/J</i>	<i>OprD</i>	<i>OprF</i> ^a	<i>OprG/H</i>
2*	<i>E. coli</i>	+++	+	-	-
5*	<i>E. coli</i>	+	+	-	-
9	<i>C. sakazakii</i>	-	-	-	-
11	<i>S. paucimobilis</i>	-	-	-	-
18	<i>E. coli</i>	-	-	-	-
19*	<i>K. pneumoniae</i>	-	-	-	-
23	<i>E. coli</i>	-	-	-	-
33	<i>En. cloacae</i>	-	-	-	-
35	<i>E. coli</i>	-	-	-	-
53	<i>E. coli</i>	-	-	-	-
10	<i>K. pneumoniae</i>	-	-	-	-
67	<i>E. coli</i>	-	-	-	-
68	<i>E. coli</i>	++	+++	-	+
76*	<i>P. stuartii</i>	++	-	-	+
85	<i>E. coli</i>	++	+++	-	+
89*	<i>S. sonnei</i>	-	-	-	-
91	<i>En. cloacae</i>	-	-	+	-
93	<i>E. coli</i>	-	-	+	-
95	<i>En. cloacae</i>	+	+	+	+
97	<i>E. coli</i>	-	-	-	-
98*	<i>K. pneumoniae</i>	-	-	-	-
105*	<i>E. coli</i>	++	+++	-	+

*Carrier of carbapenemase genes by PCR assay

^a *OprF* in *Pseudomonas* isolate is synonymous to *OmpC/F*

Note: +++= high level of protein, ++= moderate level of protein, += low/reduced level of protein, -= no amount of protein

3.3.8 Sequenced data and phylogenetic analysis of carbapenemase positive genes

The sequence data obtained from Eurofins Genomics (Ebersberg, Germany) of the three different carbapenemase genes (OXA-48, VIM-1 and NDM-1) identified in this study, including controls, were searched for their corresponding nucleotide homologies using BLAST available at the National Centre for Biotechnology Information (NCBI) website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The sequences were evaluated against known

sequences which yielded >97% identity with previously characterised CR bacteria in the GenBank database.

3.3.9 Phylogenetic relationships of carbapenemase genes

Phylogenetic trees were constructed using the Neighbor-Joining technique (Saitou and Nei 1987), where evolutionary history and taxa were taken from 500 replicates to prepare the bootstrap consensus tree confidence limits (Felsenstein 1985). Less than 50% of the replicates collapse and conform to divisions of the corresponding branches. The developed distances were measured using the maximum composite likelihood (MCL) technique (Tamura, Nei and Kumar 2004) and calculated in units of the number of base substitutions per position. The phylogenetic trees were generated using sequenced data of 19 nucleotides for OXA-48, 16 for VIM-1, and 30 for NDM-1 in the analysis. Codon sites included were first + second + third and noncoding areas. Indistinct and ambiguous sites were removed for each sequence pair. There were 326, 392 and 447 sites for OXA-48, VIM-1 and NDM-1, respectively, and the data were analysed using molecular evolutionary genetics analysis software version 6 (MEGA6) (Tamura *et al.* 2013). Details of the phylogenetic relationships are shown in Figures 3.7, 3.8 and 3.9.

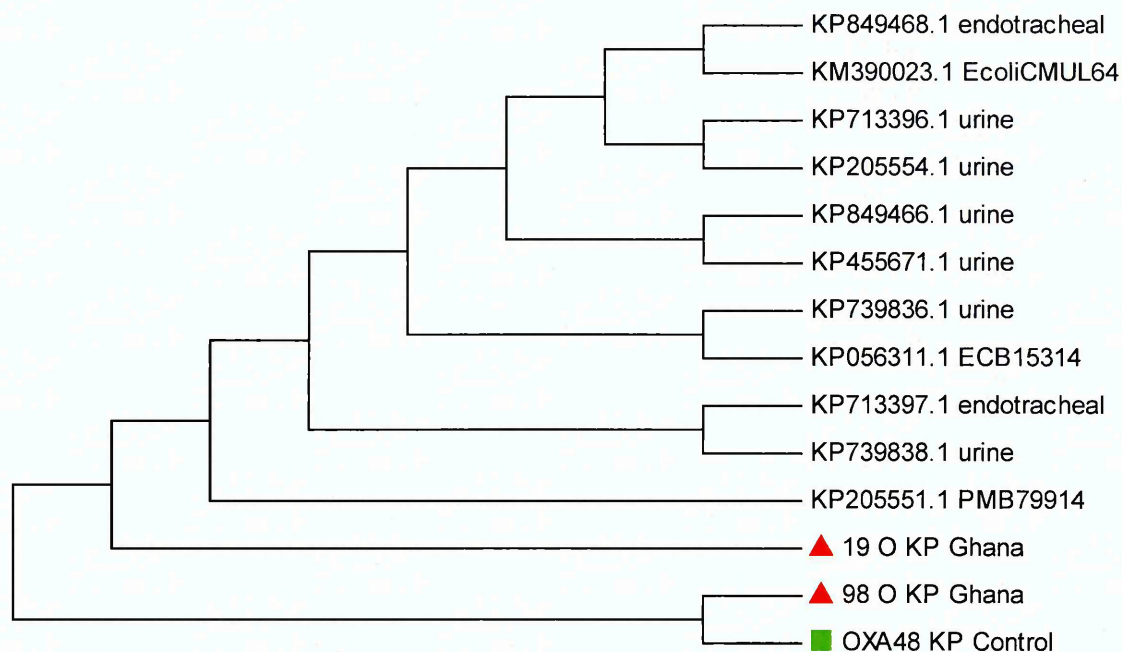


Figure 3.7 Phylogenetic trees for OXA-48 positive gene with OXA-48 genes of known sequenced organisms

Note: 'O' attached to isolate number depicts OXA-48, Red= OXA-48 carriers from Ghana, Green= control strain NCTC 13442 (OXA-48 +ve) *Klebsiella pneumoniae* strain, Uncoloured= established GenBank strains. Clusters of Ghanaian OXA-48 positive *Klebsiella pneumoniae* isolates are unique which have no link when compared with the cluster-grouped strains from the GenBank.

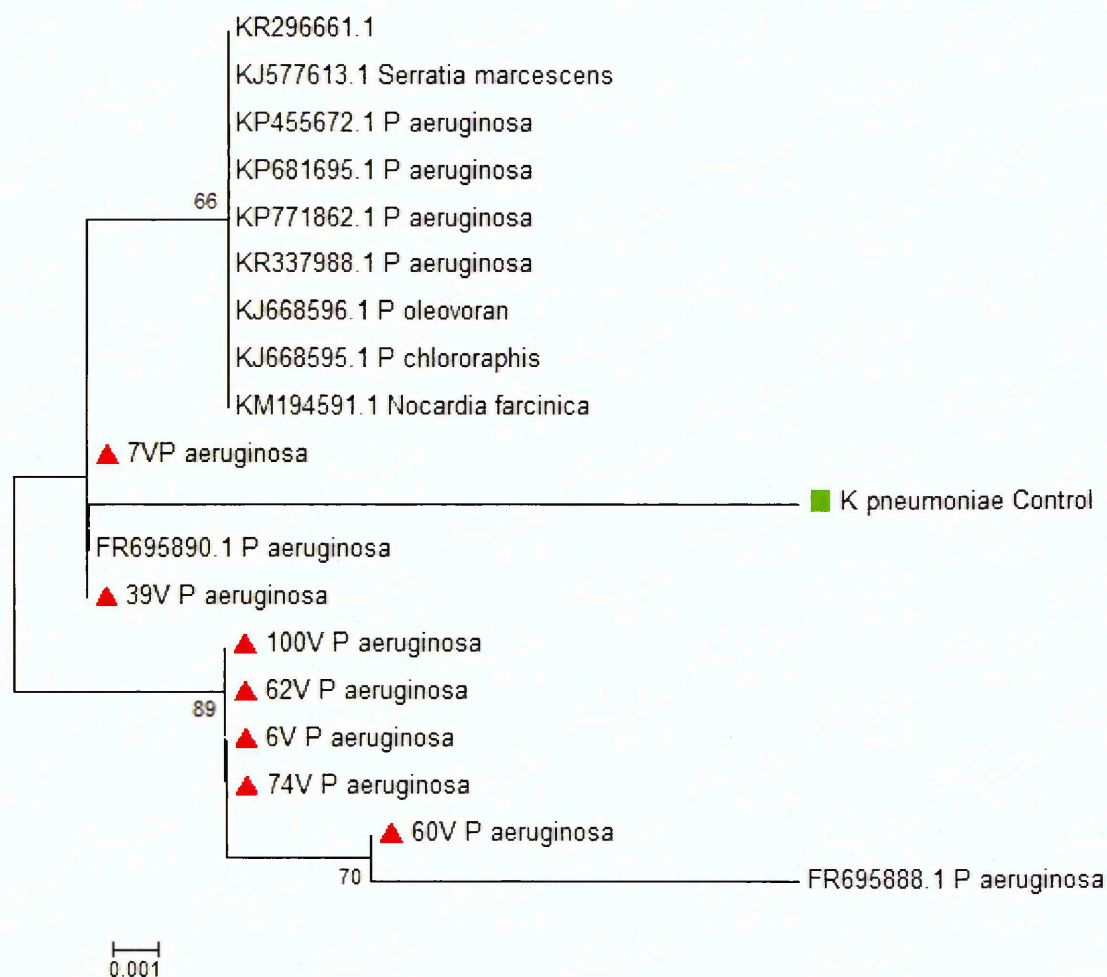


Figure 3.8 Phylogenetic trees for VIM-1 positive gene with VIM-1 genes of known sequenced organisms

Note: 'V' attached to isolate number depicts VIM-1, Red= VIM-1 carriers of carbapenemase gene from Ghana, Green= VIM-1 (NCTC 13439) *Klebsiella pneumoniae* control strain, Uncoloured= established GenBank strains. Clusters of Ghanaian VIM-1 positive *Pseudomonas* isolates are unique and have no link when compared with the cluster-grouped strains from the GenBank

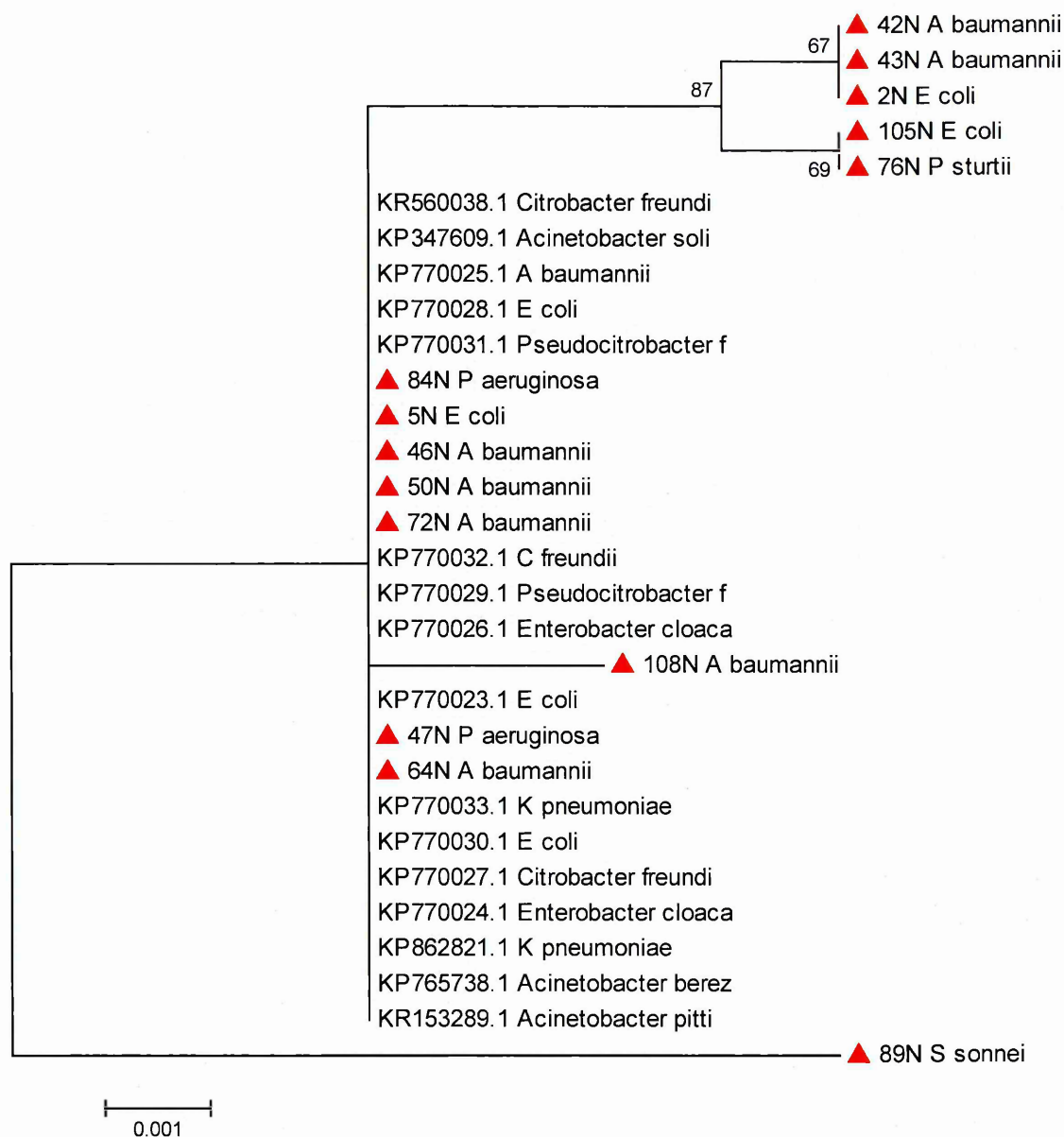


Figure 3.9 Phylogenetic trees for NDM-1 positive gene with NDM-1 genes of known sequenced organisms

Note: 'N' attached to isolate number depicts NDM-1 gene, Red= carriers of carbapenemase gene from Ghana, Uncoloured= established GenBank strains. Clusters of diverse isolates from Ghana indicating NDM-1 gene carriers are unique and have no link when compared with the cluster-grouped strains from the GenBank

3.4 Discussion

Regrettably, to date not a single search study for the detection of carbapenemase resistance genes has been performed in Ghana, despite World Health Organisation awareness campaign launched in 2011 for member-countries to curb antimicrobial resistance (WHO 2015). This study sought to quantify the presence, prevalence and distribution of carbapenemase producers, and to find out the types of carbapenemase genes emerging among the CR isolates from four selected hospitals for the first time in Ghana.

To date, no reference studies have been carried out on carbapenemase producers in Gram-negative bacteria in Ghana, even in the most recent laboratory-based nationwide study on antimicrobial resistance (Opintan *et al.* 2015). Lack of interest or opportunity due to logistical problems which constantly persist when carrying out molecular research, non-availability of common tools, reagents, control strains and primers are some of the difficulties that discourages researchers. Nonetheless, comparable molecular studies have been extensively carried out in Ghana, mostly hospital-based research studies on clonal lineage of serogroup B *Salmonella* by PCR using enterobacterial repetitive intergenic consensus (ERIC) technique and repetitive element (REP)-PCR and also a detection of *mecA* gene in methicillin-resistant *Staphylococcus aureus* isolates (Mills-Robertson *et al.* 2003, Egyir *et al.* 2014).

This study had an overall carbapenem-resistant prevalence of 2.9% (111 of 3840) from total Gram-negative bacterial pathogens while 23.4% (26/111) of the CR isolates were PCR positive for known carbapenemase genes. It is worth noting that carbapenem antimicrobials are excluded from the Ghana Ministry of Health essential drug list (Ghana Essential Medicines List 2010) following strict adherence to antimicrobial

usage policy. However, the effectiveness of meropenem and its extended use for children was later recommended for patients in hospitals with serious infections. Presumably, the low-level usage of carbapenems may have contributed to this remarkably low prevalence rate of resistance in the country's hospitals. The present overall prevalence rate of carbapenem resistance was extremely low when compared with a similar study in tertiary hospitals in Nigeria where, 5.5% (10/182) carbapenemase-producers were carrying recognised carbapenemase genes (Ogbolu and Webber 2014), mainly, MBL carbapenemases (OXA-48 and KPC genes among Enterobacteriaceae group were not detected).

With the enforcement policy on antimicrobial usage without prescription in the country, this present study reported much higher carbapenem resistance with the disc diffusion susceptibility testing than expected. The highest carbapenem resistance was 100% to both meropenem and doripenem in five different CR isolates (*Klebsiella pneumoniae*, *Enterobacter cloacae*, *Cronobacter sakazakii*, *Shigella sonnei* and *Sphingomonas paucimobilis*) and 90.3% only to imipenem for *Acinetobacter baumannii* isolates. Resistance to the three carbapenems (imipenem, meropenem and doripenem), 85.7% for *Pseudomonas putida* and 82.3% for *Pseudomonas aeruginosa* isolates were observed. Within the CR isolates studied, only *Escherichia coli* CR isolates that showed the lowest prevalence in the phenotypic resistance, 75% for doripenem, 66.7% for meropenem and 58.3% for imipenem. Similarly, previous studies on carbapenem resistance in two separate areas showed substantially decreased rates, 9.3% (Akinduti *et al.* 2012) and 40.0% (Motayo *et al.* (2013) carried out within the boundaries of unregulated usage of antimicrobials in Nigeria.

In MIC E-test assays, 56.8% of CR isolates showed complete resistance to imipenem, meropenem and ertapenem at ≥ 32 $\mu\text{g/ml}$, of which 24.3% were found in *Pseudomonas aeruginosa* isolates and 18.9% in *Acinetobacter baumannii* isolates. Ertapenem resistance of 2.7% was for carriers of carbapenemase-producing genes in Enterobacteriaceae isolates but all were susceptible *in vitro* to meropenem. The findings confirmed the Anderson *et al.* (2007) observation that *in vitro* ertapenem resistant strains may remain susceptible to other carbapenems (Anderson *et al.* 2007). However, for non-fermenting strains coded with carbapenemase genes, 14.4% ertapenem resistance was found to be susceptible *in vitro* than to imipenem in this study.

In this present study, sensitivity and specificity of phenotype based tests (MHT and BADST) showed no corresponding results with the molecular assay (PCR-based) employed (see Table 3.6). Determination was calculated using the formulas (Section 2.6.3) in Chapter 2 designed by Pasteran *et al.* (2010) to evaluate the reliability of MHT and BADST when compared with the PCR-based results obtained for carbapenemase resistance genes (Table 3.5). Thus, the MHT method performed poorly with the calculation for Class B MBL-containing isolates. However, a 100% sensitivity score for serine type (OXA-48) was confirmed by PCR assay. There was only MHT positivity for two OXA-48-producing *Klebsiella pneumoniae* isolates and one NDM-1-producing *Acinetobacter baumannii* (metallo- β -lactamase type). Though, no KPC-1-producing isolate was detected to evaluate sensitivity of MHT technique. Sensitivity for NDM-1 was only 6.3%. This is consistent with previous studies by Nordmann, Naas and Poirel (2011) and Doyle *et al.* (2012) who confirmed low sensitivity rates of less than 12% for MBL detection, partly due to false-positivity as a result of ESBLs co-existing with porin loss in association of pAmpC mimicking carbapenemase activity. Previous studies by

Galani *et al.* (2008) and Hara *et al.* (2013) also confirmed MHT was unable to detect weak VIM and NDM carbapenemase genes, however, the majority of VIM-1 and NDM-1-producing genes were detectable in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* CR isolates, respectively (Galani *et al.* 2008, Hara *et al.* (2013). Due to differences within the continent of Africa, high prevalence rates of carbapenemase activity have been reported in some African countries when the MHT method was used, 33.5% in Nigeria (Yusuf *et al.* 2012) and 22.4% Uganda (Okoche *et al.* 2015) compared with low prevalence of 2.8% observed during an outbreak situation in a Moroccan hospital (El Wartiti *et al.* 2012). Currently, the MHT technique is considered a first line phenotype-based test for investigating carbapenemase producers where six to eight organisms can be evaluated simultaneously on a 90 mm Mueller-Hinton agar plate. In low income countries, the method may be used as initial test for phenotypic screening of OXA-48-like activity in suspected *Klebsiella pneumoniae* CR isolates as confirmed in this study before more specific method can be employed for detection of carbapenemase activity.

The positivity, sensitivity and specificity of boronic acid-based inhibition testing showed limited correlation with the PCR assay results. Pasteran *et al.* (2009) and Tsakris *et al.* (2009) described the boronic acid test as the most specific and sensitive (with meropenem and imipenem discs) for detection of KPC in *Klebsiella pneumoniae* isolates, only when the corresponding organisms existed without the presence of AmpC β -lactamase enzyme (Pastern *et al.* 2009, Tsakris *et al.* 2009). The inconsistency could have come from the presence of metallo- β -lactamases in strains harbouring *AmpC* genes (ACC, CIT, DHA, EBC, FOX and MOX) which according to Hara *et al.* (2013) are largely undetectable by MHT and BADST techniques. Helmy and Wasfi (2014) described the overall sensitivity and specificity of 65% and 74% respectively for

boronic acid tests which is considered relatively low for detection of AmpC β -lactamases in isolates from urinary tract infections. However, to improve phenotypic detection rate of AmpC enzymes, Helmy and Wasfi (2014) suggested the use of AmpC disc test and inhibitor-based test with the use of cloxacillin while Woodford *et al.* (2014) recommended temocillin MIC test for OXA-48-like enzymes. Another method which could have phenotypically helped in detection of Class A and B MBL enzymes was the use of Carba NP (Dortet, Poirel, Nordmann 2012b), though, prior to this study the test reagents were not available for use to improve the rates of detection.

In all, the study detected most of the CR bacterial resistance to carbapenems from wound 47/111 (42.3%) and urine 31/111 (27.9%) samples. Though, the encoding genes were unevenly distributed among seven pathogens, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates were in the majority (Figure 3.5). The magnitude of resistance to carbapenems among Gram-negative bacteria including *Pseudomonas aeruginosa* and *Acinetobacter* species is increasing in numbers worldwide and studies have shown several acquired infections from these organisms in recent times (Saranathan *et al.* 2014, Mathlouthi *et al.* 2015).

In this study, only 21.6% of the CR isolates were confirmed as Class B carbapenemases (NDM-1 and VIM-1 genes) from non-fermenting organisms, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in Ghanaian hospitals. Interestingly, in the age group categories a trend was observed for both the CR isolates and carbapenemase-encoding genes. The age group from 3 days to 9 years were found to be harbouring relatively high numbers of NDM-1 encoding gene in *Acinetobacter baumannii* isolates, mainly in females while in the male counterparts two age groups were observed with a greater

prevalence; 3 days to 9 years and 50 to 59 with higher prevalence of VIM-1-encoding genes in *Pseudomonas aeruginosa* isolates (Figure 3.5). Accordingly, the distribution of carbapenemase producing organisms based on sex, the females recorded significant rise for NDM-1 gene in *Acinetobacter baumannii* when compared with the males having reduced number in *Pseudomonas aeruginosa* each for NDM-1 and VIM-1 genes, respectively. However, much higher values were observed in recent study, 26.9% in males and 23.3% for females based on sex distribution assessment of MBLs in Nigeria (Yusuf *et al.* 2013).

Furthermore, a European surveillance study reported carbapenem resistance of 23.4% in *Pseudomonas aeruginosa* and 20.4% in *Acinetobacter* species (Zarb 2012), while in an earlier worldwide study by Sacha *et al.* (2008) found a multitude of class B carbapenemases in only *Pseudomonas aeruginosa* isolates. In contrast, Class B gene prevalences of 9.9% and 4.5% were significantly higher for NDM-1 and VIM-1 according to this study, from wound swabs, while only 3.6% and 1.8% was observed from urinary specimens for both genes, respectively. Of all the clinical specimens, wound, urine, sputum, aspirates and stool pathogens showed a considerable amount of carbapenemase activity in both phenotype- and genotype-based investigations, while no carbapenemase presence was detected in isolates from cerebrospinal fluid, ear, catheter tip, vaginal and eye samples. Though, other studies reported different pathogens from urine where considerably higher numbers of Class B genes were detected, 27% in France (Nordmann *et al.* 2009), 91.7% in India (Deshpande *et al.* (2010) and in Nigeria 55% was observed only in *Pseudomonas aeruginosa* isolates raising the threat of these resistance genes (Yusuf *et al.* 2013). More recent studies have also shown Class B genes (mainly VIM and IMP) have a similar prominence, 33% of *Pseudomonas aeruginosa*

harboured *bla*VIM gene in Iranian hospital (Aghamiri *et al.* 2014) while 36% of *Acinetobacter baumannii* carried *bla*IMP-1 gene in India (Saranathana *et al.* 2014).

The first ever OXA-48 producing strains to be detected in Ghanaian hospitals were from Effia-Nkwanta Regional hospital in the Western region and AngloGold Ashanti Mines hospital in the Ashanti region of Ghana according to this study. OXA-48 gene was first detected in Turkey in 2004 and had since spread to Middle East and beyond (Carrër *et al.* (2010) including European countries such as Belgium (Cuzon *et al.*, 2008), France (Cuzon *et al.*, 2011) and the Netherlands (Kalpoe *et al.*, 2011). This study observed OXA-48 encoding genes in two *Klebsiella pneumoniae* isolates from a female patient aged 37 years and a 45 year old male patient with wound and sputum infections respectively. A study carried out in Senegal identified the emerging danger of OXA-48 genes in their hospitals and community attributed to movement of the same major OXA-48 encoding plasmid spreading across the borders of Africa and the Middle East (Moquet *et al.* 2011). Notably, none of the CR isolates from Ghana, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* which showed imipenem resistance were positive for OXA-48 encoding gene.

Interestingly, no IMP-1 and KPC-1 genes were detected from Ghanaian CR strains. Detection of IMP-1 producing genes in Africa have been described as rare since its presence have been exclusively and predominantly observed in China, Japan, and Australia (Pitout, Nordmann and Poirel 2015). A study by Lee *et al.* (2014) suggested that success of *bla*KPC spread depended on certain clones of plasmids particularly suited to *Klebsiella pneumoniae* isolates than in other Gram-negative bacterial pathogens. The occurrence was demonstrated to ascertain the peculiar characteristics on

ancestral plasmids that existed among the ST258 strains following transposition and insertion of KPC gene. Nonetheless, *Klebsiella pneumoniae* isolates in Korea with similar multidrug resistant types from Italy and the USA were observed as good vehicles for KPC-encoding genes (Lee *et al.* 2014). Another review on molecular epidemiology of KPC-producing genes reported that extensive recovery were detected only in *Klebsiella pneumoniae* (Munoz-Price *et al.* 2013) and findings have also been confirmed in a recent study describing *Klebsiella pneumoniae* as the most influential species, in its ability to cause antimicrobial resistance and swiftly disseminate (Pitout, Nordmann and Poirel 2015). In this present study, there were only 3 *Klebsiella pneumoniae* CR isolates in which 2 were found to be positive for OXA-48 gene.

Prior to this study, there was strong expectation for KPC-1 gene detection from the collection of Gram-negative bacterial pathogens following the revelation expressed by Chen, Anderson and Paterson (2012) that KPC-encoding genes in particular, were highly mobile, diverse and KPC-producing strains may spread within hospitals as well as in the community environment (Chen, Anderson and Paterson 2012). Apart from being the first to be detected almost two decades ago, its spread has been long expected across Africa and Australia. The dissemination has been extremely slow due to location differences, limited travels and also with contrasting epidemiological patterns in these regions (Chen, Anderson and Paterson 2012). These assertions have been confirmed in an earlier review, which attributed the spread largely through importation of KPC resistance genes through frequent international travels and those seeking medical attention abroad. Importation of carrier isolates has been critical in the dissemination of KPCs after its recovery in 1996 across USA and the world at large (Munoz-Price *et al.* 2013).

Importation of resistance genes has been observed in a few countries in North and South Africa where tourists and migrants travel from abroad for excursions and also seek special medical attention. These countries yearly experienced a number of visits from abroad in the form of tourism and other recreational activities. In this regard, the impact of KPC spread in such countries in Africa has not risen to appreciable heights reflected in the importation pattern, especially in South Africa (Brink *et al.* 2012), Egypt (Metwally *et al.* 2013) and Tanzania (Mushi *et al.* 2014).

Ghana's position on the continent of Africa, south of the Sahara, attracted limited number of people traveling in groups for international tourism and foreigners coming in from abroad to seek medical attention. Notwithstanding, other risk factors such as unregulated, frequent exposure to antimicrobials most especially selective use of carbapenems may have existed in the country. In this present study, regardless of the low number of *Klebsiella pneumoniae* CR isolates obtained, equally important Gram-negative bacterial pathogens including non-fermenters, *Acinetobacter baumannii* and *Pseudomonas* species could have acquired KPC-1 resistance genes. However, no single detectable strain was harbouring the KPC-1 gene. Several of these KPCs have been reported within this Gram-negative population in other countries (Villegas *et al.* 2007, Sacha *et al.* 2009, Robledo *et al.* 2011).

According to Manenzhe *et al.* (2014) detection of carbapenemase resistance genes has been poorly reported in Africa. Studies compiled from numerous articles both published and non-published from databases across Africa revealed prevalence levels of carbapenemase producers in hospitals ranged from 9% to 60% in sub-Saharan Africa with no surveillance data assessment from Ghana and 2.3% to 67.7% in North Africa

(Manenzhe *et al.* 2014). Compared with surveillance studies outside Africa, several rates of carbapenemase producers have been reported ranging from less than 1% for more than twenty countries in Europe including Germany (Poirel *et al.* 2011a, Ehrhard *et al.* 2014) and Spain (Miro *et al.* 2013), 1.3% in Italy, 17% in Cyprus, with much higher levels recorded in Greece 43.5% (Poirel *et al.* 2011a). China has prevalence rate of 33.3% with mainly NDM-1-producing CRE (Qin *et al.* 2014).

The significance of ESBL-types of β -lactamases and differential expression of porins served as a pacesetter for carbapenem resistance in many Gram-negative bacterial pathogens. In achieving resistance, two major mechanisms are responsible; horizontal transfer of plasmid-borne genes and mutation or disruption due to modification of existing genes within the organism. Additionally, non-enzymatic factors such as efflux pump systems seemed to have combinatorial interactions with plasmid β -lactamases to effect high-level of resistance (Peleg and Hooper 2010, Robledo, Aquino and Vazquez 2011). This is in agreement with this present study, where all the CR isolates were found to express β -lactamase activity.

The use of SDS-PAGE indicated different expression of porins for all CR isolates investigated. Predominantly, reduction of OMPs was detected largely among the *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates which formed the majority of the CR isolates. The SDS-PAGE analysis in comparison with the high-level β -lactamase activity results by nitrocefin assay, reduced susceptibility by disc diffusion method and various levels of carbapenem MICs may have significantly affected the detection of large number of carbapenemase non-PCR positives. Notably, three *Enterobacter cloacae* isolates were among the few Enterobacteriaceae isolates including each strain of *Cronobacter sakazakii* and *Sphingomonas paucimobilis* also expressed

presence of β -lactamase activity, high-level carbapenem MICs and reduced porin levels in this study. High numbers of carbapenemase negative isolates, according to PCR assay in this present study have β -lactam hydrolysing activity and that may be due to the presence of an unknown carbapenemase, an AmpC or a coincidental non-carbapenemase β -lactamase. The findings are in agreement with Thomson (2010) that false positive results are possibly due to AmpC producers, commonly with reduced susceptibility to imipenem than with other carbapenems (Thomson 2010).

The analysis of the outer membrane proteins (OMPs) on SDS-PAGE of all the CR isolates were specifically compared with *Pseudomonas aeruginosa* control strains with or without *OprD* (~44 kDa) expression which affects the susceptibility to carbapenems. In this study, OMPs of the *Pseudomonas aeruginosa* isolates produced decreased amounts of *OprD* or the porin was completely lost, except the 5.9% (3 out of 51) with high-level of porin. The majority of the isolates which had loss of porins were among the presumptive carbapenemase non-PCR positives. It is worth mentioning here that the effect of *OprD* reduction or loss could be one of the mechanisms that explain the ease with which *Pseudomonas aeruginosa* is known to become resistant. High-level production of β -lactamase activity by nitrocefin assays were prevalent in all the CR isolates studied. The effect and presence of these non-plasmid determinant enzymes may contribute significantly to carbapenem resistance coupled with the loss of *OprD* gene (Mathlouthi *et al* 2015). Essentially, in most MDR *Pseudomonas aeruginosa* isolates chromosomally encode resistance mechanisms in association with hyperexpression of AmpC, overproduction of the MexAB efflux system or both without harbouring carbapenemase resistance gene which could inactivate the *OprD* gene. The effect of these resistance mechanisms and with the presence of β -lactamase enzymes may have resulted in the high number of carbapenemase non-PCR positives. According

to Yang *et al.* (2012) porins play a significant role in increasing Gram-negative bacterial resistance to carbapenem antimicrobials, importantly, presence of ESBL or AmpC type enzymes is an essential element for resistance among Enterobacteriaceae group and non-fermenting *Pseudomonas* species (Yang *et al.* 2012). This recognition was also observed in CR isolates in this present study. Thus, all the 7 VIM-1-producing *Pseudomonas aeruginosa* isolates were carriers of TEM-1 and SHV-1 enzymes, 6 carbapenemase non-PCR positives out of 7 *Pseudomonas putida* isolates including the only VIM-1-positive isolate had reduced *OprD* or loss of porin by the SDS-PAGE assessment in this study. Pirnay *et al.* (2002) emphasised that the major determinant of resistance to imipenem particularly, in *Pseudomonas aeruginosa* isolates in the absence of MBL could be due to mutational change of the *oprD* gene (Pirnay *et al.* 2002). It is worth noting that most *Pseudomonas aeruginosa* carbapenemase non-PCR positives, including other CR isolates, showed decreased production of *oprD* gene which could be mutation-driven resistance. This phenomenon was observed on the electrophoretic patterns of the SDS-PAGE analysis with a reduction or loss of outer membrane porin D.

A recent study by Fusté *et al.* (2013), also on multidrug-resistant *Pseudomonas aeruginosa* isolates, revealed a similar reduction of the *OprD*, the main uptake of carbapenems (Fusté *et al.* (2013), while in another study in a Brazilian hospital by Cavalcanti *et al.* (2015) markedly reduced expression of *OprD* was observed in MBL-negative *Pseudomonas* isolates. This is in agreement with this present study, which has demonstrated that *OprD* loss and VIM-1 production could be one of the possible mechanisms emerging in MBL negative-PCR *Pseudomonas aeruginosa* isolates from Ghanaian hospitals.

For *Acinetobacter baumannii* isolates, 96.8% (30 out of 31) expressed loss of *OprF* in *Acinetobacter baumannii* isolates which included the NDM-1-positive producers. One carbapenemase non-PCR positive isolate with high-level expression of porin in this present study was observed and the mechanism of resistance remains unclear. It should be mentioned that the selectivity of the outer membrane permeability barrier is different for organisms such as *Acinetobacter* species or *Pseudomonas aeruginosa* compared with those in the Enterobacteriaceae group. The *OmpAb* of ~40 kDa, an outer membrane protein of *Acinetobacter baumannii* accounts for approximately 30-40% of the total OMPs (Jyothisri, Deepak and Rajeswari 1999) as compared with that of *Pseudomonas aeruginosa*. Li, Plésiat and Nikaido (2015) emphasised that these organisms do not produce the classical *Escherichia coli*-type trimeric porins that provide an increased influx of small drugs. The main nonspecific porin in these organisms is a homology of *Escherichia coli* *OmpA*, it functions by adding structural fitness usually connected to the outer membrane underlying peptidoglycan (Li, Plésiat and Nikaido 2015). *Acinetobacter baumannii* has a close homology to *Escherichia coli* *OmpA* and *Pseudomonas aeruginosa* *OprF*, which is the major outer membrane protein (Sugawara and Nikaido 2012). Of all the *Acinetobacter baumannii* isolates analysed, the major and significant band on SDS-PAGE was ~35 kDa. The results strongly suggest that the outer membrane of *Acinetobacter baumannii* contains reduced amounts of low-permeability porins, a situation similar to the *OprF* of *Pseudomonas aeruginosa*.

Other membrane proteins were analysed and also compared with the *Pseudomonas aeruginosa* control strains since the porins are closely related to the absence or presence of *OmpA*, *OmpAb*, *OmpC* and *OmpF* porins which have similar resistance to β -lactams and other antimicrobials. In this study, SDS-PAGE analysis for *Escherichia coli*,

Enterobacter aerogenes, *Klebsiella pneumoniae* and others in the Enterobacteriaceae group had *OmpC* and *OmpF* absent or markedly reduced at band 35 kDa and 37 kDa, respectively compared with control strains. The presence of *OmpA* in most of the Enterobacteriaceae group, for instance, in *Enterobacter aerogenes*, is the major source of stabilization within the bacterial membrane, which leads to a reduction in the expression of *OmpC* and *OmpF* porins and eventually a reduced susceptibility to β -lactams and other antimicrobials (Dupont *et al.* 2004, Amaral *et al.* 2014). Previous studies explained that the level of expression of *OmpC* and *OmpF* porins may not necessarily control the permeability of the outer membrane to nitrogen and glucose uptake under nutrient restriction, but may also be due to differentially control by the concentration of certain antimicrobials within the environment (Randall and Woodward 2002, Castillo-Keller, Vuong and Misra 2006). These studies are in agreement with this present study analysis which accounted for the high number of carbapenemase non-PCR positives.

Sequence data from carbapenemase producers were used to generate each phylogenetic trees for OXA-48, VIM-1 and NDM-1 positive genes with the respective isolates and control strains used in the study compared with GenBank established strains to establish reliability of phylogenetic relationship. Unfortunately, two of the PCR-positive carbapenemase genes, VIM-1 positive *Pseudomonas putida* (CR number 12) and NDM-1 positive *Acinetobacter baumannii* (CR number 27), including the positive NDM-1 control strains failed to yield substantial sequenced data to be added for their genetic relationships.

The phylogenetic trees showed an unevenly high level of heterogeneity among the CR isolates detected harbouring carbapenemase encoding genes, probably due to resistance

pattern differences to carbapenems, type of specimen, genus and species types. These features were observed from the three phylogenetic trees in this study. Clusters were distinctive from the controls used and those in the GenBank, signalling further studies may reveal a novel variant or variants from each carbapenemase-producing gene detected from Ghana.

The phylogenetic distribution of the two OXA-48 gene-positive *Klebsiella pneumoniae* isolates is interesting, the organisms were from different regional hospitals, different sexes, specimens and age differences, nonetheless, the two OXA-48 encoding genes were closely related. This accession has been confirmed in a study where OXA-48 carbapenemase-positive isolates with a variant type was found to be different from ST101 detected in Tunisia (Cuzon *et al.* 2011) where the same variant was previously reported in an outbreak in Spain (Pitart *et al.* 2011).

Of the 8 positive VIM-1 encoding genes, all were found to be imipenem-resistant, 7 *Pseudomonas aeruginosa* isolates and a strain of *Pseudomonas putida* by both disc diffusion and E-test method. Five *Pseudomonas aeruginosa* isolates were from wounds, 2 from urine, and the only *Pseudomonas putida* was from a sputum specimen. This *Pseudomonas putida* isolate could not produce sequenced data for the phylogenetic tree due to limited nucleotide generation during sequencing. Of the *Pseudomonas aeruginosa* isolates, the phylogenetic tree exhibited close relatedness, while 2 accessed numbers from the GenBank (FR695890 and FR695888) described as harbouring partial VIM-2 genes which were clonally related to 6 VIM-1-positive genes in the Ghanaian isolates except one which showed close relatedness to a couple of the accessed numbers from the GenBank (KP337988, KP681695 and KP771862). Comparatively, 66% and

89% sequence homology indicated the overall close relatedness of the positive VIM-1 carbapenemase genes detected in this study.

There was partial relatedness to one VIM-1 positive *Pseudomonas aeruginosa* isolate with a VIM-2 *Nocardia farcinica* isolate (KM194591) and an allele of VIM-41 *Pseudomonas aeruginosa* (KP771862) in subclass B1 metallo- β -lactamase from GenBank. VIM-1 gene similarities can be deceptive as genes are often carried on plasmids associated with class I integrons and plasmids are easily transferred between *Pseudomonas aeruginosa* isolates (Aghamiri *et al.* 2014). A previous study by Sanchez-Romero *et al.* (2012) also suggested some sort of linkages of common ESBL genes with a VIM-1 gene that occurred in clonal distribution of VIM-1-producing *Klebsiella pneumoniae* isolates and may be the cause of clustering together confirming the closeness of VIM-1-positive *Pseudomonas aeruginosa* isolates in the phylogenetic tree in this study (Figure 3.7).

Similarly, sequence types of NDM-1 genes mainly from *Acinetobacter baumannii* isolates were different from the majority of GenBank accessed numbers used for the phylogenetic tree. Nonetheless, two sequenced types were closely related to international clones, 89N *Shigella sonnei* was linked to *Citrobacter freundii* (KP770032) and 108N *Acinetobacter baumannii* to both *Escherichia coli* (KP770023) and *Enterobacter cloacae* (KP770026) as observed in Figure 3.8. These close relationships may be due to the association of the NDM-1 gene shared in common with different ESBL genes suggested in a previous study carried out in Morocco involving NDM-1-producing *Klebsiella pneumoniae* isolates (Poirel *et al.* 2011a).

This study raises concerns with the high levels of ESBLs which may have influenced the presence of carbapenem resistance determinants among the clinical CR isolates investigated. Although, both the phenotypic and genotypic methods yielded results comparable to other studies abroad. As a first time study, modified Hodge test and boronic-acid disc synergy method have shown inconsistencies or weaknesses in determination of carbapenemase activity in carbapenem-resistant Enterobacteriaceae group, *Pseudomonas* and *Acinetobacter* species. Relatively low sensitivity and specificity results from phenotype based assays clearly needed much improved and more diverse testing assays: examples, Carba NP, AmpC disc test, inhibitor-based test with the use of cloxacillin and temocillin MIC testing for determination of all classes of β -lactamases. PCR-based assays have shown reliability by characterising the prevailing types of carbapenemase-encoding genes, highlighting the prevalence and distribution levels among the different CR isolates, which otherwise 23 carbapenemase producers would have gone undetected by phenotypic tests used in this study.

In the SDS-PAGE analysis, there were substantial numbers of unrelated porin expression among the CR isolates investigated. However, banding patterns of porins were assessed or evaluated with in-house MBL positive and negative *Pseudomonas aeruginosa* control strains because they formed the majority of the CR isolates, and its *OprF* is synonymous to *OmpA* and *OmpC* or *OmpF* in *Acinetobacter baumannii* and those in the Enterobacteriaceae group, respectively. In particular, *OmpC/F* or *OmpK35/37* expression was deficient which could be due to mutation and the presence of a large collection of non-fermenters highly resistant to cephalosporins and carbapenems studied mainly *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates. These organisms have mutational complexities, structural differences, diminishing permeability or altered porin expressions compared with *Escherichia coli*

and *Klebsiella pneumoniae* isolates which were relatively few in numbers in this study. Furthermore, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates were in the majority being the carriers of carbapenemase genes; NDM-1, and VIM-1, while OXA-48 gene was only found in *Klebsiella pneumoniae* isolates by PCR assays, first to be identified in Ghana.

4. Plasmid analysis of carbapenem-resistant Gram-negative bacteria

4.1 Background and Aims

The increased use of β -lactam antimicrobials in the late 1980s has resulted in increased bacterial resistance identified in many Enterobacteriaceae species with the acquisition of ESBL encoding genes and more recently in the expression of mobile plasmid-encoded metallo- β -lactamase genes (Arya 2011, Fallah *et al.* 2011). These resistance genes have been observed in many clinical isolates, especially in non-fermenting organisms such as *Pseudomonas* and *Acinetobacter* species which have been prominent in harbouring high levels of multiple resistance mechanisms to first-line antimicrobials in addition to carbapenems (Peleg and Hooper 2010, Saranathan *et al.* 2014). There are five common acquired MBL-types designated as GIM, SIM, SPM, IMP and VIM. These resistance genes occur frequently in Gram-negative bacterial strains and a more recently identified type is NDM-1, classified as class B in Ambler's molecular classification (Queenan and Bush 2007). Of the MBL-types, VIM and IMP are widespread and commonly found in non-fermenting Gram-negative bacteria, *Pseudomonas aeruginosa* and *Acinetobacter* isolates (Ellington *et al.* 2007, Peleg and Hooper 2010).

Since the discovery of the NDM-1 gene in 2008 its moveable plasmids have spread rapidly into the population of different Gram-negative bacterial pathogens (Struelens *et al.* 2010). At present, over six different NDM variants have been reported in Gram-negative bacterial isolates. Alternatively, over expression of OXA β -lactamases (Class

D β -lactamases) have been detected including all variants in *Acinetobacter baumannii* CR isolates (Chen *et al.* 2011, Cornaglia, Giamarellou and Rossolini 2011, Fallah *et al.* 2011, Saranathan *et al.* 2014). As discussed in Chapter 3, chromosomally determined DNA characterisation revealed the prevalence of three carbapenemase producing genes; NDM-1, VIM-1 and OXA-48 in six different carbapenem-resistant isolates. In this Chapter, the presence of common plasmid-encoding β -lactamases was investigated in order to establish the prevalence and distribution among the carbapenem-resistant isolates from Ghana. The aim of the study was to perform plasmid analysis by conjugation and curing experiments in order to ascertain the effect of plasmid-borne β -lactamases in CR isolates. In addition, to determine the presence of common ESBL β -lactamases (TEM-1, SHV-1) associated with carbapenemases to establish the prevalence and distribution among the carbapenem-resistant isolates.

The specific objectives were to:

- Perform plasmid DNA extractions and estimate the nucleic acid content using Nano NanoDrop® ND-1000 spectrophotometer
- Determine the prevalence and distribution of common ESBL-gene types (TEM-1 and SHV-1) and carbapenemase genes among the study isolates
- Determine the presence of plasmid-mediated carbapenem resistance genes and to elucidate their role in plasmid resistance by conjugation and curing experiments
- Carry out susceptibility testing of plasmid-cured isolates to carbapenem antimicrobials to establish the resistance profiles
- Establish plasmid band sizes of cured CR isolates with restriction enzyme digestion on gel electrophoresis

4.2 Method summary

Plasmid DNA isolation of 111 CR isolates was performed and nucleic acid content estimated using a NanoDrop® ND-1000 spectrophotometer to establish plasmid DNA concentrations in each carbapenem-resistant isolate. Amplified plasmid DNA products were analysed on agarose gel electrophoresis to establish the locations of the plasmid resistance genes. Acquisition and elimination of plasmid-borne carbapenem resistance genes were performed by conjugation and curing experiments using CR isolates as donor and Top 10 *Escherichia coli* cells as recipient. Susceptibility testing was performed on all cured CR isolates to establish the level of resistance by both disc diffusion and E-test assays to show plasmid-associated loss of resistance. Finally, to establish the differences or similarities in the fingerprints of the encoded plasmids generated by digestion with or without restriction endonucleases to compare the respective cured CR isolates of the 26 carbapenemase-positive gene carriers chromosomally detected and described in Chapter 2 to establish the presence of plasmid-borne resistance genes. The full description is in Chapter two of this study.

4.3 Results

4.3.1 Plasmid DNA concentration analysis

The extraction procedure described in Chapter 2 (Section 2.8.1) was used to determine the concentrations of the nucleic acid content of all plasmid DNA samples including *Escherichia coli* (ATCC 25922) negative control strain and two positive control strains, *Klebsiella pneumoniae* NDM-1 (NCTC 13443) and *Pseudomonas aeruginosa* VIM-1 (NCTC 13437) for this study. The NanoDrop® ND-1000 spectrophotometer was used to determine the estimated nucleic acid content of each plasmid DNA sample. Values

ranging from 1.8 ng/l to 48.5 ng/l were determined in the samples. For positive carbapenemase producing strains, values ranging from 2.3 to 20.4 ng/l were found of which the highest was in the *Klebsiella pneumoniae* isolate and the lowest observed in one of the *Pseudomonas aeruginosa* isolates. The highest value of 48.5 ng/l was obtained from the carbapenemase non-producing *Acinetobacter baumannii* isolate found in the urine of a female patient aged 29 years. Details of the plasmid DNA concentrations are shown in Table 4.1.

4.3.2 Plasmid DNA analysis by gel electrophoresis

Gel electrophoresis analysis of the plasmid DNA samples revealed that all the 111 CR isolates were harbouring one or multiple plasmids with sizes ranging averagely between 10 kb and ~95 kb when compared with the known 100 kb DNA molecular marker. The gel electrophoretic analysis of the Plasmid DNA reaction products revealed that the number of band lanes averagely ranged from 1 to 7. A representative gel showing one or more band locations of plasmid-borne carbapenemase resistance from different types of CR isolates used can be seen in Figure 4.1.

Table 4.1 Quantification of plasmid DNA in carbapenem-resistant isolates using the Nano-Drop Spectrophotometer (ND-1000)

Cn	Conc. in ng/l	Cn	Conc. in ng/l	Cn	Conc. in ng/l	Cn	conc. in ng/l	Cn	Conc. in ng/l	Cn	Conc. in ng/l
S1	6.1	S21	4.7	S41	7.7	S61	8.8	S81	3.6	S101	7.5
S2	12.8*	S22	7.7	S42	5.2*	S62	20.0*	S82	5.2	S102	6.3*
S3	18.7	S23	3.4	S43	7.5*	S63	5.8	S83	10.2	S103	15.5
S4	5.7	S24	4.1	S44	4.3	S64	17.6*	S84	8.9*	S104	10.2
S5	6.6*	S25	9.9	S45	10.0	S65	8.5	S85	8.9	S105	15.9*
S6	2.4*	S26	6.3	S46	4.2*	S66	8.2	S86	5.1	S106	7.6
S7	2.3*	S27	8.9*	S47	5.2*	S67	7.9	S87	9.0	S107	12.4
S8	12.8	S28	8.2	S48	6.4	S68	13.0	S88	26.5	S108	9.9*
S9	11.2	S29	4.7	S49	5.9	S69	9.1	S89	8.5*	S109	8.3
S10	4.7	S30	6.6	S50	5.0*	S70	13.7	S90	4.8	S110	14.4
S11	6.0	S31	3.9	S51	10.3	S71	7.6	S91	14.9	S111	11.9
S12	5.8*	S32	3.3	S52	3.8	S72	11.8*	S92	5.2	NDM-1	19.3
S13	4.8	S33	10.9	S53	5.2	S73	11.7	S93	7.6	VIM-1	10.6
S14	3.7	S34	3.9	S54	7.1	S74	7.0*	S94	5.4	EC-ve	12.6
S15	4.0	S35	7.7	S55	19.5	S75	48.5	S95	18.3		
S16	9.9	S36	6.7	S56	5.9	S76	18.1*	S96	9.4		
S17	16.6	S37	1.8	S57	9.0	S77	8.6	S97	7.5		
S18	11.5	S38	7.1	S58	23.1	S78	13.0	S98	20.4*		
S19	19.7*	S39	3.2*	S59	2.4	S79	4.0	S99	7.3		
S20	7.7	S40	2.2	S60	2.9*	S80	4.5	S100	6.5*		

* Carbapenemase-positive gene carriers

Note: Cn= code number, S= sample, NDM-1= New Delhi metallo- β -lactamase-1 *Klebsiella pneumoniae* (NCTC 13443), VIM-1= Verona integron-encoded metallo- β -lactamase-1 *Pseudomonas aeruginosa* (NCTC 13437), EC-ve= *Escherichia coli* (ATCC 25922) negative control



Figure 4.1 Plasmid DNA profiling on agarose gel electrophoresis

Note: M= 100 kb DNA marker, Nc= negative control *Escherichia coli* ATCC 25922, Tc= Top 10 *Escherichia coli* competent cell, lane: 1 to 6= plasmid DNA samples, Bk= Blank sample.

4.3.3 Plasmid transfer analysis

Conjugation experiments were performed for both carbapenemase non- and positive-producers (donor cells) mating-out into *Escherichia coli* Top 10 competent cells (recipient cell) using the technique described in Chapter 2 (Section 2.8.4). Either double or multiple bands were observed in all the plasmid DNA extracts. For the 26 carbapenemase positive-producers and the cured isolates, carriers of multiple plasmids were observed while carbapenemase non-producers showed either single or multiple plasmids, indicating the presence of unknown plasmids among the CR isolates. PCR amplicons from plasmid DNA were visualised on agarose gels. This was performed to demonstrate the presence of common ESBL-type β -lactamase encoding genes (TEM-1 and SHV-1) and carbapenemase encoding genes (KPC-1, NDM-1, VIM-1, IMP-1 and OXA-48) using plasmid DNA. In total, TEM-1 ESBL demonstrated 100% prevalence in plasmids from all the CR isolates while only 3.6% (4/111) were without the SHV-1 ESBL (sample code numbered: 11, 67, 70 and 89). Details of the sample numbers, type

of isolate and source of specimens of the CR isolates are listed in Appendix 1. The prevalence and distribution of common ESBL type β -lactamases investigated and plasmid-borne carbapenemase gene carriers detected and the type of CR isolates are shown in Tables 4.2 and 4.3.

Table 4.2 Beta-lactamase genes amplified from plasmid DNA identified in carbapenem-resistant isolates

Ambler Class	β -lactamase type tested	Types found in isolates (n) %
A	TEMs, SHVs	TEMs (111) 100, SHVs (107) 96.4
	SME-1, KPC-1	SME-1 (0) 0.0, KPC-1 (0) 0.0
B	IMP-1, VIM-1, Ccr-A, NDM-1	IMP-1 (0) 0.0, VIM-1 (8) 7.2, Ccr-A (0) 0.0, NDM-1 (16) 14.4
C	None	None
D	OXA-48	OXA-48 (2) 1.8

Note: n= number of CR isolate

Table 4.3 Distribution of carbapenemase genes carried on plasmids in carbapenem-resistant Gram-negative isolates

β-lactamase type	CR isolates n=111 (%)	Ps.a	A.b	E.c	Ps.p	K.p	En.c	C.z	P.s	S.s	S.p
		51	31	12	7	3	3	1	1	1	1
TEM-1	111 (100)	51	31	12	7	3	3	1	1	1	1
SHV-1	107 (96.4)	50	31	11	7	3	3	1	1	0	0
NDM-1	16 (14.4)	2	8	3	1	0	0	0	1	1	0
VIM-1	8 (7.2)	7	0	0	1	0	0	0	0	0	0
OXA-48	2 (1.8)	0	0	0	0	2	0	0	0	0	0

Note: n= total number of CR isolate, Ps.a= *Pseudomonas aeruginosa*, A.b= *Acinetobacter baumannii*, E.c= *Escherichia coli*, Ps.p= *Pseudomonas putida*, K.p= *Klebsiella pneumoniae*, En.c= *Enterobacter cloacae*, C.z= *Cronobacter zakasakii*, P.s= *Providencia stuartii*, S.s= *Shigella sonnei*, S.p= *Sphingomonas paucimobilis*

In the samples tested, 96.4% (107/111) of the CR isolates co-harboured both TEM-1 and SHV-1 genes. However, three of the carbapenemase non-PCR positives' harboured the TEM-1 gene alone. The three isolates included *Sphingomonas paucimobilis* (isolate 11), *Escherichia coli* (isolate 67) and *Pseudomonas aeruginosa* (isolate 70) and one carbapenemase-positive gene carrier, *Shigella sonnei* (isolate 89). Bands for KPC-1 and IMP-1 were not identified in the plasmid DNA samples with the primer sequences used. All of the 26 carbapenemase-positive gene carrying isolates harboured both the ESBL genes (TEM-1 and SHV-1) except the *Shigella sonnei* strain which harboured only the TEM-1 ESBL gene. Representative samples of group A & B and C & D (Figures 4.2a & 4.2b) are shown for comparison, comprising plasmid DNA analysis for both carbapenemase-positive gene carriers and carbapenemase deficient isolates on agarose

gel electrophoresis. Figure 4.3 shows the presence of two β -lactamases (TEM-1 and SHV-1) carried on plasmids on agarose gel electrophoresis, where TEM-type ESBL genes were identified in all samples (1 to 11) while SHV-type ESBL gene was identified in all samples except in sample number 11.

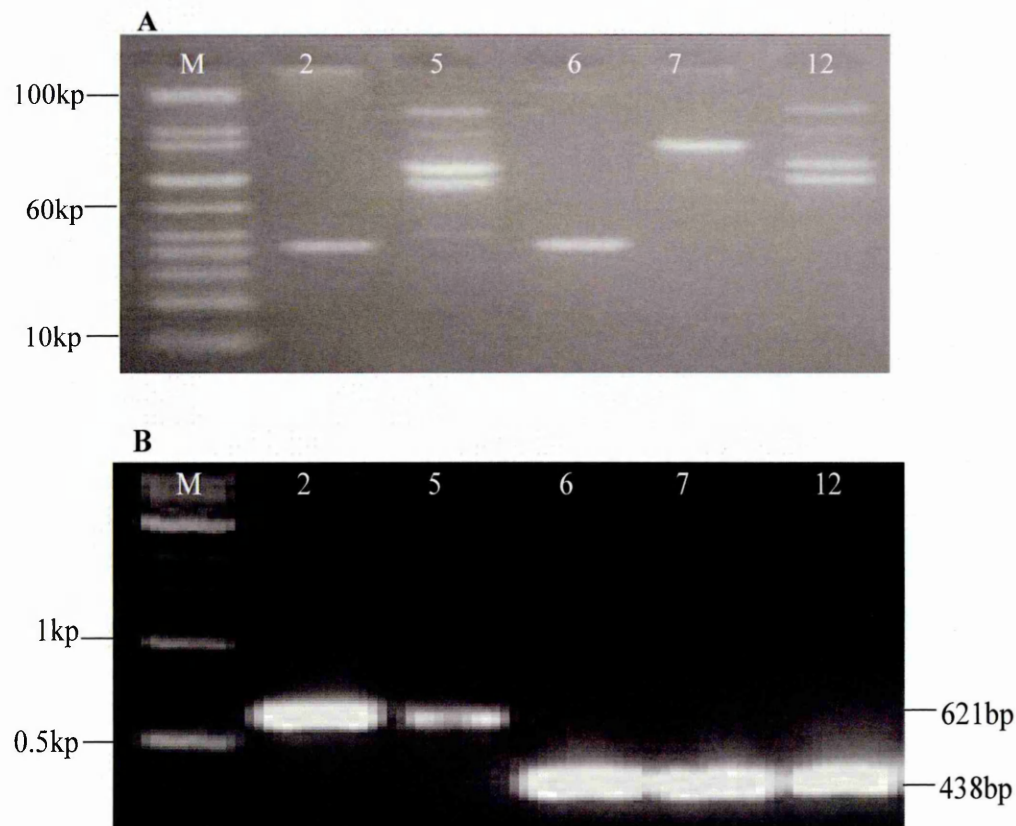


Figure 4.2a Amplified carbapenemase genes from plasmids

Note: M= marker ladder, NC= NDM-1 positive (NCTC 13443) control strain. (A, plasmid on gel), five plasmid DNA extracts each (2, 5, 6, 7, and 12) showing different band patterns. In (B, PCR product on gel) corresponding plasmid DNA extracts each amplified with known positive carbapenemase gene primers showing; lanes 2 and 5 for NDM-1 gene (621bp); lanes, 6, 7 and 12 for VIM-1 gene (438bp), respectively.

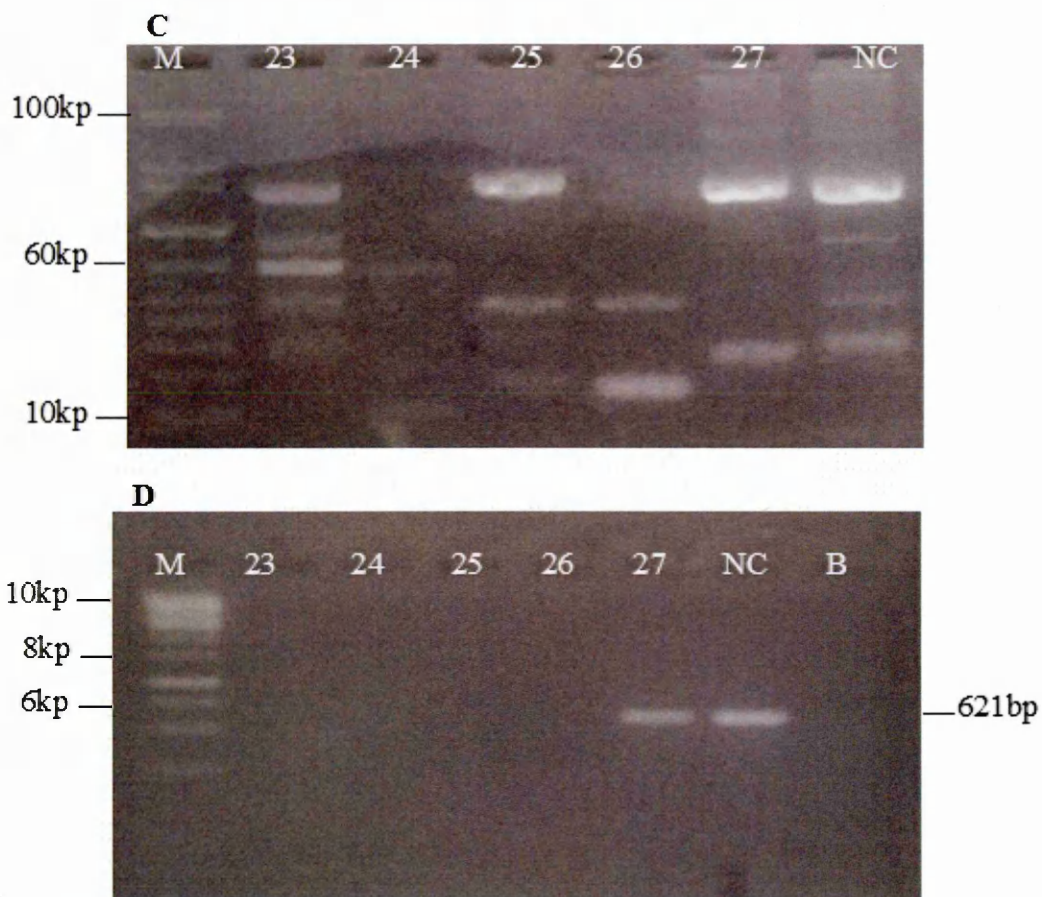


Figure 4.2b Amplified carbapenemase genes from plasmids

Note: M= marker ladder, NC= NDM-1 positive (NCTC 13443) control strain. In (C plasmid on gel), five plasmid DNA extracts (23, 24, 25, 26, and 27) showing different plasmid band patterns. In (D, PCR product on gel), corresponding plasmid DNA extracts amplified with known positive carbapenemase gene primers showing only lane 27 is encoded with NDM-1 gene (621bp).

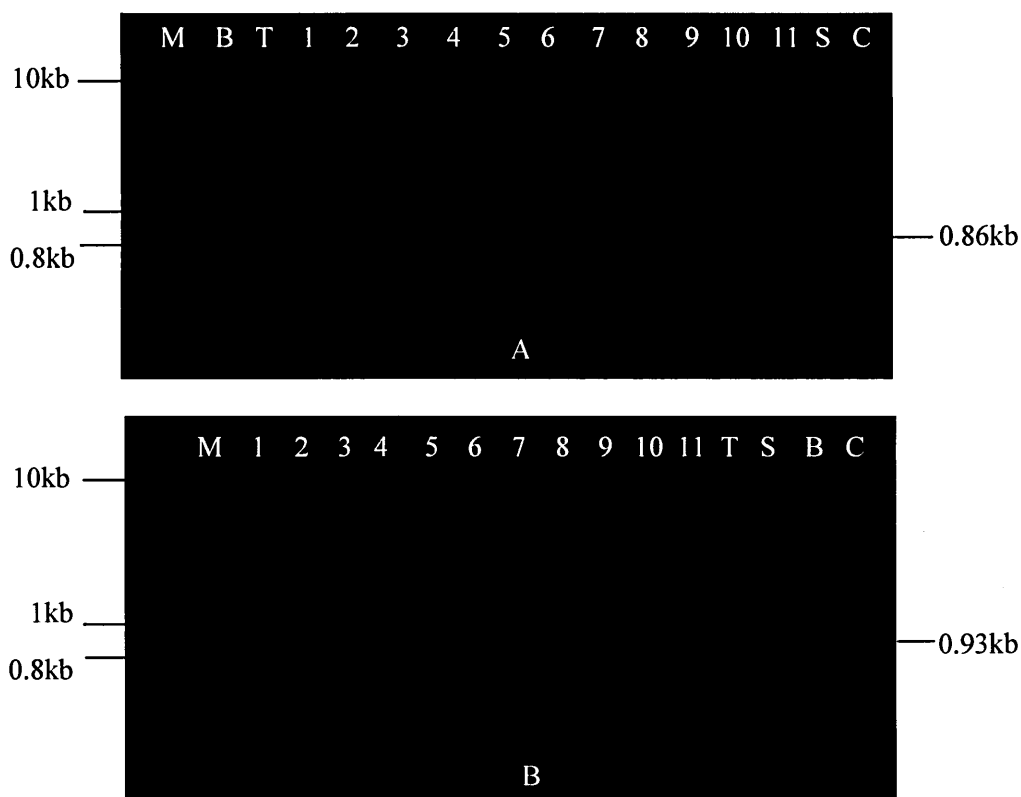


Figure 4.3 Plasmid profiling of two β -lactamase genes on gel electrophoresis

Note: M= 10Kb DNA marker, B= Blank, S= SHV-type ESBL gene (*Klebsiella pneumoniae* NCTC 165032 positive control), 1-11= CR sample numbers, T= TEM-type ESBL gene (*Escherichia coli* NCTC 13351 positive control), C= negative ESBL (*Escherichia coli* ATCC 25922) control strain. In (A) gel showing TEM-type ESBL gene is showing in all the CR isolate numbers from 1 to 11 while in (B) gel SHV-type ESBL gene is showing in all except CR isolate number 11.

4.3.4 Resistance pattern of cured plasmid-isolates to carbapenems

The 26 cured carbapenemase-positive isolates were tested using disc diffusion susceptibility to imipenem, meropenem and doripenem agents. The resistance patterns were compared with the original CR isolates tested earlier (see Appendix 2) in which 15 out of the 26 cured isolates were reduced to intermediate resistance or remained resistant to one carbapenem (Table 4.4a). Ten cured isolates were susceptible and only one cured isolate retained the same level of resistance to one or two carbapenems used.

Further inhibition analysis revealed that 8 of the cured carbapenemase-positive gene carriers exhibited complete susceptibility to all the carbapenems. These isolates were from 4 *Pseudomonas aeruginosa*, 2 *Acinetobacter baumannii* and 1 *Escherichia coli* and *Shigella sonnei* isolates. However, 3 of the cured isolates, *Klebsiella pneumoniae*, *Pseudomonas putida* and *Providencia stuartii* remained resistant to all the carbapenems tested.

Comparatively, significant numbers of the original carbapenemase non-PCR positives exhibited substantially increased levels of susceptibility 44/111 (39.6%), and 34/111 (30.6%) displayed intermediate resistance to one or two carbapenems tested when compared with cured isolates encoded with carbapenemase genes. A representative example of an original (uncured) CR isolate compared with the corresponding cured variant of the same isolate was tested together (Figure 4.4). The cured isolate showed complete susceptibility to both imipenem and meropenem discs.

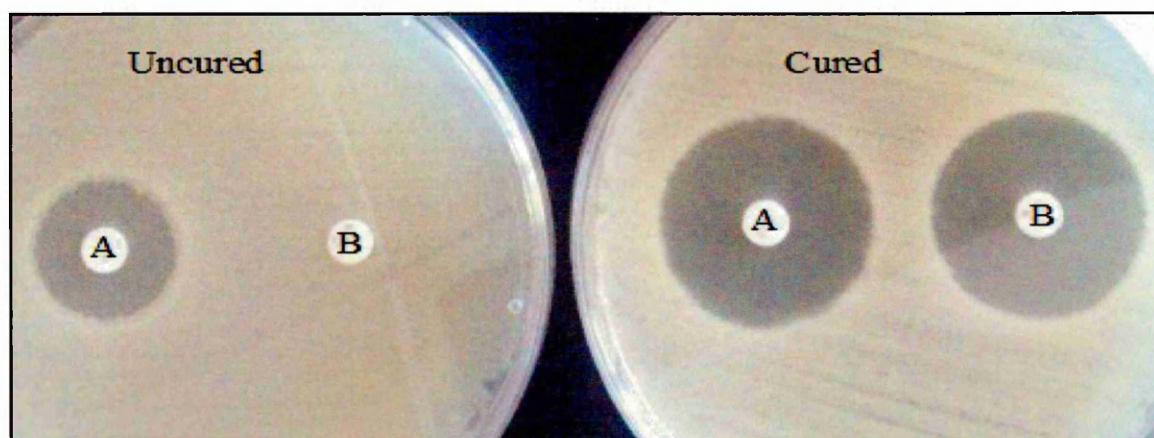


Figure 4.4 Comparison between resistance pattern of an uncured plasmid-borne isolate and corresponding cured susceptibility testing

Note: Uncured {A= imipenem, intermediate resistance; B= meropenem, total resistance}; Cured {A and B showing complete susceptibility to imipenem and meropenem respectively}

4.3.5 Resistance profile of cured isolates by the E-test assay

The E-test resistance patterns of all the cured isolates tested for both carbapenemase non-PCR positives and carbapenemase-positive gene carriers showing various MIC levels are listed in Table 4.4a. The differences observed in the disc diffusion resistance patterns were consistent with those observed in the MIC assay results. The original CR isolates were found to have relatively high MIC values (see Appendix 2 and Table 4.4a). The MIC levels were reduced more towards imipenem and meropenem than ertapenem, where some maintained the same high-level of resistance as evidenced in the original MIC values (Table 4.4a). It can be seen that the MICs of the cured isolates were either reduced by 2- or 4-fold, the majority of the carbapenemase non-PCR positives were found to be harbouring TEM-1 and SHV-1 genes (Table 4.4b).

Table 4.4a Plasmid profiling of carbapenem-resistant isolates before conjugation and cured experiments

Isolate code	Name of isolate	Sensitivity testing		MIC ($\mu\text{g/ml}$)						Plasmid DNA		
				Original			Cured			Original number of bands	Band loss after cured	After cured (size range in ~ kb)
		Original	Cured	Imi	Mer	Ert	Imi	Mer	Ert			
1	<i>P. aeruginosa</i>	R	In	>32	>32	>32	32	16	>32	4	3	60
2*	<i>E. coli</i>	R	In	>32	>32	>32	16	8	16	9	3	70 to 85
3	<i>P. aeruginosa</i>	R	S	4	16	>32	4	16	32	5	4	55
4	<i>A. baumannii</i>	R	R	>32	>32	>32	>32	>32	>32	8	7	55
5*	<i>E. coli</i>	R	S	2	3	16	0.12	0.5	2	7	3	60 to 95
6*	<i>P. aeruginosa</i>	R	In	>32	>32	>32	16	16	8	4	2	75 to 85
7*	<i>P. aeruginosa</i>	R	In	>32	>32	>32	8	8	16	7	6	45 to 75
8	<i>A. baumannii</i>	R	In	>32	>32	>32	16	16	16	8	6	45 to 80
9	<i>C. sakazakii</i>	R	S	>32	>32	>32	4	4	16	7	5	40 to 70
10	<i>P. aeruginosa</i>	R	S	>32	>32	>32	4	8	8	8	5	45 to ≥ 100
11	<i>S. paucimobilis</i>	R	S	>32	>32	>32	2	4	4	9	5	45 to ≥ 100
12*	<i>P. putida</i>	R	S	>32	>32	>32	4	4	8	5	5	-
13	<i>P. aeruginosa</i>	R	S	16	>32	>32	2	8	16	6	5	≥ 100

Isolate code	Name of isolate	Sensitivity testing		MIC (µg/ml)						Plasmid DNA		
				Original			Cured			Original number of bands	Band loss after cured	After cured (size range in ~kb)
		Original	Cured	Imi	Mer	Ert	Imi	Mer	Ert			
14	<i>P. aeruginosa</i>	R	S	8	4	>32	1	1	4	9	7	45 to ≥100
15	<i>P. aeruginosa</i>	R	S	>32	12	>32	4	2	8	9	7	45 to ≥100
16	<i>P. aeruginosa</i>	R	S	16	4	>32	2	0.5	16	9	6	90 to ≥100
17	<i>P. aeruginosa</i>	R	In	>32	>32	>32	32	16	16	6	5	≥100
18	<i>E. coli</i>	R	In	16	>32	>32	4	8	32	6	6	-
19*	<i>K. pneumoniae</i>	R	S	16	8	>32	2	1	16	6	5	60
20	<i>A. baumannii</i>	R	In	>32	>32	>32	16	16	32	9	6	70 to ≥100
21	<i>P. aeruginosa</i>	R	S	2	16	>32	0.5	2	8	8	4	30 to 75
22	<i>A. baumannii</i>	R	In	>32	16	>32	16	16	32	9	6	35 to 95
23	<i>E. coli</i>	R	S	>32	>32	>32	4	4	16	9	6	30 to 95
24	<i>P. aeruginosa</i>	R	S	>32	>32	>32	12	16	32	7	5	20 to 70
25	<i>P. aeruginosa</i>	R	S	>32	>32	>32	8	8	16	7	5	30 to 70
26	<i>P. aeruginosa</i>	R	S	>32	>32	>32	4	16	32	7	3	30 to 70
27*	<i>A. baumannii</i>	R	S	6	>32	>32	2	8	16	9	4	30 to 95
28	<i>P. aeruginosa</i>	R	In	>32	>32	>32	32	16	16	6	5	95
29	<i>A. baumannii</i>	R	In	>32	>32	>32	8	16	32	9	5	35 to ≥100

Isolate code	Name of isolate	Sensitivity testing		MIC (µg/ml)						Plasmid DNA		
				Original			Cured			Original number of bands	Band loss after cured	After cured (size range in ~kb)
		Original	Cured	Imi	Mer	Ert	Imi	Mer	Ert			
30	<i>A. baumannii</i>	R	S	>32	16	>32	8	8	32	7	4	30 to 75
31	<i>Ps aeruginosa</i>	R	S	4	16	>32	2	2	16	7	4	30 to 70
32	<i>P. aeruginosa</i>	R	S	>32	>32	>32	4	4	16	9	3	20 to 95
33	<i>En. cloacae</i>	R	S	32	>32	>32	4	4	16	9	4	30 to 95
34	<i>A. baumannii</i>	R	S	8	16	>32	2	2	16	9	5	30 to 95
35	<i>E. coli</i>	I	S	0.38	0.50	0.50	0.38	0.50	0.50	9	4	40 to 95
36	<i>A. baumannii</i>	R	In	>32	>32	>32	8	16	32	6	3	30 to 60
37	<i>P. aeruginosa</i>	R	In	16	>32	>32	4	32	32	9	3	30 to 95
38	<i>A. baumannii</i>	R	In	>32	>32	>32	16	16	32	7	4	30 to 75
39*	<i>P. aeruginosa</i>	R	In	>32	>32	>32	16	16	32	7	3	30 to 75
40	<i>P. aeruginosa</i>	R	In	>32	>32	>32	32	8	32	8	4	30 to 80
41	<i>P. aeruginosa</i>	R	In	>32	>32	>32	16	32	>32	7	3	30 to 70
42*	<i>A. baumannii</i>	R	In	>32	>32	>32	16	16	32	9	4	30 to 95
43*	<i>A. baumannii</i>	R	In	>32	>32	>32	16	8	32	9	4	30 to 95
44	<i>P. putida</i>	R	S	>32	>32	>32	4	4	16	8	4	20 to 80
45	<i>A. baumannii</i>	R	In	>32	>32	>32	16	8	32	9	4	30 to 95

Isolate code	Name of isolate	Sensitivity testing		MIC (µg/ml)						Plasmid DNA		
				Original			Cured			Original number of bands	Band loss after cured	After cured (size range in ~ kb)
		Original	Cured	Imi	Mer	Ert	Imi	Mer	Ert			
46*	<i>A. baumannii</i>	R	In	>32	>32	>32	32	16	32	9	4	30 to 95
47*	<i>P. aeruginosa</i>	R	In	>32	>32	>32	8	8	32	9	4	30 to 95
48	<i>P. aeruginosa</i>	R	S	>32	>32	>32	8	16	16	7	5	30 to 70
49	<i>A. baumannii</i>	R	In	>32	>32	>32	16	16	16	9	4	30 to 95
50*	<i>A. baumannii</i>	I	S	4	16	>32	2	4	8	9	4	30 to 95
51	<i>A. baumannii</i>	R	In	>32	>32	>32	16	16	32	9	5	50 to 95
52	<i>P. aeruginosa</i>	R	S	12	12	>32	2	2	16	8	3	40 to 80
53	<i>E. coli</i>	R	S	16	>32	>32	2	8	16	9	4	30 to 95
54	<i>P. aeruginosa</i>	R	S	4	>32	>32	2	4	16	7	5	40 to 70
55	<i>K. pneumoniae</i>	R	S	8	16	>32	2	2	8	7	4	10 to 75
56	<i>A. baumannii</i>	R	In	16	>32	>32	8	32	32	7	1	20 to ≥100
57	<i>P. aeruginosa</i>	R	S	4	>32	>32	2	4	8	9	4	40 to 95
58	<i>P. aeruginosa</i>	I	S	8	4	>32	0.5	2	16	9	3	25 to 90
59	<i>P. aeruginosa</i>	R	S	6	3	>32	2	1	16	9	3	25 to 90
60*	<i>P. aeruginosa</i>	R	In	>32	>32	>32	16	8	32	7	3	30 to 70
61	<i>P. aeruginosa</i>	R	In	>32	16	>32	16	8	32	9	3	25 to 90

Isolate code	Name of isolate	Sensitivity testing		MIC (µg/ml)						Plasmid DNA		
				Original			Cured			Original number of bands	Band loss after cured	After cured (size range in ~kb)
		Original	Cured	Imi	Mer	Ert	Imi	Mer	Ert			
62*	<i>P. aeruginosa</i>	R	In	>32	8	>32	16	8	32	9	3	30 to 95
63	<i>P. aeruginosa</i>	R	In	16	>32	>32	8	32	32	8	6	30 to 85
64*	<i>A. baumannii</i>	R	In	8	>32	>32	8	16	32	9	4	30 to ≥100
65	<i>P. aeruginosa</i>	R	In	>32	>32	>32	32	16	32	9	3	10 to ≥100
66	<i>P. aeruginosa</i>	R	In	>32	>32	>32	16	16	32	8	2	30 to 75
67	<i>E. coli</i>	I	S	2	0.125	16	0.5	0.125	4	8	2	30 to 75
68	<i>E. coli</i>	I	S	4	0.19	4	1	0.12	16	9	1	25 to 90
69	<i>A. baumannii</i>	R	S	16	32	>32	2	8	16	9	3	30 to 95
70	<i>P. aeruginosa</i>	I	S	4	0.50	>32	2	0.50	4	7	2	10 to 70
71	<i>P. aeruginosa</i>	R	S	16	8	>32	4	2	16	8	4	30 to ≥100
72*	<i>A. baumannii</i>	R	In	>32	>32	>32	8	32	32	9	5	30 to ≥100
73	<i>A. baumannii</i>	R	In	>32	16	>32	8	16	16	9	6	30 to ≥100
74*	<i>P. aeruginosa</i>	R	In	>32	>32	>32	16	16	32	9	5	30 to ≥100
75	<i>A. baumannii</i>	R	R	>32	>32	>32	>32	>32	>32	8	5	40 to 75
76*	<i>P. stuartii</i>	I	S	4	0.25	8	1	0.25	8	9	3	20 to ≥100
77	<i>A. baumannii</i>	R	In	>32	>32	>32	16	16	32	9	4	30 to 95

Isolate code	Name of isolate	Sensitivity testing		MIC (µg/ml)						Plasmid DNA		
				Original			Cured			Original number of bands	Band loss after cured	After cured (size range in ~ kb)
		Original	Cured	Imi	Mer	Ert	Imi	Mer	Ert			
78	<i>P. putida</i>	R	S	>32	>32	>32	8	8	16	5	3	20 to 50
79	<i>P. aeruginosa</i>	R	R	>32	>32	>32	>32	>32	>32	8	6	50 to 80
80	<i>P. aeruginosa</i>	R	S	8	16	>32	2	4	16	9	4	20 to 90
81	<i>P. aeruginosa</i>	R	S	0.38	6	16	0.12	2	8	9	3	30 to 95
82	<i>A. baumannii</i>	R	In	32	>32	>32	16	8	16	7	4	30 to 70
83	<i>P. putida</i>	R	S	0.38	16	32	0.38	4	16	6	3	20 to 60
84*	<i>P. aeruginosa</i>	R	S	8	4	16	2	2	8	8	5	30 to ≥100
85	<i>E. coli</i>	I	S	0.38	0.25	4	0.38	0.25	2	8	2	30 to 80
86	<i>P. aeruginosa</i>	R	R	>32	>32	>32	>32	>32	>32	8	4	30 to 75
87	<i>P. aeruginosa</i>	R	S	>32	32	>32	8	8	16	9	3	30 to 95
88	<i>P. putida</i>	R	In	>32	>32	>32	16	16	32	8	4	20 to ≥100
89*	<i>S. sonnei</i>	R	S	1.5	4	32	1.5	2	16	8	3	30 to 80
90	<i>P. putida</i>	R	S	>32	>32	>32	4	4	8	5	2	10 to 50
91	<i>En. cloacae</i>	R	R	>32	>32	>32	>32	>32	>32	8	4	20 to 75
92	<i>P. putida</i>	R	In	>32	>32	>32	16	32	32	5	3	20 to 50
93	<i>E. coli</i>	R	In	>32	>32	>32	>32	>32	>32	9	6	25 to 95

Isolate code	Name of isolate	Sensitivity testing	MIC (µg/ml)							Plasmid DNA		
			Original			Cured				Original number of bands	Band loss after cured	After cured (size range in ~ kb)
		Original	Cured	Imi	Mer	Ert	Imi	Mer	Ert			
94	<i>A. baumannii</i>	R	S	6	12	>32	2	4	4	9	4	30 to 95
95	<i>En. cloacae</i>	R	In	32	>32	>32	16	32	32	5	2	15 to 50
96	<i>P. aeruginosa</i>	R	S	16	8	32	4	4	16	8	4	30 to 80
97	<i>E. coli</i>	R	In	16	>32	>32	8	32	16	9	3	30 to 95
98*	<i>K. pneumoniae</i>	R	S	16	8	>32	4	4	16	9	3	30 to 95
99	<i>P. aeruginosa</i>	R	R	>32	>32	>32	>32	>32	>32	9	4	30 to 95
100*	<i>P. aeruginosa</i>	R	In	>32	>32	>32	16	8	32	8	3	30 to 75
101	<i>P. aeruginosa</i>	R	In	8	8	>32	2	4	16	8	2	30 to 80
102*	<i>A. baumannii</i>	R	In	>32	>32	>32	16	8	32	9	4	30 to 95
103	<i>A. baumannii</i>	R	S	1	4	32	1	2	16	8	3	30 to 75
104	<i>P. aeruginosa</i>	R	In	16	>32	>32	16	16	32	8	4	30 to 75
105*	<i>E. coli</i>	R	S	0.75	2	32	0.50	2	4	8	4	15 to 75
106	<i>A. baumannii</i>	R	In	>32	>32	>32	8	16	16	9	3	10 to ≥100
107	<i>A. baumannii</i>	R	S	16	>32	>32	4	16	16	8	3	25 to ≥100
108*	<i>A. baumannii</i>	R	R	>32	>32	>32	>32	>32	>32	8	3	30 to ≥100
109	<i>P. aeruginosa</i>	R	In	>32	>32	>32	16	16	16	9	6	15 to ≥100

Isolate code	Name of isolate	Sensitivity testing		MIC (µg/ml)						Plasmid DNA		
				Original			Cured			Original number of bands	Band loss after cured	After cured (size range in ~ kb)
		Original	Cured	Imi	Mer	Ert	Imi	Mer	Ert			
110	<i>P. aeruginosa</i>	R	In	>32	>32	>32	16	8	32	6	2	25 to 60
111	<i>P. aeruginosa</i>	R	S	16	>32	>32	4	4	16	9	3	30 to ≥100
Control	<i>E. coli</i> Top 10	S	S	0.125	0.12	0.50	0.125	0.12	0.50	-	-	-
Control	<i>E. coli</i> (ATCC 25922 isolate)	S	S	0.12	0.125	0.50	0.12	0.125	0.50	-	-	-

*Positive carbapenemase-producers. Note: Imi= imipenem, Mer= meropenem, Ert= etapenem, R= resistant, S= sensitive, In= intermediate, -= No band seen

Table 4.4b Plasmid profiling of carbapenemase non-PCR positives and minimum

inhibitory concentrations with common β -lactamase genes

Isolate number	Name Carbapenem-resistant isolate	Type of ESBL		Carbapenemase non-PCR positives					
				MIC (μ g/ml) before curing			MIC (μ g/ml) after curing		
		TEM	SHV	Imp	Mer	Ert	Imp	Mer	Ert
1	<i>P. aeruginosa</i>	+	+	>32	>32	>32	32	16	>32
3	<i>P. aeruginosa</i>	+	+	4	16	>32	4	16	32
4	<i>A. baumannii</i>	+	+	>32	>32	>32	>32	>32	>32
8	<i>A. baumannii</i>	+	+	>32	>32	>32	16	16	16
9	<i>C. sakazakii</i>	+	+	>32	>32	>32	4	4	16
10	<i>P. aeruginosa</i>	+	+	>32	>32	>32	4	8	8
11	<i>S. paucimobilis</i>	+	-	>32	>32	>32	2	4	4
13	<i>P. aeruginosa</i>	+	+	16	>32	>32	2	8	16
14	<i>P. aeruginosa</i>	+	+	8	4	>32	1	1	4
15	<i>P. aeruginosa</i>	+	+	>32	12	>32	4	2	8
17	<i>P. aeruginosa</i>	+	+	16	4	>32	2	0.5	16
18	<i>E. coli</i>	+	+	>32	>32	>32	32	16	16
20	<i>A. baumannii</i>	+	+	16	>32	>32	4	8	32
21	<i>P. aeruginosa</i>	+	+	2	16	>32	0.5	2	8
22	<i>A. baumannii</i>	+	+	>32	16	>32	16	16	32
23	<i>E. coli</i>	+	+	>32	>32	>32	4	4	16
24	<i>P. aeruginosa</i>	+	+	>32	>32	>32	12	16	32
25	<i>P. aeruginosa</i>	+	+	>32	>32	>32	8	8	16
26	<i>P. aeruginosa</i>	+	+	>32	>32	>32	4	16	32
28	<i>P. aeruginosa</i>	+	+	>32	>32	>32	32	16	16
29	<i>A. baumannii</i>	+	+	>32	>32	>32	8	16	32
30	<i>A. baumannii</i>	+	+	>32	16	>32	8	8	32
31	<i>P. aeruginosa</i>	+	+	4	16	>32	2	2	16
32	<i>P. aeruginosa</i>	+	+	>32	>32	>32	4	4	16
33	<i>En. cloacae</i>	+	+	32	>32	>32	4	4	16
34	<i>A. baumannii</i>	+	+	8	16	>32	2	2	16
35	<i>E. coli</i>	+	-	0.38	0.50	0.50	0.38	0.50	0.50
36	<i>A. baumannii</i>	+	+	>32	>32	>32	8	16	32
37	<i>P. aeruginosa</i>	+	+	16	>32	>32	4	32	32
38	<i>A. baumannii</i>	+	+	>32	>32	>32	16	16	32
40	<i>P. aeruginosa</i>	+	+	>32	>32	>32	32	8	32
41	<i>P. aeruginosa</i>	+	+	>32	>32	>32	16	32	>32
44	<i>P. putida</i>	+	+	>32	>32	>32	4	4	16
45	<i>A. baumannii</i>	+	+	>32	>32	>32	16	8	32
48	<i>P. aeruginosa</i>	+	+	>32	>32	>32	8	16	16
49	<i>A. baumannii</i>	+	+	>32	>32	>32	16	16	16
51	<i>A. baumannii</i>	+	+	>32	>32	>32	16	16	32
52	<i>P. aeruginosa</i>	+	+	12	12	>32	2	2	16
53	<i>E. coli</i>	+	+	16	>32	>32	2	8	16
54	<i>P. aeruginosa</i>	+	+	4	>32	>32	2	4	16
55	<i>K. pneumoniae</i>	+	+	8	16	>32	2	2	8

Isolate number	Name Carbapenem-resistant isolate	Type of ESBL		Carbapenemase non-PCR positives					
				MIC ($\mu\text{g/ml}$) before curing			MIC ($\mu\text{g/ml}$) after curing		
		TEM	SHV	Imp	Mer	Ert	Imp	Mer	Ert
56	<i>A. baumannii</i>	+	+	16	>32	>32	8	32	32
57	<i>P. aeruginosa</i>	+	+	4	>32	>32	2	4	8
58	<i>P. aeruginosa</i>	+	+	8	4	>32	0.5	2	16
59	<i>P. aeruginosa</i>	+	+	6	3	>32	2	1	16
61	<i>P. aeruginosa</i>	+	+	>32	16	>32	16	8	32
63	<i>P. aeruginosa</i>	+	+	16	>32	>32	8	32	32
65	<i>P. aeruginosa</i>	+	+	>32	>32	>32	32	16	32
66	<i>P. aeruginosa</i>	+	+	>32	>32	>32	16	16	32
67	<i>E. coli</i>	+	-	2	0.125	16	0.5	0.125	4
68	<i>E. coli</i>	+	+	4	0.19	4	1	0.12	16
69	<i>A. baumannii</i>	+	+	16	32	>32	2	8	16
70	<i>P. aeruginosa</i>	+	-	4	0.50	>32	2	0.50	4
71	<i>P. aeruginosa</i>	+	+	16	8	>32	4	2	16
73	<i>A. baumannii</i>	+	+	>32	16	>32	8	16	16
75	<i>A. baumannii</i>	+	+	>32	>32	>32	>32	>32	>32
77	<i>A. baumannii</i>	+	+	>32	>32	>32	16	16	32
78	<i>P. putida</i>	+	+	>32	>32	>32	8	8	16
79	<i>P. aeruginosa</i>	+	+	>32	>32	>32	>32	>32	>32
80	<i>P. aeruginosa</i>	+	+	8	16	>32	2	4	16
81	<i>P. aeruginosa</i>	+	+	0.38	6	16	0.12	2	8
82	<i>A. baumannii</i>	+	+	32	>32	>32	16	8	16
83	<i>P. putida</i>	+	+	0.38	16	32	0.38	4	16
85	<i>E. coli</i>	+	+	0.38	0.25	4	0.38	0.25	2
86	<i>P. aeruginosa</i>	+	+	>32	>32	>32	>32	>32	>32
87	<i>P. aeruginosa</i>	+	+	>32	32	>32	8	8	16
88	<i>P. putida</i>	+	+	>32	>32	>32	16	16	32
90	<i>P. putida</i>	+	+	>32	>32	>32	4	4	8
91	<i>En. cloacae</i>	+	+	>32	>32	>32	>32	>32	>32
92	<i>P. putida</i>	+	+	>32	>32	>32	16	32	32
93	<i>E. coli</i>	+	+	>32	>32	>32	>32	>32	>32
94	<i>A. baumannii</i>	+	+	6	12	>32	2	4	4
95	<i>En. cloacae</i>	+	+	32	>32	>32	16	32	32
96	<i>P. aeruginosa</i>	+	+	16	8	32	4	4	16
97	<i>E. coli</i>	+	+	16	>32	>32	8	32	16
99	<i>P. aeruginosa</i>	+	+	>32	>32	>32	>32	>32	>32
101	<i>P. aeruginosa</i>	+	+	8	8	>32	2	4	16
103	<i>A. baumannii</i>	+	+	1	4	32	1	2	16
104	<i>P. aeruginosa</i>	+	+	16	>32	>32	16	16	32
106	<i>A. baumannii</i>	+	+	>32	>32	>32	8	16	16
107	<i>A. baumannii</i>	+	+	16	>32	>32	4	16	16
109	<i>P. aeruginosa</i>	+	+	>32	>32	>32	16	16	16
110	<i>P. aeruginosa</i>	+	+	>32	>32	>32	16	8	32
111	<i>P. aeruginosa</i>	+	+	16	>32	>32	4	4	16
Control	<i>E. coli</i> Top 10	-	-	0.125	0.12	0.50	0.125	0.12	0.50

Note: Imi= imipenem, Mer= meropenem, Ert= ertapenem, += positive, -= negative

4.3.6 Plasmid DNA restriction pattern of cured carbapenemase-positive gene carriers

Analysis of both undigested and restriction enzyme (*EcoRI* and *HindIII*) digested plasmids of the cured 26 PCR-positive CR isolates were analysed by agarose gel electrophoresis in order to study plasmid resistance gene patterns. The restriction patterns were found to be non-identical in almost all of the cured PCR-positive CR isolates with their corresponding differences in band sizes ranging from 3 to > 6 bands, indicating that the isolates were harbouring diverse types of plasmids. However, profiles of a few cured CR isolates encoded with NDM-1 and VIM-1 genes respectively showed unclear similarities when carefully examined. The indication here is that dependent upon the type of gene (NDM-1 or VIM-1), those few CR isolates may have common shared genes but remain genetically unrelated. A representative restriction analysis of plasmid-cured isolates of the carbapenemase-positive gene carriers from the Top 10 *Escherichia coli* competent cells depicting different plasmid band sizes are shown in Figure 4.5 and 4.6. Gel (A) was without restriction enzymes digestion, showing relatively low number of band lanes with no significant relatedness or similarities, while in gel (B) with digested endonucleases (*EcoRI* and *HindIII*) displaying different β -lactamase genes in the cured CR isolates with increased number of band lanes (Figure 4.5).

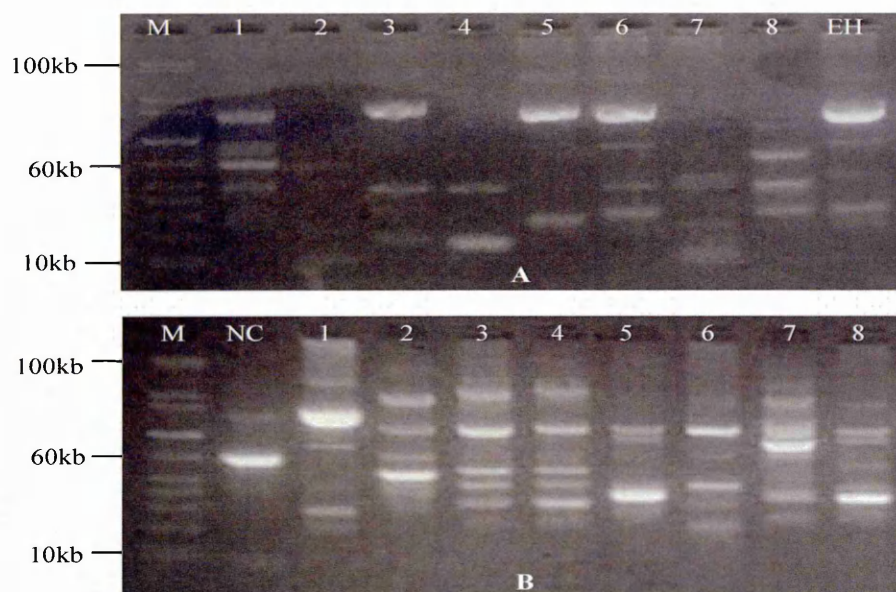


Figure 4.5 Undigested and digested restriction enzyme profiling from cured carbapenemase-positive gene carriers

Note: M= DNA marker; lanes, 1 to 8= plasmid-cured carbapenemase-positive producers; EH= *EcoRI*- *HindIII*-digested marker; NC= *Escherichia coli* Top 10 recipient cells. Gel A: (undigested) profile; lane 1 through to 8 representing CR numbers (27, 39, 42, 43, 46, 47, 50 and 60) are showing different band patterns of the cured carbapenemase-positive producers. Gel B (digested) profile; corresponding to the same respective cured carbapenemase producers in (gel A) showing diverse plasmid band patterns, suggesting that they harbour different plasmids

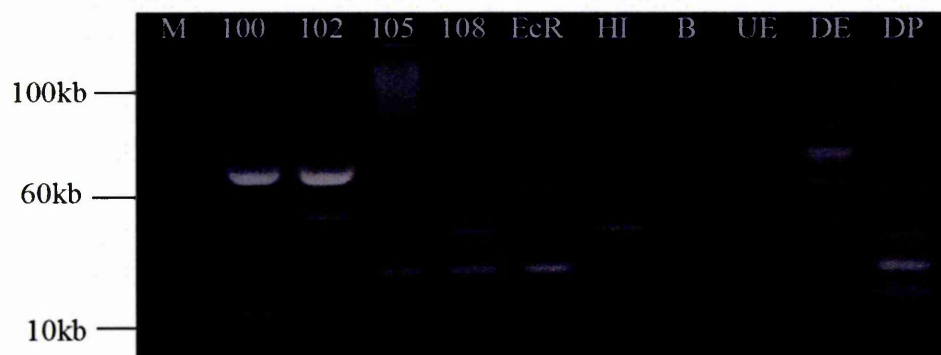


Figure 4.6 Profiling of restriction enzyme digestion from cured carbapenemase-positive gene carriers

Note: M= DNA ladder marker; Lanes, 100, 102 105 and 108= plasmid-cured carbapenemase producers; EcR= *EcoRI*-digested; HI= *HindIII*-digested; B= blank; UE= undigested *Escherichia coli* Top 10 recipient cells; DE= digested *Escherichia coli* Top 10 recipient cells; DP= digested *Pseudomonas aeruginosa* (ATCC 27852) control strain. In this gel, all the plasmid-cured carbapenemase producers are showing diversity in band patterns (see also appendix 5).

4.4 Discussion

Plasmids are sometimes considered as non-essential elements found in normal bacterial growth. However, the spread of plasmids and acquired antimicrobial resistance genes are a major clinical challenge (Pitout and Laupland 2008). TEM and SHV families are commonly detected from Enterobacteriaceae species in Africa, while CTX-M ESBL types are predominantly found in East Asia and Europe (Dhillon and Clark 2012).

Since the discovery of TEM and SHV types in the 1980s, the enzymes have been widely reported in Egypt (Borg *et al.* 2006), South Africa (Bamford *et al.* 2011), and recently in Ghana (Opintan *et al.* 2015). Expressions of these common ESBLs have been also detected in common non-fermenting organisms, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Jacoby and Munoz-Price 2005, Meyer *et al.* 2010, Heizmann *et al.* 2013). Infections caused by multidrug-resistant Gram-negative bacteria, including *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates, have been found to co-harbour ESBLs, AmpC-type β -lactamases and plasmid-encoded carbapenemase resistance genes (Kanj and Kanafani 2011, Armand-Lefèvre *et al.* 2013, Carattoli 2013). The presence of these resistance genes poses diagnostic and therapeutic challenges. Early recognition or detection of these resistance genes makes them more preventable and their spread into susceptible pathogens to cause severe infections in many sensitive areas such as ICUs and transplantation units in many health-care facilities can be controlled.

The aim of this study was to perform plasmid analysis on CR isolates from Ghanaian hospitals to ascertain the presence, prevalence and distribution of plasmid-borne β -lactamases including carbapenemase resistance genes. The main findings have shown high-levels of common ESBLs probably due to frequent exposure to drugs such as penicillins and third generation cephalosporins. Similar resistance mechanisms have been observed in other studies in which the loss of porin expression or ESBL enzymes in association with ampC β -lactamases and expression of common efflux pumps play a role in the acquisition of carbapenemase resistance in Gram-negative bacterial pathogens (Armand-Lefèvre *et al.* 2013, Meletis 2016).

In this study, high-levels of the common traditional enzymes, TEM- and SHV-ESBL types were detected in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates, which may have contributed to the resistance of carbapenem antimicrobials. Several studies have reported that bacterial production of ESBL enzymes serve as the first-step in the development of bacterial resistance. Genetically, *bla*TEM and *bla*SHV types are derived from a simple point mutation and largely linked to cross infection in health-care facilities because of persistent exposure to β -lactams without the use of carbapenems and cephamycins (Shah *et al.* 2004, Varkey, Balaji and Abraham 2014).

Plasmids act as intermediaries in the dissemination of mobile elements, aiding antimicrobial resistance especially among Gram-negative bacterial pathogens (Raj 2012), and movements of these resistance genes have been the focus of intense study in global antimicrobial resistance (Nordmann, Naas and Poirel 2011, Pitout, Nordmann and Poirel 2015). Plasmid profiling revealed that carbapenemase genes co-existed with the TEM-1 and SHV-1 β -lactamase enzymes which also may have compounded the resistance to carbapenems. The effect of the β -lactamase enzymes was evidently

observed in all the cured CR isolates by the reduction in susceptibility testing to one or two of the carbapenems when compared with the original CR isolates tested. This notable behaviour confirms the mobile resistance genes were located on plasmids including TEM- and SHV-type genes and this finding is in agreement with the Raj (2012) study that found a single plasmid that when lost during curing experiments at an elevated temperature of 45°C, plasmid-borne resistance was eliminated (Raj 2012). In contrast, in another curing experiment conducted by Saranathan *et al.* (2014) the loss of three or more plasmids did not eliminate multidrug resistance in two cured *Acinetobacter baumannii* isolates. The resistance pattern of the two isolates remained the same when tested with ceftriaxone and carbapenems after acridine orange was used in the curing process (Saranathan *et al.* 2014). Although, curing agent (acridine orange) was not used in this present study, the findings here seemed to suggest that there were inherent chromosomal resistance possibly in association with plasmid-borne multidrug resistance genes that augment the resistance in *Acinetobacter baumannii* isolates. It is now well understood that ESBL encoding genes that co-exist in poly-microbial infections may easily disseminate into other genera or within species of the same strain causing outbreaks in hospitals or in community settings (Dhillon and Clark 2012). The risks of outbreaks in such environments cannot be overemphasised; strains harbouring these resistance genes may disseminate, especially in ICUs, during mechanical ventilation, emergency surgery and during urinary catheterisations (Enoch, Birkett and Ludlam 2007, Meletis *et al.* 2012).

There are other β -lactamases such as CTX-M and AmpC-types that have clinical relevance and present a threat to growing number of Gram-negative bacterial resistance observed in severe infections in recent times (Naas *et al.* 2011, Ma *et al.* 2015). Frequent urinary pathogens, usually from urethral catheterisation have been identified

as having high-levels of multidrug resistance largely driven by plasmid β -lactamase enzymes (Motayo *et al.* 2013). These two enzymes, CTX-M and AmpC-type β -lactamases were not determined due to unavailability of control strains during the study. However, these two β -lactamase enzymes in combination with non-enzymatic mechanisms (e.g. efflux pump systems) have been found in most Gram-negative bacterial pathogens and may be contributing to the increasing array of resistance to carbapenems. A recent study in India, found a prevalence of 84% for *bla*CTX-M-15 among ESBL isolates, either alone or co-harboursing *bla*NDM-1-producing gene (Khajuria *et al.* 2014). Previous studies have reported TEM-1 and CTX-M-15 as the most frequently encountered ESBL enzymes detected in the NDM-1-possessing Enterobacteriaceae in India (Lascols *et al.* 2011, Khan and Nordmann 2012), Africa (Poirel *et al.* 2011b) and Norway (Samuelsen *et al.* 2011). Interestingly in this study, 7.2% of plasmid NDM-1 resistance among *Acinetobacter baumannii* isolates, in which *bla*TEM-1 and *bla*SHV-1 co-existed as additional enzymes probably contributing to CR Gram-negative bacterial resistance, is reported for the first time.

In this present study, plasmid profiling has shown that resistance genes were carried on plasmids. On average, the original plasmid-bands of seven were reduced to four or three after the curing experiments. However, the majority of the profiles obtained from diverse species of CR pathogens studied, including the cured carbapenemase-positive gene carriers, showed different patterns of band sizes from plasmids. The indication was that most of the CR isolates were carrying different unknown plasmids from their various hospitals where they were isolated. In susceptibility testing by the disc diffusion method, all cured isolates showed a significant reduction in MIC to either one or two carbapenems tested. MIC assays performed against carbapenems (imipenem, meropenem and ertapenem) were observed to have 2- or 4-fold reductions in MIC

values, except in one of the cured carbapenemase-producing *Acinetobacter baumannii* isolates and eight (7.2%) of the cured carbapenemase non-PCR positives that maintained the same MIC levels of >32 µg/ml as previously tested in the original state. The results of the MIC assays were suggestive of chromosomal mediated resistance, ineffective curing or that there were no relevant plasmids for this isolate. Additionally, there could be some mimicking effects of non-plasmid mediated resistance and/or AmpC-type of β-lactamases that co-existed with the carbapenemase resistance genes. Other studies have suggested that the presence and alteration of specific outer membrane proteins may significantly contribute to carbapenemase resistance (Landman, Bratu, Quale 2009, Mataseje *et al.* 2011).

Other important findings include the observation that no two plasmid-borne carbapenemase-producing genes were detected in a single CR isolate, and the absence of *blaKPC-1* and *blaIMP-1* genes in all the plasmid DNA samples. These two carbapenemase resistance genes, due to their limited spread, are scarcely experienced in most studies conducted in Africa (Manenzhe *et al.* 2014). This phenomenon across the African continent is in agreement with the present study.

In this study, restriction enzyme digestion profiling of the cured carbapenemase-positive gene carriers exhibited different plasmid-mediated resistance genes except in a few CR isolates depending on the species. Notably, there are isolates commonly encountered from the reservoir of intestinal flora which were among the CR isolates studied, especially, the cured carbapenemase-positive gene carriers: *Providencia stuartii*, *Klebsiella pneumoniae*, *Escherichia coli* and *Shigella sonnei* isolates. These organisms may easily acquire plasmid-borne resistance genes from different sources of specimen

within the four different regional hospital centres and may pose challenges in the evaluation of band size similarities. This study was able to identify a small number of cured CR isolates that have some unclear identity on the agarose gel electrophoresis. The band patterns were commonly linked to *bla*NDM-1- and *bla*VIM-1-encoded genes rather than to the OXA-48 gene with slight differences in the band sizes of the digested plasmid-mediated DNA occurring in *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates. This finding is in agreement with a study conducted by Rasheed *et al.* (2008) in which two different multidrug-resistant Gram-negative bacterial pathogens harbouring a common plasmid from two different care-receivers in the same hospital were found at identical band sizes. This indication showed that there was some evidence of horizontal dissemination of plasmid-borne carbapenemase resistance genes between the two clinically important pathogens by restriction digestion (Rasheed *et al.* 2008).

Globally, plasmid analysis has increased awareness of the various types of plasmids in most outbreak situations circulating within a particular region or area (Tato *et al.* 2010). Plasmids investigations are crucial in clinically relevant pathogens usually by PCR-based assays and sought to reveal differences or similarities on agarose gel electrophoresis during an outbreak situation to assist in the development of an immediate treatment action plan. Currently, the spread of carbapenemase resistance genes are largely linked to horizontal transfer of an array of plasmid-borne β -lactamase genes. Several epidemiology studies, for instance, have recognised a high diversity of plasmid resistance genes linked to the *bla*NDM genes, particularly, *IncA/C* types mainly spreading into the Enterobacteriaceae group (Poirel *et al.* 2011a, Carattoli *et al.* 2012). Others such as *IncFII*, *IncH*, *IncL/M* and *IncN* types have also been found linked to the appearance of *bla*NDM, often in association with clinically important antimicrobial resistance genes notably, CMY-type β -lactamases for cephalosporin

resistance, *qnrA* for quinolone resistance, and *rmtA* and *rmtC* also responsible resistance genes to aminoglycosides (Pitout, Nordmann and Poirel 2015). The capture of *bla*VIM-1 has been commonly detected in integrons harbouring *aadA* and *aacA4* cassettes, usually such plasmids were found in the population of *Pseudomonas* or Enterobacteriaceae which are widely disseminated in Europe (Tato *et al.* 2010). In contrast, several laboratory-based studies have reported that the detection of the VIM-2 gene is limited to plasmid-mediated *Pseudomonas aeruginosa* isolates: 100% (57/57) in Kenya (Pitout *et al.* 2008), 100% (30/30) in Tunisia (Ktari *et al.* 2011), 73% (11/15) in South Africa (Jacobson *et al.* 2012) and 58% (7/12) in the Ivory Coast (Jeannot *et al.* 2013). On a different note, OXA-48-producing isolates have been recognised to have only one type of plasmid-borne OXA-48a at a range size of 62 kb which belongs to the *IncL/M* group and currently found in many geographical locations which are different from those observed with the *bla*NDM genes (Poirel, Bonnin and Nordmann 2012).

There were several limitations to this plasmid study analysis, some plasmid-encoded genes of interest could not be determined because of the non-availability of resources and materials. Firstly, lack of experimental reagents and various primer-sets to extend the investigations to plasmid-mediated CTX-M and *AmpC* β -lactamase genes, including relevant antimicrobial resistance genes of significance in the health care delivery system such as: *qnrS* and *qnrB* for fluoroquinolone resistance and *RmtA* and *RmtC* for aminoglycoside resistance genes. Other detailed investigations into aminoglycoside-modifying enzymes (AMEs) for aminoglycoside resistance gene cassettes such as acetyltransferases AAC (3)-Ia (*aacC1*), nucleotidyl transferases ANT (2'')-Ia (*aadB*), ANT(3'')-Ia (*aadA1*) and phosphotransferases APH (3')-Via (*aphA6*), conferring gentamicin resistance in *Acinetobacter baumannii* isolates have been critically evaluated (Aliakbarzade *et al.* 2014). These resistance genes are on plasmids, otherwise,

further investigations may have revealed associations with carbapenemase-producing genes since ciprofloxacin and gentamicin or both used in combination have been frequently used to manage highly resistant Gram-negative bacterial infections in Ghanaian hospitals (Opintan *et al.* 2015). Secondly, standard control strains on plasmids such as pCTX-M, pAmpCs and pAMEs were unavailable during the conjugation experiments. These control strains may have served as markers for obtaining reasonable band lanes to match with the unknown plasmid-borne resistance genes for more comprehensive comparison of the various plasmid bands on gels.

The results generated from this study, suggest that genes encoding for most the plasmids were unknown at different band locations. Further studies are needed in the transfer experiments in order to identify various resistance genes which may incapacitate carbapenem antimicrobials. An area of interest is the mechanism of spread of resistance genes than growth and division of the initial transconjugant cells. By simple conjugation method, the number of transconjugant cells over time intervals that would retain the resistance genes could be identified. The findings may assist determination of conjugation frequency rate and also put in place control measures of any possible outbreak of CR isolates that may occur in hospitals.

In this study, common plasmid resistance genes and some unknown resistance genes may have contributed to resistance of carbapenemases in both carbapenemase non-PCR positives and carbapenemase-positive gene carriers. The curing method by using high temperature was simple and successfully eliminated most plasmids with reduced susceptibility to the carbapenems tested. The diversity of the CR isolates investigated was broad, those in association with carbapenemase resistance genes showed substantial prevalence of unknown plasmid-borne patterns with a high-level of ESBL enzymes. The findings of the transfer experiments had also shown that if strict measures are not taken to curb infection, rapid spreading of resistance genes may spread from carriers to commensal organisms living in a poly-microbial environment (e.g. the gastrointestinal tract) by conjugation. In order to curb the spread of infections, clinicians would be advised to separate infected patient from contact with uninfected persons in the hospital. The presence of these resistance genes is attributable not to clonal spread, but rather, may aide the dissemination of plasmids among genetically diverse isolates that are found in the hospital facilities.

5. Genetic relatedness of carbapenem-resistant isolates from Ghana using ERIC-PCR technique

5.1 Background

The enterobacterial repetitive intergenic consensus (ERIC) technique is a molecular method that has been added to the methods used for the epidemiological analysis and genotyping of bacterial species. The application of ERIC offers a greater potential for the study of the development of bacterial interspersed repetitive arrangements because sequences are lengthier without targeting a specific region of the genome. In addition, it offers detailed information to enable the comparative analysis in a wider range of bacterial species (Wilson and Sharp 2006, Stefańska, Rzewuska and Binek 2008).

It is noteworthy that the ERIC technique has been used in different areas of research including veterinary microbiology (Guimarães *et al.* 2011), food microbiology (Munoz *et al.* 2011) and in particular, various clinical areas dealing with human infections within the hospital environment (Rai *et al.* 2014, Durmaz *et al.* 2015). Indeed, ERIC-PCR can be used to study human pathogens, in both Gram-positive and -negative bacterial isolates in order to determine their genetic diversity, and occasionally extended to bacterial pathogens in the animal kingdom. Further, genomic DNA fingerprints from ERIC-PCR have proved to be useful for the investigation of other organisms apart from those in the Enterobacteriaceae family such as *Aeromonas* species (Szczuka and Kaznowski 2004), *Staphylococcus aureus* (Sabat *et al.* 2006) and *Haemophilus parasuis* (Wei *et al.* 2011). More recently, the application of ERIC-PCR has been used to investigate opportunistic pathogens, *Pseudomonas aeruginosa* and *Acinetobacter*

baumannii isolates capable of causing outbreaks in hospitals worldwide (Urban, Segal-Maurer and Rahal 2003, Nigro, Post and Hall 2011, Siqueira *et al.* 2013, Freeman *et al.* 2015). Notably, isolates from *Pseudomonas* and *Acinetobacter* can easily acquire multiple resistance to a panel of antimicrobials including carbapenems, share common genes, transfer resistant traits both intra and interspecies, and the encoded genes they carry are extremely mobile, particularly in *Pseudomonas aeruginosa* (Armand-Lefevre *et al.* 2013, Meletis 2016). Good reproducibility and applicability in the determination of closed relatedness have been reported in several studies on a variety of pathogens, which share the same homogeneities. For most Gram-negative bacterial strains, diversity/similarity among pathogens have traditionally been determined using antimicrobial resistance patterns, basic microbiological methods or genotyping methods such as by gene expression using microarray technology, multilocus sequence typing (MLST), pulsed field gel electrophoresis (PFGE) and Enterobacterial repetitive intergenic consensus (ERIC). Irrespective of the species they belong to, the ERIC technique has been used to study *Enterobacter sakazakii* (Ye *et al.* 2009), *Pseudomonas aeruginosa* (Stehling, Leite and Silveira 2010, Kidd *et al.* 2011) and *Acinetobacter baumannii* isolates (Viana *et al.*, 2011).

Results presented in Chapters 3 and 4 have identified the presence of three types of carbapenemase genes (NDM-1, VIM-1 and OXA-48) that were associated with the common plasmid-borne ESBL β -lactamases (TEM-1 and SHV-1) and unknown resistance genes of importance in the profiling analysis of plasmids which have not yet been identified. In most cases, the ERIC assay has generated DNA patterns, which enable the differentiation of plasmid-borne resistance genes found in pathogenic organisms. Several studies have shown remarkable successes using ERIC-PCR assay to distinguish bacterial strains among diverse species (Xia *et al.* 2012, Ramazanzadeh,

Zamani and Zamani 2013, Martins *et al.* 2014, Filgona, Banerjee and Anupurba 2015, Abdalhamid *et al.* 2016). To date, there are no reports on the investigation of the presence of β -lactamase determinants in CR isolates and their clonal relatedness or variabilities in Ghana. This study aimed to broadly determine the genetic relatedness or variability in both carbapenemase non-PCR positive and PCR-positive isolates by species, and separately assess clonal similarities same for all the PCR-positive species using fingerprint patterns generated by ERIC-PCR amplifications.

The specific objectives were to:

- Perform genomic DNA typing of carbapenem-resistant isolates using ERIC-PCR assay technique
- Determine the various genetic diversities of all CR isolates with regard to the presence of the common ESBL β -lactamases and carbapenemase resistance genes acquired in the four regional hospitals
- Determine clonal similarities of CR isolates in relation to the bacterial species and source of specimens
- Detect the genetic relatedness of all PCR-positive species linked with the resistance genes identified.

5.2 Method summary

Genomic DNA samples of all CR isolates were amplified using the ERIC-PCR assay technique to construct the dendrograms. Positions of the band lanes on each agarose gel electrophoresis were normalised using the standard 1 kb DNA molecular marker. The location of each given band was located as one and no band as zero. The nearest band patterns of each bacterial species were used to analyse the similarity or variability matrix calculated by the number of base differences. Dendrograms of ERIC-PCR fingerprint patterns were assembled for both PCR carbapenemase negatives and carbapenemase positive gene carriers together and separately for all carbapenemase positive gene carriers based on each species using Gel ComparII image analysis software (version 6.6.11, Applied Maths, Kortrijk, Belgium), and applied the unweighted pair group method with arithmetic mean (UPGMA) cluster method. The full description is in Chapter two of this study.

5.3 Results

In this study, the ERIC molecular setup described in Chapter 2 section 2.9 was used. All genomic DNA samples and their relevant controls were successfully amplified using the PCR method. The presence of DNA banding patterns representing known or unknown resistance genes in all of the CR isolates, including 10 different Gram-negative bacterial pathogens comprising: *Pseudomonas*, *Acinetobacter*, *Klebsiella*, *Enterobacter*, *Providencia*, *Shigella*, *Cronobacter*, *Sphingomonas* species and *Escherichia coli* were analysed. Gel electrophoretic patterns revealed high level of variation in which a majority of the fingerprints resulted in single or multiple DNA yields with 1 to 8 bands ranging from 50 to 800 bp. Band patterns of 93 complex dissimilarities were visually distinguished from the 111 CR isolates studied, while the remaining 18 showed band

similarities in pairs: sample number 1 & 14, 12 & 18, 26 & 36, 33 & 35, 38 & 39, 41 & 45, 42 & 43, 58 & 59 and 94 & 97. Figure 5.1 shows a representative band patterns in which sample number 58 exhibits band relatedness to sample 59 by visual inspection. Other band patterns of CR isolates and control strains by ERIC-PCR profiling are shown on agarose gel electrophoresis (see Appendix 6).

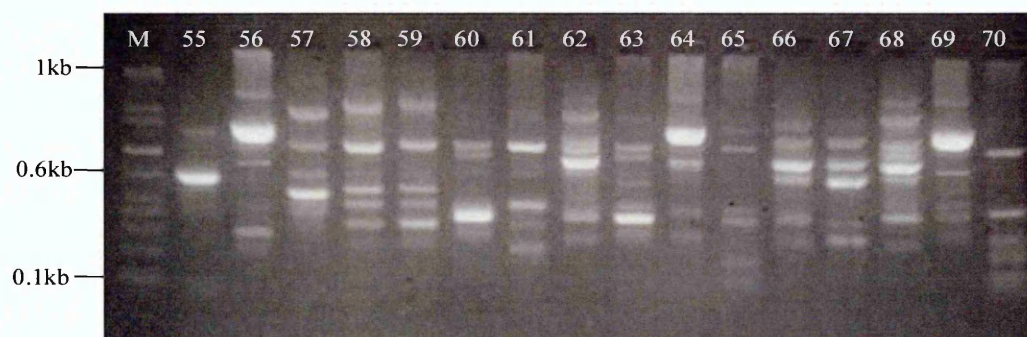
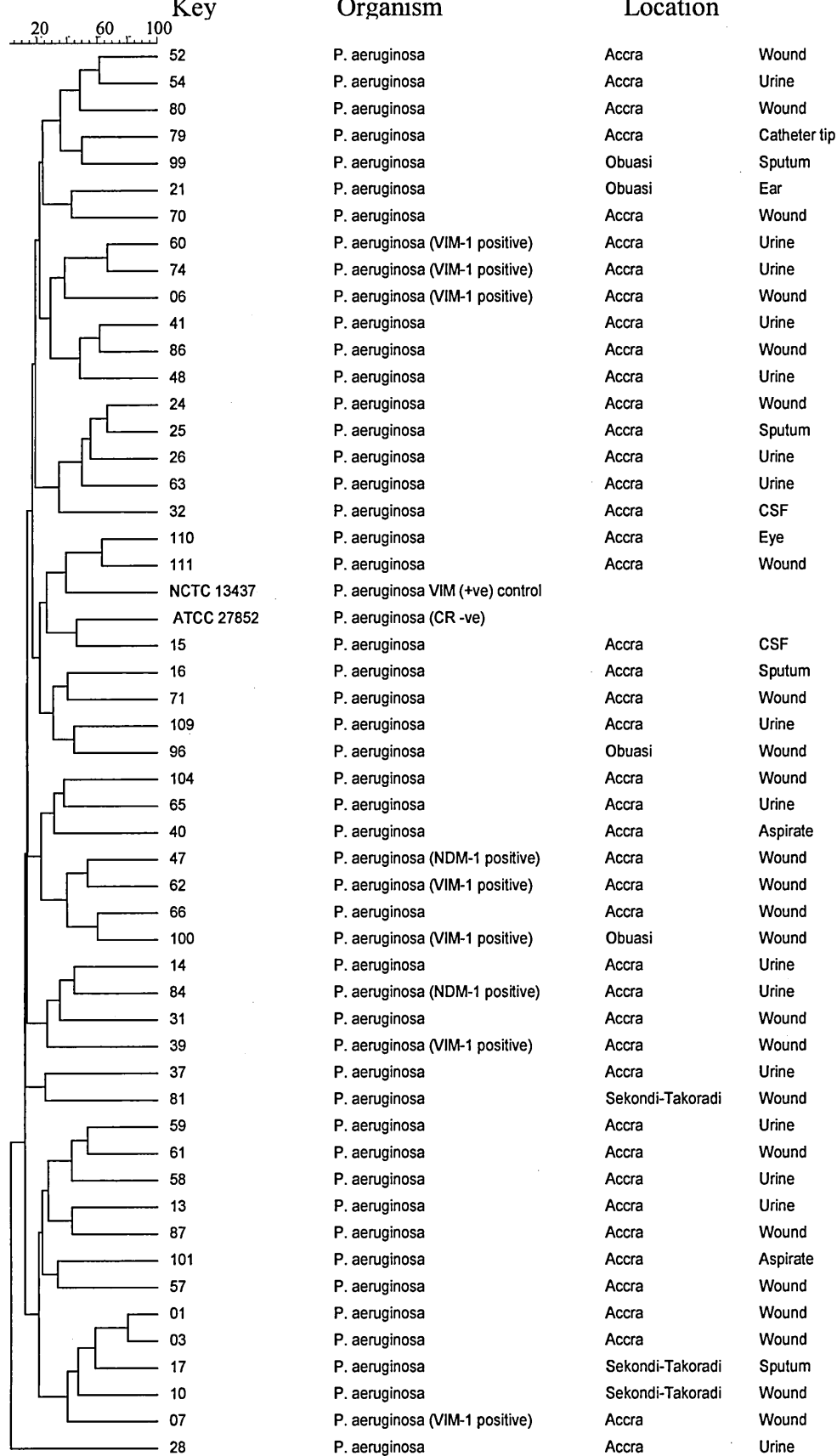


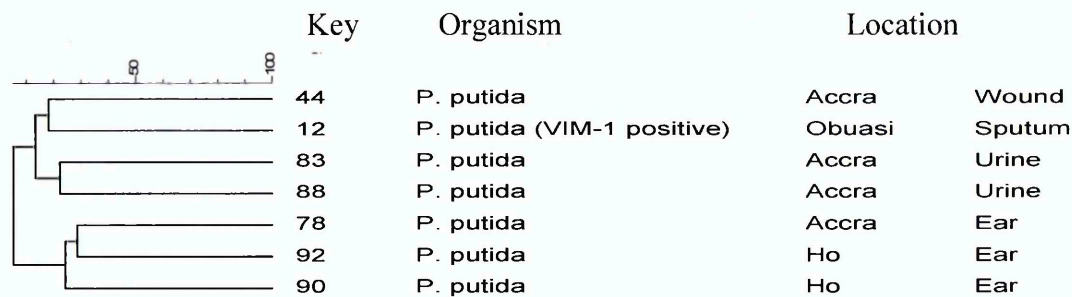
Figure 5.1 Representative example of ERIC fingerprints of different carbapenem-resistant isolates on agarose gel electrophoresis

Note: M= DNA 1 kb marker, 55 through to 70= CR isolate number listed in Appendix 1. By visual inspection sample numbers 58 & 59 are showing close relatedness on the gel.

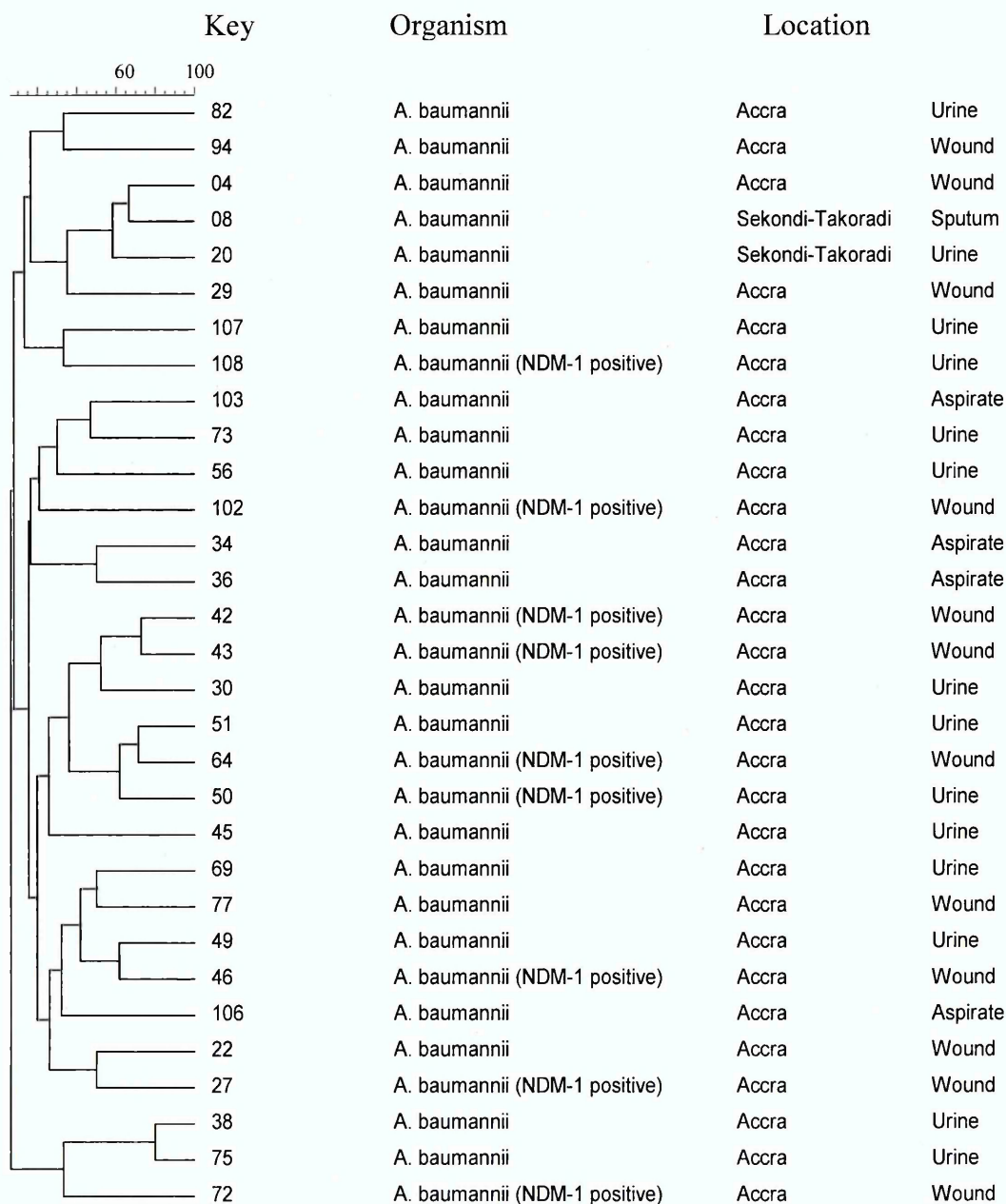
The dendrogram data from the computer-designed analysis for both carbapenemase non-PCR positives and PCR-positive CR isolates were evaluated together to analyse the genetic diversity or close relatedness.



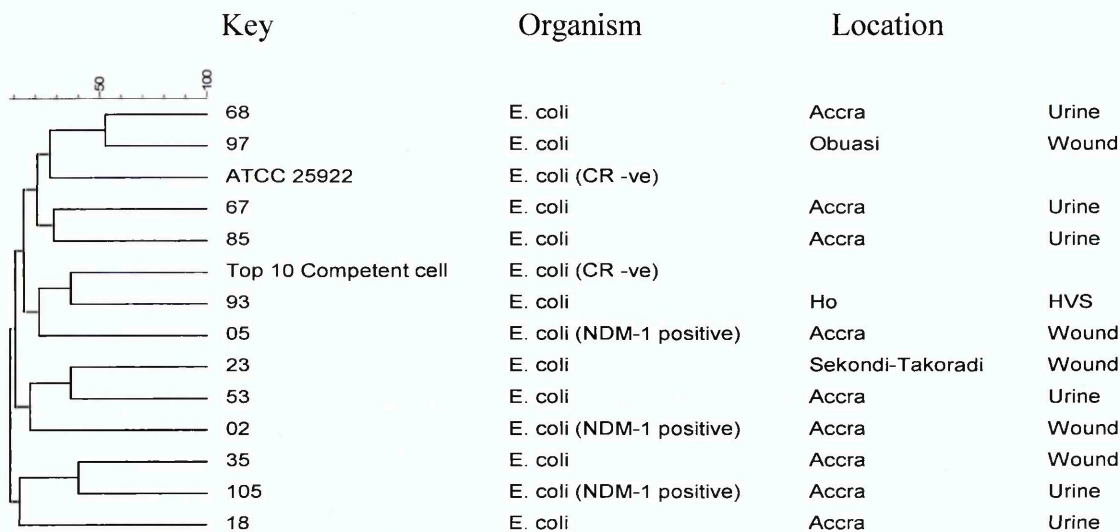
Group A: *P. aeruginosa* CR isolates with control strains



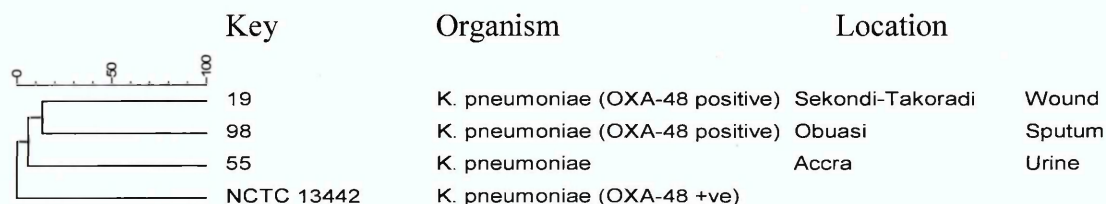
Group B: *P. putida* CR isolates



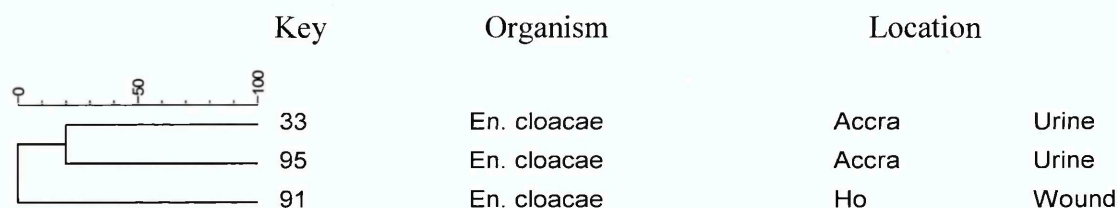
Group C: *A. baumannii* CR isolates



Group D: *E. coli* CR isolates with control strains



Group E: *K. pneumoniae* CR isolates with control strain



Group F: *En. cloacae* CR isolates

Figure 5.2 Dendrogram generated from ERIC-PCR genomic DNA products for

both carbapenemase non-PCR positives and carbapenemase-positive gene carriers

Note: Key= isolate number or control strain/type number and CR= carbapenem-resistant. Regarding the type of isolate, resistance gene, hospital location and source of specimen, the following groupings based on type of species: Group A (*P. aeruginosa*) 60 & 74 (VIM-1 positive) and 1 & 3; Group B (*P. putida*) 83 & 88; Group C (*A. baumannii*) 34 & 36, 42 & 43 (NDM-1 positive), 38 & 75 and 67 & 85; and Group F (*En. cloacae*) 33 & 95 are showing close relatedness in cluster-pairs.

Table 5.1 Genetic relatedness for both carbapenemase non-PCR positives and carbapenemase-positive gene carriers

Sample code number ^a	Name of carbapenem-resistant isolate	Regional hospitals				Type of specimen	ESBL β -lactamase genes		Carbapenemase genes		
		KBTH	ENRH	AGMH	HRH		TEM	SHV	NDM	VIM	OXA
60* & 74*	<i>P. aeruginosa</i>	+	-	-	-	Urine	+	+	-	+	-
1 & 3	<i>P. aeruginosa</i>	+	-	-	-	Wound	+	+	-	-	-
83 & 88	<i>P. putida</i>	+	-	-	-	Urine	+	+	-	-	-
34 & 36	<i>A. baumannii</i>	+	-	-	-	Aspirate	+	+	-	-	-
42* & 43*	<i>A. baumannii</i>	+	-	-	-	Wound	+	+	+	-	-
38 & 75	<i>A. baumannii</i>	+	-	-	-	Urine	+	+	-	-	-
67 & 85	<i>E. coli</i>	+	-	-	-	Urine	+	- ^b & +	-	-	-
33 & 95	<i>En. cloacae</i>	+	-	-	-	Urine	+	+	-	-	-

^a Cluster-paired sample numbers

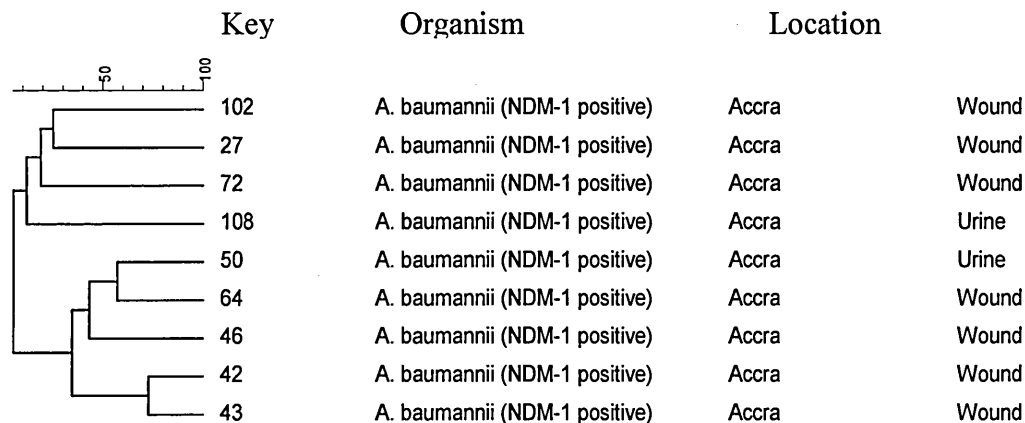
*Positive carbapenemase-positive gene carriers

^b SHV negative gene for isolate number 67

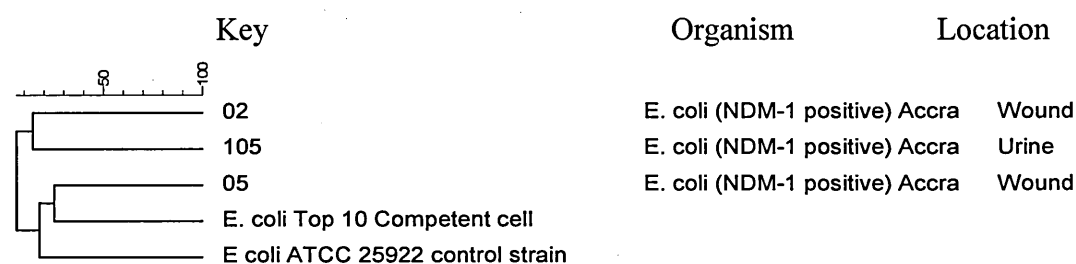
Note: += found in both, -= not found in both

For easier comparison and recognition, Table 5.1 is showing similarity details analysed from Figure 5.2. There was great dissimilarity among all the CR isolates, except for 6 of the *Acinetobacter baumannii*, 4 *Pseudomonas aeruginosa*, 2 each of *Pseudomonas putida*, *Escherichia coli* and *Enterobacter cloacae* isolates which demonstrated cluster-pair similarities, respectively. One cluster-pair each of *Acinetobacter* and *Pseudomonas aeruginosa* isolates were identified to carry NDM-1 gene (number 42 & 43 from wound samples) and VIM-1 gene (number 60 & 74 from urine samples), while the other cluster-pairs were carbapenemase non-PCR positive carriers (numbers: 1 & 3, 83 & 88, 34 & 36, 38 & 75, 67 & 85 and 33 & 95) with different ages; 58 & 31, 4 & 44, 13 & 55, 35 & 29, 1¹/₅₂ & 33 and 4 & 30 years, respectively. Interestingly, the close relatedness among the bacterial species were commonly observed from male patients (see Appendix 1) from a regional hospital (Korle Bu Teaching hospital, Greater Accra). Overall ERIC data obtained from diverse clinical specimens indicate that the majority of the CR isolates were not horizontally transmitted within the hospital, since few have shown similarities.

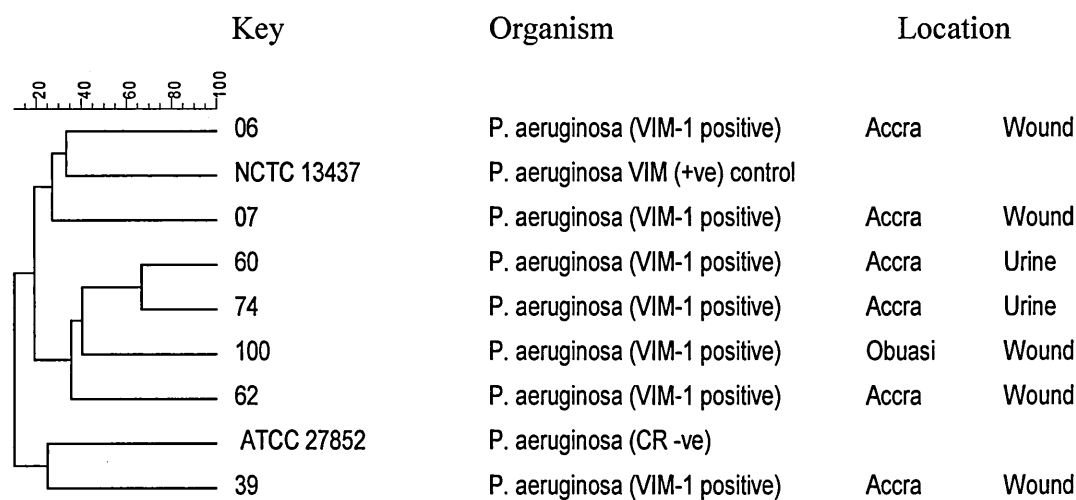
High genetic dissimilarity was also observed among the 26 PCR-positive carbapenemase carriers with few distinguishable patterns based on species of CR isolates (Figure 5.3). However, 2 cluster-pairs of *Acinetobacter baumannii* and cluster-pair *Pseudomonas aeruginosa* isolates were harbouring NDM-1 and VIM-1 genes, respectively.



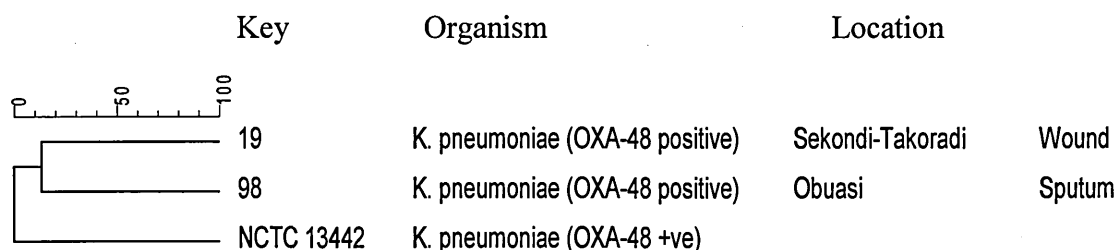
Group A: *A. baumannii* NDM-1 positive gene carriers



Group B: *E. coli* NDM-1 positive gene carriers with control strains



Group C: *P. aeruginosa* VIM1-1 positive gene carriers with control strains



Group D: *K. pneumoniae* OXA-48 positive gene carriers with control strain

Figure 5.3 Dendrogram generated from ERIC-PCR genomic DNA products for carbapenemase-positive gene carriers

Note: Key= isolate number or control strain/type number. Regarding the groupings based on type of species, type of resistance gene, hospital location and source of specimen, the following groupings based on type of species: Group A (*A. baumannii* NDM-1 positive) 102 & 27, 42 & 43 and 50 & 44 in urine and wound respectively; Group B (*E. coli* NDM-1 positive) 02 & 105 in wound and urine respectively; Group C (*P. aeruginosa* VIM-1 positive) 60 & 74 are showing close relatedness in cluster-pairs

For easier comparison, Table 5.2 illustrates the actual relatedness analysed from Figure 5.3. Strikingly, the only cluster-pair of OXA-48 carrying *Klebsiella pneumoniae* isolates were genetically related from male patients, however, they were observed to have come from different age groupings, specimens and hospitals in this study.

Table 5.2 Genetic relatedness among the carbapenemase-positive gene carriers

Sample code number ^a	Name of carbapenem-resistant isolate	Regional hospitals				Type of specimen	ESBL β -lactamase genes		Carbapenemase genes		
		KBTH	ENRH	AGMH	HRH		TEM	SHV	NDM	VIM	OXA
102 & 27	<i>A. baumannii</i>	+	-	-	-	Wound	+	+	+	-	-
42 & 43	<i>A. baumannii</i>	+	-	-	-	Wound	+	+	+	-	-
50 & 64*	<i>A. baumannii</i>	+	-	-	-	Urine & Wound	+	+	+	-	-
02 & 105*	<i>E. coli</i>	+	-	-	-	Wound & Urine	+	+	+	-	-
60 & 74	<i>P. aeruginosa</i>	+	-	-	-	Urine	+	+	-	+	-

^a Cluster-paired sample numbers

* Closely related but different specimens respectively

Note: += found in both, -= not found in both

5.4 Discussion

Recently, molecular characterisation based on ERIC fingerprinting has become a new area of interest for the analysis of bacterial similarity or variability found in ecological niches. In this context, ERIC-PCR is described as a simple, reliable, reproducible, cost-effective and discriminatory genotyping method appropriate for use in rapidly clustering genetically related microbial pathogens responsible for both hospital and community acquired infections (Wilson and Sharp 2006, Sabat *et al.* 2013, Durmaz *et al.* 2015). Other potential genotyping applications include: detection of strain diversity, gene mapping, epidemiology studies and taxonomic similarities (Guimarães *et al.* 2011).

The aim of the present study was to analyse the genetic relatedness or variability among CR isolates from Ghana. The study analysis has shown a high degree of genetic diversity among the CR isolates using the ERIC-PCR technique. ERIC-PCR fingerprints have proved the existence and expression of MBL-types namely; NDM-1- and VIM-1-type of genes following genomic DNA optimisation of both carbapenemase non-PCR positives and carbapenemase positive gene carriers. A study by Abdalhamid *et al.* (2016) described the expression of the two genes as highly transmissible on mobile elements that can easily spread from one patient to another in a health-care environment (Abdalhamid *et al.* 2016).

The difference in genetic diversity that existed between carbapenemase non-PCR positives and carbapenemase-positive producers was observed in the non-fermenting isolates including those in the Enterobacteriaceae group. ERIC-PCR typing showed distinguishable fingerprints for the 111 CR isolates. In assessing the patterns of all fingerprints, 83.8% (93/111) were observed to have substantial variability among the 10 diverse CR isolates recovered from the four hospitals. However, a small number of the

non-fermenting, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates were observed to have close relatedness. Of significance to this study, the ERIC-PCR profiling has shown the diversity that existed among the various species of pathogens within the CR isolates. Notably, in the carbapenemase-positive gene carriers, the only two cluster-pairs NDM-1 positive *Acinetobacter baumannii* and cluster-pair VIM-1 positive *Pseudomonas aeruginosa* isolates were only detected from the Korle Bu Teaching Hospital (KBTH) in the Greater Accra region (Table 5.1 and 5.2) and none of these cluster relations were detected in the three other regional hospitals studied.

The presence of these MBL-types of resistance genes, coupled with ESBL production and the unknown number of other resistance genes encoded in *Acinetobacter baumannii* isolate is of major concern in a hospital environment (Bassetti, Ginocchio and Mikulska 2011). The two isolates, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* have been described as environmental and opportunistic pathogens naturally adaptable in hospitals to cause serious infections, with mortality rates ranging from 18% to 61% (Nordmann and Poirel 2014). Conversely, clonal transfer of resistance genes are commonly associated with these non-fermenting isolates in several studies worldwide (Stehling, Leite and Silveira 2010, Kaase *et al.* 2011, Armand-Lefèvre *et al.* 2013, Diene and Rolain 2014, Mathlouthi *et al.* 2015).

The virulent characteristics of *Acinetobacter baumannii* and *Pseudomonas* species are known to cause serious infections, and have the propensity to colonise most health-care facilities and also the capacity to harbour antimicrobial determinant genes. Most vulnerable patients such as the elderly in ICUs, and children and babies admitted in NICU may be prone to infections caused by these organisms (Hewitt *et al.* 2013, Berezin, Solórzano and the Latin America Working Group on Bacterial Resistance

2014). An evidence based study carried out in the paediatric and NICU wards recovered emerging carriers of OXA-type carbapenemase genes in *Pseudomonas* and *Acinetobacter* species (Siqueira *et al.* 2013), while both species have been found as carriers of the MBL-type genes, commonly in *Acinetobacter* isolates (Bush and Jacoby 2010, Roca *et al.* 2012).

Berezin, Solórzano and the Latin America Working Group on Bacterial Resistance (2014) described the infections of *Pseudomonas* and *Acinetobacter* species as most critical when additionally associated with resistance genes for fluoroquinolones, tetracyclines, sulphonamides, and aminoglycosides encoded on the same moveable genetic elements (Berezin, Solórzano and the Latin America Working Group on Bacterial Resistance 2014). The presence of ESBL enzymes in these non-fermenting isolates is their common risk factors for carbapenemase resistance. Further, ESBL production becomes problematic when in association with carbapenemase resistance genes, usually identified with a reduced susceptibility to third-generation cephalosporins and quinolones, whereas on the contrary, in the Enterobacteriaceae group moderate susceptibility is observed in the same antimicrobial drug classes when phenotypically assessed (Armand-Lefèvre *et al.* 2013, Meletis 2016).

In the present study, a computer-generated profiling of ERIC-PCR revealed that 14.4% (16/111) of both the carbapenemase non-PCR positive and carbapenemase-positive isolates which displayed multiple band patterns comprising; 9 (8.1%) isolates of *Acinetobacter baumannii*, 4 (3.6%) *Pseudomonas aeruginosa*, 2 (1.8%) each of *Pseudomonas putida*, *Escherichia coli* and *Enterobacter cloacae* respectively showed cluster patterns. These few species were indicating a related origin of dissemination, especially those from the *Pseudomonas* and *Acinetobacter baumannii* species in this

study. A similar study conducted by Siqueira *et al.* (2013) found that small pocket groupings of *Pseudomonas* and *Acinetobacter* encoded with carbapenemase resistance genes were detected showing clonal similarities by the ERIC-PCR amplification technique in a Brazilian hospital (Siqueira *et al.* 2013). Of note, these findings were comparable to those in this present study, in which close relatedness were found 3 cluster-pair patterns of *Acinetobacter baumannii* isolates recovered from aspirate, wound and urine, and 2 cluster-pair *Pseudomonas aeruginosa* isolates from urine and wound specimens. Interestingly, the relatedness was identified in the same hospital, Korle Bu Teaching Hospital in the Greater Accra region. The significance of the findings attest to the fact that the hospital is the largest hospital in the study receives the largest number of patients and serves as the largest tertiary and referral centre in the whole country. However, close relatedness of ERIC-PCR fingerprints was unexpectedly observed between the 2 PCR-positive OXA-48 *Klebsiella pneumoniae* isolates since both were recovered from different hospitals; AGMH and ENRH, while the sample sites were also different; sputum and wound, respectively.

The findings presented here suggest high genetic diversity existed among the CR isolates may have harboured other unknown resistance genes that can potentially cause cross-transmission, together with the small number of positive NDM-1 *Acinetobacter* and VIM-1 *Pseudomonas aeruginosa* isolates identified as closely related in cluster-pair patterns by ERIC-PCR fingerprints. However, these resistance gene are emerging and may require further infection control measures to be implemented in the care facility. The significance of the genetic relatedness of the few cluster-pairs identified by ERIC-PCR has given an indication of the relatedness of carbapenemase genes in dissemination. Besides the common ESBLs (TEM-1 and SHV-1) detected, various banding patterns may be associated with other antimicrobial resistance genes. It is

noteworthy and of concern, that large numbers of non-PCR carbapenemase-positive *Acinetobacter* and *Pseudomonas* isolates may possibly be associated with non-carbapenemase-related resistance features or unknown resistance genes that can also disseminate into different bacterial isolates within the same health-care facility. Further studies on non-carbapenemase-related resistance need to be systematically carried out.

The multiple plasmid bands in these nosocomial non-fermenting pathogens were observed in this study, which have the capacity to harbour many resistance genes, make them a clinical concern as well as major public health threat. The multi-resistant nature of these bacterial pathogens to commercially available antimicrobials subsequently renders their treatment extremely challenging. Of clinical significance is the emergence of plasmid-encoded *AmpC* cephalosporinases. These *AmpC* enzymes are resistance determining attributes' to carbapenems that may be prevalent in those detected as carbapenemase non-PCR positive *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates. The production of *AmpC* enzymes may be implicated in both carbapenemase-positive producers and carbapenemase non-PCR positives and may have the ability to spread to other clinically relevant pathogens in the same hospital setting.

In summary, ERIC-PCR fingerprints have shown a great diversity among the species of Gram-negative bacterial pathogens and specimen collection sites in this study. There was a small number of cluster-pairs from both carbapenemase non-PCR positives and carbapenemase-positive gene carriers that exhibited close genetic relatedness, particularly in *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates. The presence of the carbapenemase genes in clinical isolates from Ghana is revealed for the first time. Therefore, necessary steps should be taken to contain the spread of resistance genes, most especially frequent transfers of patients to affected areas or wards within

the hospitals should be avoided. These study findings underpin the need to implementing serious and preventive measures to control resistance-gene dissemination into the Ghanaian population. The emerging worldwide threat of drug resistance requires that all countries make all effort to understand the trends of resistance patterns at a local and national level. Although current efforts can prevent the emergence of new resistance patterns they may not reverse existing patterns in the Ghanaian population.

6. Overall discussion, conclusion and future work

6.1 General discussion

This study aimed to apply various phenotype- and genotype-based techniques to identify and characterise carbapenemase resistance in Gram-negative bacterial pathogens in Ghana. A diverse number of carbapenemase resistance genes have been reported worldwide including many African countries. In Ghana, no known extensive studies have been carried out and published on carbapenemase resistance genes using clinical isolates.

It is noteworthy that the first VIM-2 gene found in *Pseudomonas aeruginosa* was isolated in Norway from a Ghanaian patient (Samuelsen *et al.* 2009), but since then there has been no follow-up studies to ascertain the presence and existence of the VIM-2 resistance gene in the Ghanaian population (Manenzhe *et al.* 2014). Indeed, since the detection of the VIM-2 gene in 2009, no molecular studies have been carried out on carbapenem resistance mechanisms in the country. To the knowledge of the author, this present study is the only research study that has explored the carbapenemase determining genes in clinical isolates. Thus, the presence of the VIM-2 gene had confirmed that the occurrence of VIM-types may have existed long ago among some species of *Pseudomonas* in the Ghanaian population.

The primary approach was to apply basic phenotypic tests and genotyping techniques available in Ghana that would easily detect and characterise the presence of carbapenemase resistance genes for the first time in the country. The emphasis was that Ghana as a developing country is faced with numerous constraints and resources deficiencies for investigating important health-related studies. In this regard, engaging in more sophisticated and laborious techniques may require collaborative support from a funding agency or the Ghana Government. Long delays of securing funding support and lack of sponsorship from funding agencies are some of the cumulative setbacks for carrying out meaningful and extensive studies in Ghana. The resources available were only to support conventional methods of culture and susceptibility testing, inexpensive phenotype-based tests that could easily be applied and the use of basic genotypic assay such as PCR technique. Importation difficulties, cumbersome procedures and unstable currency exchange rate systems are major challenges, compounded with unusual delays at the ports on receipt of purchased chemicals, reagents and consumables. Sometimes importation items are rendered unreliable, when reagents that require freezing at an ambient temperature of -20 °C at the ports are usually kept under poor conditions due to frequent electricity power outages.

In Ghana, carbapenems are not used in the management of patients with serious bacterial infections except meropenem, which is strongly recommended because the usage of the drug could be extended to cover children over the age three, and strictly given to patients whose laboratory reports show complete resistance to third or fourth generation cephalosporins and quinolones (Ghana Essential Medicines List 2010). High resistance observed in the disc diffusion and the MIC investigations are clearly in agreement with the assertion that the detection of carbapenemases may not necessarily be exclusively due to carbapenems but may be caused by a combination of additional

resistance mechanisms (Baroud *et al.* 2013). In this present study, over 76.6% (85/111) of the CR isolates were found to be carbapenemase non-PCR positives which needed further investigation. In Africa, the presence of different carbapenemase resistance genes have recently been reported in Morocco (Barguigua *et al.* 2013), Egypt (Fouad *et al.* 2013), Ivory Coast (Jeannot *et al.* 2013), Nigeria (Olaitan *et al.* 2013), and South Africa (Govind *et al.* 2013).

The findings following the phenotype-based assays, the molecular characterisation, outer membrane protein profiling, plasmid profiling and ERIC-PCR analysis of the CR isolates, suggested that the hospital environments may act as a reservoir of resistance determining genes. Of particular relevance are those CR species namely: *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas putida*, *Providencia stuartii* and *Shigella sonnei* that harbour the various resistance genes. The majority of the resistance genes were found in non-fermenting *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates identified from different clinical specimens obtained from four geographically and demographically unrelated regional hospitals in Ghana. These two species were significantly from wound, urine, sputum and aspirate specimens, however, no CR isolates were cultured from blood samples. Thus, poor environmental conditions in health-care facilities may have contributed to their presence and at the same time may act as a reservoir for the carbapenemase resistance determining genes. This is because these species are ubiquitous environmental organisms that are often found in the hospital setting. Presently, there is no epidemiological record to ascertain this fact since this present study is the first to reveal the prevalence and distribution of carbapenemase genes in the country's hospitals.

In the present study, phenotype-based tests only revealed CR resistance patterns to carbapenems and few numbers were detected as having carbapenemase activity. Most previous studies conducted on bacterial resistances were based on phenotypic analysis without applying molecular techniques. Studies conducted on antimicrobial resistance in a few Ghanaian hospitals saw a rise of bacterial resistance without detectable resistance to carbapenems (Newman *et al.* 2011, Feglo *et al.* 2013). In most African countries, and in particular in Ghana, identification of bacterial resistance strains is routinely performed as an investigative tool in order to offer researchers vital information on the spread of hospital infections. The high-level of antimicrobial resistance has existed for several decades, especially on the continent of Africa due to non-compliance of drug use (WHO 2014). The emergence and occurrence of multidrug resistance has been attributed to the lack of antimicrobial drug controls. According to Habte-Gabr (2010) and WHO (2014) the problems of resistance could only be managed properly by: education of the public, training prescribers of antimicrobials, improved access to diagnostic laboratories, constant surveys to detect the emergence of resistance and the existence of a policy to regulate the use of antimicrobial agents (Habte-Gabr 2010, WHO 2014).

Indeed, several changes in investigative methodologies by different groups of experts have been made in order to standardise the procedures, and to take into account the inherent factors that constantly affect the suitability of susceptibility testing. These factors include the rates of mutations occurring in most bacterial species, reproducibility and cultivable growth medium that can support the majority of bacterial growth. Notably, the use of disc diffusion susceptibility testing alone could not have revealed the major findings following the phenotype-based tests performed for carbapenemase activity. The application of susceptibility testing techniques narrows the scope of

determination and identification of serious infection situations involving ESBLs, AmpC, and metallo- β -lactamase enzymes in Gram-negative bacilli. Apart from these phenotype-based methods, PCR-based techniques are preferred for the determination of the distribution, prevalence, plasmid analysis and clonal relationship of multidrug resistance most especially the trend of carbapenemase-producing isolates. In some parts of Africa, investigators lack the expertise and technical competency in using molecular techniques regularly in their research studies. A recent study conducted by Opintan *et al.* (2015) reported that the levels of antimicrobial resistances are of concern because of the unregulated use of antimicrobials by Ghanaians as a result of which various antimicrobial resistances are conferred. The study also detected different multidrug-resistant isolates, including Gram-negative bacilli with ESBL-producing strains in a nationwide laboratory-based survey. However, no attempt was made to explore the presence of carbapenemase resistance determining genes with phenotype- and genotype-based assays (Opintan *et al.* 2015). In this present study, the most common of these assay techniques employed were to establish the distribution and prevalence of carbapenemase genes in clinical isolates from Ghana were reliable and reproducible.

The inconsistency of MHT results were evident when this method was employed in this study, where only two isolates of *Klebsiella pneumoniae* and one *Acinetobacter baumannii* were detected as positive for OXA-48 and NDM-1 genes when compared with the twenty-six positive isolates assayed by PCR amplification. However, it was observed that the MHT was more reliable for the detection of isolates harbouring an OXA-48 gene than the BADST method. It was observed that MHT had a lower sensitivity for NDM-1 gene detection, while BADST was found to be unreliable for phenotyping isolates harbouring NDM-1, VIM-1 and OXA-48 determining genes. In contrast, the boronic acid-disc synergy test which was employed as another phenotype-

based method was inconclusive for the determination of carbapenemase activity since none of the positive results were detected by PCR assay. This could be a consequence of primer sequences that amplify specific regions of the DNA in the test isolates. Therefore, it was possible that the CR isolates were not false-positive results from the BADST, but false-negative results from the PCR-based assay. Indeed, the findings obtained from the phenotype-based tests were not comparable to the PCR amplification technique. In this study, Paterans *et al.* (2010) and Doyle *et al.* (2012) observed that MHT and BADST are efficient and more reliable for the detection of carbapenemase enzymes in the Enterobacteriaceae group (Paterans *et al.* 2010, Doyle *et al.* 2012). These findings are in agreement with those in this study, where the majority of the positive carbapenemase producers detected were mostly non-fermenting isolates. Additionally, availability of the chromogenic culture medium, ChromID CARBA (BioMérieux, France) with sensitivity and specificity of 97.4% and 99.7% respectively, for carbapenemase producers from the Enterobacteriaceae group (Cantón *et al.* 2012) could have improved detection rates during the study investigations.

PCR amplification revealed diverse species that are carriers of NDM-1, OXA-48 and VIM-1 resistance genes mainly in isolates of *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas* species, respectively, with no KPC-1 and IMP-1 gene detections. Search on databases has confirmed the finding that genes encoding KPC-1 and IMP-1 are more frequently found in North America, Europe and Asia than Africa (Manenzhe *et al.* 2014).

The VIM-1 gene was found in *Pseudomonas aeruginosa* and *Pseudomonas putida*, and OXA-48 in *Klebsiella pneumoniae* isolates. Of serious concern was the class B type of

MBLs detected among the *Acinetobacter baumannii* isolates and *Pseudomonas* species detected in different clinical specimens from four demographically unrelated regional hospitals. Their presence in this study suggests that the occurrence of carbapenemase genes have already emerged in the Ghanaian population which needs regular monitoring and implementation of a policy to detect routinely most of the carbapenemase activity phenotypically in hospital laboratories with occasional surveillance studies supported with molecular detections.

There was strong expectation for KPC-1 gene detection prior to this present study since it was known worldwide, although, none was detected in any of the CR isolates. An earlier review revealed that the spread of KPC-1 resistance genes is one of the leading causes of hospital acquired infections and it is also the most prevalent circulating gene worldwide (Nordmann, Naas and Poirel 2011). In a subsequent review by Munoz-Price *et al.* (2013) it was claimed that the KPC types were the most common encoding genes found in the countries of Europe, Asia and the Americas after its discovery over 16 years ago with little or few records of spread in Africa (Munoz-Price *et al.* 2013). The KPC-1 gene is scarcely found in African countries. KPCs are detectable in *Acinetobacter* and *Pseudomonas* species in parts of the northern regions of South America, the Caribbean and Central America. Evidently, none of the non-fermenter isolates in this study were encoded with KPC carbapenemases, which could be attributed to continental differences and locations. Most countries have reported a threat of two different types of genes co-inhibiting a positive KPC-1 organism. Several studies report the emergence of these genes in isolates of carbapenemase-producing *Klebsiella pneumoniae*, co-harboursing two carbapenemase genes: VIM-19 and KPC-2 co-producer isolate from Greece (Pournaras *et al.* 2010), NDM-1 and OXA-181 producer *K. pneumoniae* in Oman (Dortet *et al.* 2012), NDM-1 and OXA-232 producer *K.*

pneumoniae in the USA (Doi *et al.* 2014) and recently, *K. pneumoniae* harbouring NDM-1 and OXA-48 reported from Saudi Arabia (Zowawi *et al.* 2014). Fortunately, no double detectable carbapenemase genes in a single CR isolate from Ghanaian hospitals were identified in this present study.

Interestingly, no IMP-1 gene was detected which may attest to the fact that imipenem is excluded as an essential antimicrobial for clinical management of patients according to the Ghana's Essential Medicines List and National Drug Policy (Ghana's Essential Medicines List 2010). However, among the MBLs, the VIM-1 gene was only detected in the CR *Pseudomonas* species with no IMP-1 gene found. Chen *et al.* (2009) reported that the IMP gene alone does not confer increased carbapenem resistance in Enterobacteriaceae, however, they contend that care has to be taken on its spread when the drug is used on patients tested of having reduced susceptibility, and warned that the results of investigations into carbapenemase activity should be treated with caution (Chen *et al.* 2009).

The carbapenemases detected in this study were found to co-exist with TEM-1- and SHV-1-encoding genes, which indicate that there could be variants or other types of β -lactamases in existence that could not be detected. Further investigation is needed since most of the CR isolates studied are potential pathogens that can cause nosocomial infections, and harbour other undetermined plasmid-borne antimicrobial resistance determinant-genes. The presence of these genes may aid the persistence and survival of these species in the hospital environment.

In the plasmid-cured CR isolates tested, the observation that MIC levels to the carbapenems were moderately reduced by E-test assay is of major concern. The low levels of MICs indicate that there were some alternative mechanisms of resistance contributing to carbapenem resistance. Results from previous studies suggest the possible mechanisms for the incorporation of the *AmpC* genes and the alteration of porins that decrease the entry of carbapenem agents into the cell, as exhibited in most imipenem-resistant *Klebsiella pneumoniae* isolates (Miriagou *et al.* 2010, El Wartiti *et al.* 2012) or the alteration of the efflux pump systems encountered especially in *Acinetobacter* and *Pseudomonas* species.

The impact of improper use of antimicrobials have been reported in previous studies carried out in Nigeria (Okonko *et al.* 2009), Tanzania (Moyo *et al.* 2010) and recently, in Ghana (Opintan *et al.* 2015). In these studies, only traditional methods for detection of bacterial resistance were employed. Currently, pulsed-field gel electrophoresis (PFGE), and random amplification of polymorphic DNA (RAPD) are extensively used by most clinical laboratories worldwide (Sabat *et al.* 2013) for the determination of bacterial resistance profiles.

In this study, enterobacterial repetitive intergenic consensus (ERIC) by PCR amplification of genomic DNA products revealed an array of diversity among the different CR isolates studied. However, small number of closely related isolates existed within a pair each from the *Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolates. The simplicity and reproducibility of the ERIC-PCR technique is paramount in that it makes it adaptable in less endowed countries in Africa, including Ghana to investigate outbreak of infections in hospitals. Indeed, none of the traditional methods

alone may have revealed the diversity of strains in the diverse Gram-negative bacterial species studied. For future surveillance studies involving genetic characterisation, keeping epidemiologic records and monitoring isolates capable of demonstrating clonal transmission of bacteria in hospital environments, ERIC-PCR fingerprints may help the profiling of genetic variability or relatedness in any outbreak situations in Ghana.

ERIC-PCR has been used to study genetic diversity or relatedness of various bacterial species present in clinical samples. One limitation of ERIC sequences is when compared with genomic sequences. Comparatively, genomic sequences are extremely dependent on the GenBank DNA database for easy evaluation of genome assembly depending on each microbial species. Thus, isolates are easily identified by genetic features individually assessed using BLAST to determine their location, origin within the genome (chromosomal or plasmid borne) and function based on homology. In contrast, band patterns of the ERIC fingerprints are currently assessed by software license for evaluation. In spite of good reproducibility, genotyping and without having prior knowledge of target genome sequences of the Gram-negative bacterial pathogens, however, there were few limitations encountered when using ERIC-PCR technique. Carbapenemase producing and multi-resistant Gram-negative bacterial pathogens that are known to be associated with wound and urine have been investigated using ERIC-PCR. Isolates of *Acinetobacter baumannii* and *Pseudomonas* species were isolated in large numbers in all the four regional hospitals under non-outbreak conditions. Despite the differences in the source of specimens, ages and sexes, high genetic diversity was observed among the CR isolates by ERIC-PCR analysis. Of concern are the relatively small number of ubiquitous isolates *Acinetobacter baumannii* and *Pseudomonas aeruginosa* showing similarities in the same hospital, Korle Bu Teaching hospital in the

Greater Accra region. These may pose a serious threat to public health in the care facility.

The major limitations of this study are that investigations were carried out at the University of Ghana, Medical Microbiology laboratory attached to the Korle Bu Teaching hospital laboratory where collections of suspected CR isolates were frequently recovered, identified and monitored in their numbers. Large numbers of CR isolates were therefore obtained as a result of constant flow of specimens on regular basis for isolation and susceptibility testing. As a major tertiary and referral centre of the country a larger number of samples can be examined when compared with the other 3 regional hospitals that received limited samples daily and have accounted for fewer CR isolates. In addition, irregular patient attendance and low bed capacity coupled with limited resources to carry out comprehensive laboratory analysis on clinical samples may have greatly contributed to the low numbers in CR isolates. This discrepancy has limited the numbers of samples for the ERIC-PCR analysis from three of the four hospitals studied. As a result, substantial number of carbapenemase resistance genes was found at the Korle Bu Teaching Hospital.

6.2 Conclusion

In all, carbapenem resistances were relatively low with high numbers of ESBL carriage coupled with small number of PCR-positive carbapenemase gene detections. Porin loss was one of the possible major mechanisms. The CR isolates were diverse and showed limited relatedness in this study. CR isolates encoded with carbapenemase resistance genes in the four regional hospitals in Ghana following the use of common phenotype-based methods and PCR optimisation assays have been characterised. This is the first report of the occurrence of three significant carbapenemase genes; NDM-1, VIM-1 and OXA-48 in Ghanaian hospitals. These findings have provided an insight of the emerging resistance genes belonging to the Ambler Class B, two MBL-types; NDM-1 and VIM-1 and one non-MBL-type, OXA-48 in the Class D group.

Potentially, the emergence of these three resistance genes is of relevance and a threat to the medical community. It is therefore essential that early detection by “quick-to-perform” tests must be used in order to identify the presence of carbapenemase activity in real time so that the severity of infections caused by these bacterial pathogens can be minimised by putting into place preventative measures. The possibility of plasmid-borne carbapenemase genes and uncertain multidrug resistant isolates of *Acinetobacter*, *Pseudomonas* and *Klebsiella* including other nosocomial Gram-negative bacterial pathogens could be tested and identified using PCR amplification assays. ERIC-PCR optimisation assays may serve as a suitable genotyping tool for the assessment of genetic diversity or close relatedness of isolates that are found in clinical settings.

The findings reported here have broadly highlighted the emergence of carbapenemase resistance genes found in Ghanaian healthcare settings. However, further nationwide

study on a larger scale to determine the common variants of the carbapenemase genes detected in this study is required in order to ascertain their presence in the Ghanaian population for the purposes of future epidemiological records.

Finally, the present study may also contribute to the debate about which techniques in the Ghanaian context can be deployed for an inexpensive nationwide study of carbapenemase determining genes during outbreaks. Frequent investigations should be conducted to detect the CR pathogens present in susceptible environments within the hospital. It is strongly recommended that the use of specific test-kits to complement resistance patterns obtained from disc diffusion and E-test assays as screening tests for all clinical isolates resistant to carbapenems is adopted. Results from these test-kits, in effect may help care-givers to institute early interventions such as reviewing the antimicrobial treatment policy and plan patient isolation. The use of ChromID CARBA medium and Carba NP test can be introduced as the first screening method to identify weakly detectable carbapenemases and also to provide the basic information before the application of molecular assay by PCR or be used in combination in any research studies.

6.3 Future work

The detection of a large number of carbapenemase non-PCR positives has shown that resistance patterns significantly varied among the clinical isolates studied. Carbapenemases are emerging in *Acinetobacter* and *Pseudomonas* species (Mataseje *et al.* 2012), which indicate that these isolates may spread within the hospital environment and to follow the trend of ESBLs into the community population.

For meaningful future work, awareness drive and active advocacy may resolve some of the major goals since little is known in Ghana about the epidemiology and strains encoding carbapenemase resistance genes in the health-care delivery system. The importance of these resistance genes is the great risk they pose to all who work as health-care personnel and the Ghanaian population at large. The study findings can be used by the medical community, largely to the medical doctors, epidemiologists, microbiologists, researcher scientists and other health-care providers to inform practice. Serious infections cannot be ruled out in strains encoded with these resistance genes.

The first task would be on awareness creation to showcase the study findings to the Heads of Clinical Departments in their monthly meetings in form of presentations, to explain the relevance and importance of the carbapenemase resistance genes in the hospital environments. Emphasis would be placed on how to prevent the spread of the genes in the intensive care units (ICUs), cardiothoracic units (CTUs) and surgical theatres. Multicentre screening in these sensitive areas (ICUs and CTUs) would be the first target within the hospital setting.

Other audiences that deal with health-related problems such as the Ministry of Health, Ghana College of Physicians and Surgeons Council, Ghana Association of Biomedical Scientists, Ghana Registered Nurses and Midwives Association, Parliamentary Select Committee for Health and the Office of the World Health Organisation would be briefed on the study findings in support of funds for a nationwide surveillance study. A nationwide study would establish the actual level of prevalence of the isolates encoded with carbapenemase resistance genes in the country. Of note, all the 10 regional hospitals in Ghana would be involved and networked in order to have a constant flow of information for implementing the necessary objectives, a quick supply of reagents and

data collection. Target groups that may be involved in the nationwide study detection (microbiologists, biomedical scientists and research assistants) would be given intensive training on modern techniques of identifying carbapenemase production in a simplified detection outline in a form of flow-chart diagram. All suspected CR isolates would be phenotypically identified and positive isolates stored and sent for genotype typing to be collectively carried out at the collaborative institution, the Noguchi Memorial Institute for Medical Research, University of Ghana.

Specifically, consultants, doctors and other health-care providers would be advised to be vigilant and strictly stick to basic strategies to decrease person-to-person transmission of infections, including limitation of medical device use, contact precautions, hand hygiene, patient and staff contact, healthcare personnel education, antimicrobial stewardship, laboratory notification strategies, and introduction of active screening for CR isolates in every hospital.

The overall results of the study would be disseminated to the medical community and policy-makers; the Ministry of Health and the Government of Ghana. The outcome would give the nation a better understanding of the true prevalence of all types of carbapenem-resistant isolates encoded with resistance genes. As a threat to public health, strategies in regard to transmission of infections by the carrier isolates would be significantly controlled in Ghana.

With massive improvement of laboratory resources through research funding, other unanswered area of study could be tackled independently. For instance, a total of 80.2% (89/111) of the CR isolates recovered were made up of these two non-fermenting pathogens, while only 17.1% (19/111) were encoded with the NDM-1 and VIM-1

carbapenemases, leaving a number of unanswered questions. The reasons for the large number of negative results require further investigations.

This study is in agreement with the suggestion made by Perez and van Duin (2013) that various methods are to be used when investigating carbapenemase activity in a clinical laboratory. Thus, in the context of this study, future work could include the use of different testing assays, depending on the availability of the testing reagents, reliability and reproducibility of the methods that clearly detect all carbapenemase and other β -lactamase genes. Further work could be conducted on the high numbers of non-carbapenemase producers by using the Carba NP test described by Nordmann, Poirel and Dortet (2012) for rapid determination of carbapenemase-producing isolates that harbour weakly detectable genes that could not be detected by PCR assay.

Other determining genes in association with carbapenemase genes, usually with the OXA-type β -lactamases, could be investigated in detail with no prior knowledge of the underlying resistance traits that encode each CR isolate. Non-susceptibility of carbapenems in combination with the production of ESBLs or intrinsic types, CTX-M and plasmid-borne AmpC-type β -lactamases with additional loss of porin proteins poses detection difficulties. For instance, mutational loss or reduced expression of *OprD* raising the MIC levels higher than the normal breakpoints are major contributors to carbapenems resistance. There should be special emphasis on the growing number of new allele variants found among the host of clinically relevant β -lactamases. With the requisite reference control strains, future work could include the CTX-M-15 gene in particular which is found to be associated with incompatibility group FII plasmids. Broad host-range replicon plasmids such as IncL/M, IncN and IncI1 have also been shown to be involved in the dissemination and spread of CTX-M genes. The resistance

had been reported from the co-production of CTX-M-15 with OXA-48 and other carbapenemases (VIM-1 and NDM-1) increases the emergence and development of multidrug resistant pathogens (Bakthavatchalam, Anandan and Veeraraghavan 2016). Further studies could be carried out to establish the true number of positive carbapenemases from the high numbers of carbapenemase non-PCR positives obtained in this present study.

Future work on OMPs would be subjected to a panel of control strains. Isolates of the same species would have its specific control strain for easier analysis on SDS-PAGE. Thus, actual expression of OMPs could be properly analysed on the basis of its control strain. For example, uptake of antimicrobials into the periplasmic region of the cell is greatly affected by diminishing or increased permeability of the outer membrane in each individual bacterial species. Based on resistance to imipenem and meropenem, the outer membrane of *Pseudomonas aeruginosa* control strains were regulated and analysed using the presence or absence of *OprD* on SDS-PAGE.

The finding that the NDM-1 gene was the most prevalent among CR isolates could be investigated further using long-fragment real-time quantitative PCR (LF-qPCR) to determine the *in vitro* transcription of NDM-1 in order to determine the full length of the NDM-1 gene and also find any unknown variants. This innovation was described in a study by Huang *et al.* (2014) can rapidly detect NDM-1 gene when long fragment quantitative PCR technique is used (Huang *et al.* 2014), which can then be extended to other carbapenemase gene detection studies.

Genomic DNA fingerprints generated by using ERIC-PCR amplification could be performed to evaluate the genetic diversity of CR isolates of both clinical and environmental samples. This could be a comparative study to establish the possibility of similar bacterial isolates having close relatedness in the transmission of resistance genes into the Ghanaian population. For future epidemiologic studies, it is suggested that some form of real-time characterisation of various carbapenemase variants by PCR amplification, could be included in future Public Reference Laboratory investigations in the country to rapidly identify the carbapenemase variant-types for record keeping and to support further outbreak investigations in our hospitals.

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Appendices

Appendix 1 Vitek automated microbial identification results of carbapenem-resistant isolates from Ghana

Type of specimen	Isolate code	Sex	Age (Year)	Name of isolate
Wound	1	M	58	<i>P. aeruginosa</i>
Wound	2	F	26	<i>E. coli</i>
Wound	3	M	31	<i>P. aeruginosa</i>
Wound	4	F	25	<i>A. baumannii</i>
Wound	5	F	41	<i>E. coli</i>
Wound	6	F	7	<i>P. aeruginosa</i>
Wound	7	F	2½	<i>P. aeruginosa</i>
Sputum	8	M	91	<i>A. baumannii</i>
Wound	9	F	22	<i>C. sakazakii</i>
Wound	10	M	57	<i>P. aeruginosa</i>
Urine	11	M	26	<i>S. paucimobilis</i>
Sputum	12	F	23	<i>P. putida</i>
Urine	13	F	30	<i>P. aeruginosa</i>
Urine	14	F	22	<i>P. aeruginosa</i>
CSF	15	M	19	<i>P. aeruginosa</i>
Sputum	16	M	44	<i>P. aeruginosa</i>
Sputum	17	M	30	<i>P. aeruginosa</i>
Urine	18	F	57	<i>E. coli</i>
Wound	19	F	37	<i>K. pneumoniae</i>
Urine	20	F	2months	<i>A. baumannii</i>
Ear	21	F	28	<i>P. aeruginosa</i>
Wound	22	F	4months	<i>A. baumannii</i>
Wound	23	F	16	<i>E. coli</i>
Wound	24	M	79	<i>P. aeruginosa</i>
Sputum	25	F	32	<i>P. aeruginosa</i>
Urine	26	M	13	<i>P. aeruginosa</i>

Type of specimen	Isolate code	Sex	Age (Year)	Name of isolate
Wound	27	F	75	<i>A. baumannii</i>
Urine	28	M	16	<i>P. aeruginosa</i>
Wound	29	F	24	<i>A. baumannii</i>
Urine	30	M	78	<i>A. baumannii</i>
Wound	31	M	2	<i>P. aeruginosa</i>
CSF	32	F	6	<i>P. aeruginosa</i>
Urine	33	M	4	<i>En. cloacae</i>
Aspirate	34	M	13	<i>A. baumannii</i>
Wound	35	F	30	<i>E. coli</i>
Aspirate	36	M	55	<i>A. baumannii</i>
Urine	37	M	58	<i>P. aeruginosa</i>
Urine	38	M	35	<i>A. baumannii</i>
Wound	39	M	56	<i>P. aeruginosa</i>
Aspirate	40	F	36	<i>P. aeruginosa</i>
Urine	41	F	63	<i>P. aeruginosa</i>
Wound	42	M	1	<i>A. baumannii</i>
Wound	43	M	19	<i>A. baumannii</i>
Wound	44	M	22	<i>P. putida</i>
Urine	45	F	63	<i>A. baumannii</i>
Wound	46	F	3days	<i>A. buamannii</i>
Wound	47	M	1	<i>P. aeruginosa</i>
Urine	48	M	58	<i>P. aeruginosa</i>
Urine	49	F	36	<i>A. baumannii</i>
Urine	50	M	60	<i>A. baumannii</i>
Urine	51	F	33	<i>A. baumannii</i>
Wound	52	M	74	<i>P. aeruginosa</i>
Urine	53	M	24	<i>E. coli</i>
Urine	54	M	84	<i>P. aeruginosa</i>
Urine	55	F	69	<i>K. pneumoniae</i>
Urine	56	F	19	<i>A. baumannii</i>

Type of specimen	Isolate code	Sex	Age (Year)	Name of isolate
Wound	57	M	15days	<i>P. aeruginosa</i>
Urine	58	M	3	<i>P. aeruginosa</i>
Urine	59	M	38	<i>P. aeruginosa</i>
Urine	60	M	58	<i>P. aeruginosa</i>
Wound	61	M	3months	<i>P. aeruginosa</i>
Wound	62	F	70	<i>P. aeruginosa</i>
Urine	63	M	74	<i>P. aeruginosa</i>
Wound	64	F	7days	<i>A. baumannii</i>
Urine	65	F	60	<i>P. aeruginosa</i>
Wound	66	M	53	<i>P. aeruginosa</i>
Urine	67	M	13months	<i>E. coli</i>
Urine	68	F	2	<i>E. coli</i>
Urine	69	F	29	<i>A. baumannii</i>
Wound	70	M	22	<i>P. aeruginosa</i>
Wound	71	F	2	<i>P. aeruginosa</i>
Wound	72	F	5months	<i>A. baumannii</i>
Urine	73	F	32	<i>A. baumannii</i>
Urine	74	M	60	<i>P. aeruginosa</i>
Urine	75	F	29	<i>A. baumannii</i>
Wound	76	F	42	<i>P. stuartii</i>
Wound	77	M	76	<i>A. baumannii</i>
Ear	78	M	6	<i>P. putida</i>
Catheter tip	79	M	73	<i>P. aeruginosa</i>
Wound	80	F	1	<i>P. aeruginosa</i>
Wound	81	M	70	<i>P. aeruginosa</i>
Urine	82	F	88	<i>A. baumannii</i>
Urine	83	F	4	<i>P. putida</i>
Urine	84	F	1½	<i>P. aeruginosa</i>
Urine	85	F	33	<i>E. coli</i>
Wound	86	M	3	<i>P. aeruginosa</i>

Type of specimen	Isolate code	Sex	Age (Year)	Name of isolate
Wound	87	F	5	<i>P. aeruginosa</i>
Urine	88	F	44	<i>P. putida</i>
Stool	89	M	60	<i>S. sonnei</i>
Ear	90	M	21	<i>P. putida</i>
Wound	91	F	17	<i>En. cloacae</i>
Ear	92	M	65	<i>P. putida</i>
HVS	93	F	46	<i>E. coli</i>
Wound	94	F	6	<i>A. baumannii</i>
Urine	95	F	30	<i>En. cloacae</i>
Wound	96	F	70	<i>P. aeruginosa</i>
Wound	97	F	50	<i>E. coli</i>
Sputum	98	M	45	<i>K. pneumoniae</i>
Sputum	99	M	30	<i>P. aeruginosa</i>
Wound	100	F	86	<i>P. aeruginosa</i>
Aspirate	101	F	12	<i>P. aeruginosa</i>
Wound	102	F	3days	<i>A. baumannii</i>
Aspirate	103	M	27	<i>A. baumannii</i>
Wound	104	M	23	<i>P. aeruginosa</i>
Urine	105	M	3months	<i>E. coli</i>
Wound	106	F	9months	<i>A. baumannii</i>
Urine	107	F	19	<i>A. baumannii</i>
Urine	108	F	33	<i>A. baumannii</i>
Urine	109	F	40	<i>P. aeruginosa</i>
Eye	110	M	5days	<i>P. aeruginosa</i>
Wound	111	M	5	<i>P. aeruginosa</i>

Note: M= male, F= female

Appendix 2 Phenotype-based identification of carbapenem-resistant isolates

No	Sensitivity testing			ESBL testing			MIC Levels				M H T	BADST		
	I m p	M e r	D o r	Cpd+ clv	Cpd	Ratio	Px	Ip	Mp	Er		Mp	Mp+ Apb	Ratio
1	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
2	R	R	R	0	0	0	12	>32	>32	>32	-	10	11	1
3	S	R	R	0	0	0	>256	4	16	>32	-	6	6	0
4	R	R	R	8	9	1	>256	>32	>32	>32	+	6	6	0
5	R	R	R	0	0	0	>256	2	3	16	-	18	18	0
6	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	1
7	R	R	R	0	0	0	>256	>32	>32	>32	-	17	18	1
8	R	R	R	8	8	0	>256	>32	>32	>32	+	8	6	2
9	R	R	R	8	8	0	>256	>32	>32	>32	-	17	18	1
10	R	R	R	0	0	0	>256	>32	>32	>32	-	6	7	1
11	R	R	R	0	0	0	>256	>32	>32	>32		6	7	1
12	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
13	R	R	R	10	10	0	8	16	>32	>32	-	11	12	1
14	R	R	R	0	0	0	>256	8	4	>32	+	11	13	2
15	R	R	R	0	0	0	>256	>32	12	>32	-	8	10	2
16	R	R	R	0	0	0	>256	16	4	>32	-	20	20	0
17	R	R	R	0	0	0	>256	>32	>32	>32	+	6	6	0
18	R	R	R	10	10	0	12	16	>32	>32	-	12	12	0
19	R	R	R	0	0	0	>256	16	8	>32	+	14	14	0
20	R	R	R	0	0	0	16	>32	>32	>32	-	6	6	0
21	I	R	R	0	0	0	>256	2	16	>32	-	12	14	2
22	R	R	R	0	0	0	>256	>32	16	>32	+	6	6	0
23	R	R	R	0	0	0	>256	>32	>32	>32	-	6	7	1
24	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
25	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
26	I	R	R	0	0	0	>256	>32	>32	>32	-	6	8	2
27	I	R	R	9	6	3	>256	6	>32	>32	-	6	8	2

28	R	R	R	0	0	0	>256	>32	>32	>32	-	6	7	1
29	R	R	R	0	0	0	>256	>32	>32	>32	+	6	8	2
30	R	R	R	0	0	0	24	>32	16	>32	+	6	7	1
31	S	R	R	12	11	1	>256	4	16	>32	-	6	6	0
32	R	R	R	0	0	0	>256	>32	>32	>32	-	7	8	1
33	R	R	R	0	0	0	>256	32	>32	>32	-	25	31	6
34	R	R	R	0	0	0	32	8	16	>32	+	6	8	2
35	I	I	I	0	0	0	>256	0.3 8	0.5 0	0.5 0	-	20	21	1
36	R	R	R	0	0	0	>256	>32	>32	>32	+	6	6	0
37	R	R	R	0	0	0	>256	16	>32	>32	-	6	6	0
38	R	R	R	0	0	0	12	>32	>32	>32	-	6	7	1
39	R	R	R	0	0	0	>256	>32	>32	>32	-	6	8	2
40	R	R	R	0	0	0	>256	>32	>32	>32	+	6	6	0
41	R	R	R	0	0	0	32	>32	>32	>32	-	6	7	1
42	R	R	R	0	0	0	>256	>32	>32	>32	-	6	7	1
43	R	R	R	8	6	2	>256	>32	>32	>32	-	6	8	2
44	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
45	R	R	R	0	0	0	12	>32	>32	>32	-	6	8	2
46	R	R	R	0	0	0	>256	>32	>32	>32	-	6	9	3
47	R	R	R	0	0	0	>256	>32	>32	>32	-	8	8	0
48	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
49	R	R	R	13	13	0	>256	>32	>32	>32	+	6	7	1
50	I	I	R	0	0	0	>256	4	16	>32	-	6	7	1
51	R	R	R	12	13	1	>256	>32	>32	>32	+	6	8	2
52	R	R	R	0	0	0	>256	12	12	>32	-	12	13	1
53	R	R	R	10	10	0	12	16	>32	>32	-	6	7	1
54	S	R	R	0	0	0	>256	4	>32	>32	-	6	6	0
55	R	R	R	0	0	0	>256	8	16	>32	+	15	15	0
56	R	R	R	6	9	3	>256	16	>32	>32	-	6	7	1
57	R	R	R	7	8	1	>256	4	>32	>32	-	13	15	2
58	R	I	R	0	0	0	>256	8	4	>32	-	11	13	2
59	R	R	R	0	0	0	>256	6	3	>32	-	11	12	1

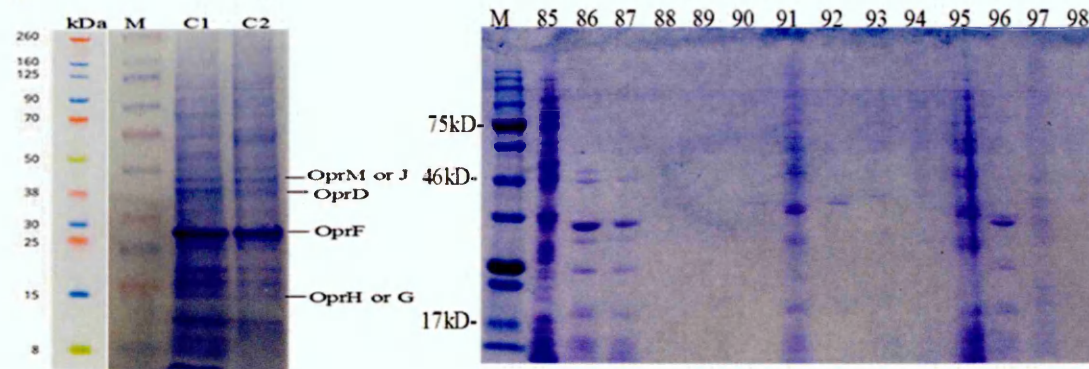
60	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
61	R	R	R	0	0	0	>256	>32	16	>32	-	6	6	0
62	R	R	R	0	0	0	>256	>32	8	>32	-	6	6	0
63	R	R	R	0	0	0	>256	16	>32	>32	-	6	8	2
64	I	R	R	0	0	0	>256	8	>32	>32	-	6	8	2
65	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
66	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
67	S	I	I	0	0	0	128	2	0.1 25	16	-	22	22	0
68	S	I	I	0	0	0	>256	4	0.1 9	4	-	22	22	0
69	R	R	R	15	15	0	>256	16	32	>32	+	6	8	2
70	S	I	I	0	0	0	>256	4	0.5 0	>32	-	29	29	0
71	R	R	R	0	0	0	>256	16	8	>32	-	10	11	1
72	I	R	R	9	6	3	>256	>32	>32	>32	-	6	8	2
73	R	R	R	0	0	0	>256	>32	16	>32	+	6	8	2
74	R	R	R	0	0	0	>256	>32	>32	>32	-	6	8	2
75	R	R	R	0	0	0	>256	>32	>32	>32	-	15	15	0
76	R	I	R	0	0	0	>256	4	0.2 5	8	-	21	22	1
77	R	R	R	0	0	0	>256	>32	>32	>32	-	8	8	0
78	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
79	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
80	R	R	R	0	0	0	>256	8	16	>32	-	6	7	1
81	S	R	S	0	0	0	>256	0.3 8	6	16	-	13	15	2
82	R	R	R	10	10	0	>256	32	>32	>32	-	6	7	1
83	S	R	R	0	0	0	>256	0.3 8	16	32	-	6	6	0
84	R	R	R	0	0	0	>256	8	4	16	-	6	7	1
85	I	I	I	0	0	0	>256	0.3 8	0.2 5	4	-	21	22	1
86	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
87	R	R	R	0	0	0	>256	>32	32	>32	-	6	8	2
88	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
89	R	R	R	0	0	0	>256	1.5	4	32	-	15	16	1
90	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0

91	R	R	R	0	0	0	>256	>32	>32	>32	-	24	31	7
92	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
93	R	R	R	0	0	0	>256	>32	>32	>32	-	6	7	1
94	R	R	R	0	0	0	>256	6	12	>32	-	9	9	0
95	R	R	R	0	0	0	>256	32	>32	>32	-	24	30	6
96	R	R	R	0	0	0	>256	16	8	32	-	9	10	1
97	R	R	R	9	9	0	12	16	>32	>32	-	6	7	1
98	R	R	R	0	0	0	>256	16	8	>32	+	15	15	0
99	I	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
100	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
101	R	R	R	0	0	0	>256	8	8	>32	+	10	10	0
102	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
103	I	R	I	0	0	0	>256	1	4	32	-	15	16	1
104	R	R	R	0	0	0	>256	16	>32	>32	-	6	6	0
105	R	R	R	0	0	0	>256	0.7 5	2	32	-	17	17	0
106	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
107	R	R	R	7	6	1	>256	16	>32	>32	+	6	6	0
108	R	R	R	0	0	0	>256	>32	>32	>32	+	6	6	0
109	R	R	R	0	0	0	>256	>32	>32	>32	+	6	6	0
110	R	R	R	0	0	0	>256	>32	>32	>32	-	6	6	0
111	R	R	R	0	0	0	>256	16	>32	>32	-	6	6	0
+vc	R	R	R	0	0	0	>250	4	>32	>32	+	9	15	6
-vc	S	S	S	34	34	0	0.50	0.1 25	0.0 12	0.0 12	-	36	36	0

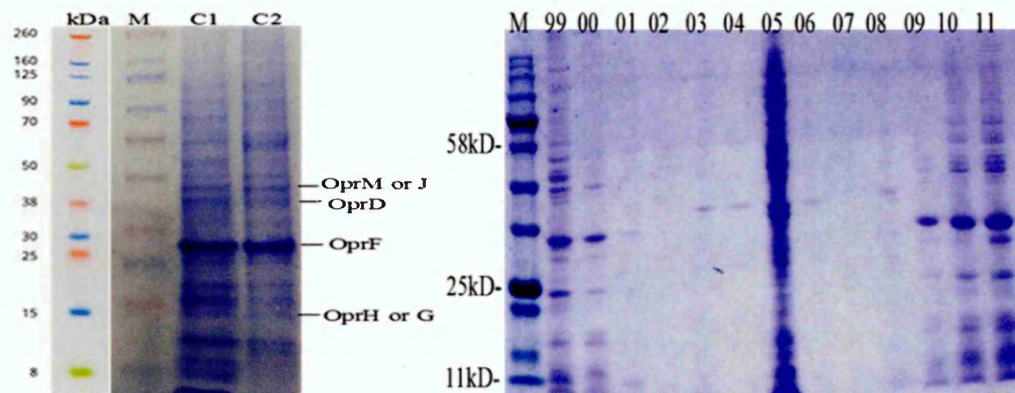
Note: S= sensitive, I= intermediate, R= resistant, +vc= positive carbapenemase *Klebsiella pneumoniae* (NCTC 13438) control strain, -vc= negative carbapenemase *Escherichia coli* (ATCC 25922) control strain, Imp= imipenem disc, Mer= meropenem disc, Dor= doripenem disc, Cpd= cefpodoxime, Cpd+clv= cefpodoxime + clavulanic acid, Px= cefpodoxime E-test strip, Ip= imipenem E-test strip, Mp= meropenem E-test strip, Er= ertapenem E-test strip, MHT= modified Hodge test, MIC= minimum inhibitory concentration, BADST= boronic acid disc synergy test, Apb= aminophenylboronic acid hydrochloride.

Appendix 3 Representative outer membrane proteins of control strains and carbapenem-resistant isolates on SDS-PAGE

A



B



Note: M= Protein marker in kDa; C1= control SHU 2048 VIM positive *Pseudomonas aeruginosa* strain; C2= control SHU 2054 MBL negative *Pseudomonas aeruginosa* strain; 85-111 represent sample numbers; *OprM/J* (48/49kDa)= major intrinsic multidrug efflux proteins; *OprD* (44kDa)= specific for β -lactam imipenem resistance; *OprF* (35kDa)= major porin for structural stabilisation of peptidoglycan; *OprH/G* (22/23kDa)= polycationic antimicrobial uptake and putatively in iron uptake

Appendix 4 Carbapenem MIC levels and major porins in carbapenem-resistant

isolates

Isolate Code	Name of isolate	A	B	C	Major porins	
					OprD ^a	OprF ^b
1	<i>P. aeruginosa</i>	+	-	-	H	M
4	<i>A. baumannii</i>	+	-	-	-	-
6*	<i>P. aeruginosa</i>	+	-	-	R	-
7*	<i>P. aeruginosa</i>	+	-	-	-	-
8	<i>A. baumannii</i>	+	-	-	-	-
9	<i>C. sakazakii</i>	+	-	-	-	-
10	<i>P. aeruginosa</i>	+	-	-	-	-
11	<i>S. paucimobilis</i>	+	-	-	-	-
12*	<i>P. putida</i>	+	-	-	-	-
16	<i>P. aeruginosa</i>	+	-	-	R	M
20	<i>A. baumannii</i>	+	-	-	-	-
23	<i>E. coli</i>	+	-	-	-	-
24	<i>P. aeruginosa</i>	+	-	-	-	R
25	<i>P. aeruginosa</i>	+	-	-	-	R
26	<i>P. aeruginosa</i>	+	-	-	-	-
29	<i>A. baumannii</i>	+	-	-	-	-
32	<i>P. aeruginosa</i>	+	-	-	-	R
33	<i>En. cloacae</i>	+	-	-	-	-
36	<i>A. baumannii</i>	+	-	-	-	-
37	<i>P. aeruginosa</i>	+	-	-	R	-
38	<i>A. baumannii</i>	+	-	-	-	-
39*	<i>P. aeruginosa</i>	+	-	-	-	-
40	<i>P. aeruginosa</i>	+	-	-	R	R
41	<i>P. aeruginosa</i>	+	-	-	R	-
42*	<i>A. baumannii</i>	+	-	-	R	-
43*	<i>A. baumannii</i>	+	-	-	-	-
44	<i>P. putida</i>	+	-	-	-	-

Isolate Code	Name of isolate	A	B	C	Major porins	
					OprD ^a	OprF ^b
45	<i>A. baumannii</i>	+	-	-	-	-
46*	<i>A. baumannii</i>	+	-	-	-	-
47*	<i>P. aeruginosa</i>	+	-	-	-	-
48	<i>P. aeruginosa</i>	+	-	-	-	-
49	<i>A. baumannii</i>	+	-	-	-	-
51	<i>A. baumannii</i>	+	-	-	-	-
53	<i>E. coli</i>	+	-	-	-	-
56	<i>A. baumannii</i>	+	-	-	M	M
60*	<i>P. aeruginosa</i>	+	-	-	R	-
61	<i>P. aeruginosa</i>	+	-	-	-	-
62*	<i>P. aeruginosa</i>	+	-	-	R	-
63	<i>P. aeruginosa</i>	+	-	-	-	-
64*	<i>A. baumannii</i>	+	-	-	-	-
65	<i>P. aeruginosa</i>	+	-	-	-	-
72*	<i>A. baumannii</i>	+	-	-	-	-
74*	<i>P. aeruginosa</i>	+	-	-	-	M
75	<i>A. baumannii</i>	+	-	-	-	-
77	<i>A. baumannii</i>	+	-	-	-	-
78	<i>P. putida</i>	+	-	-	-	-
79	<i>P. aeruginosa</i>	+	-	-	-	M
82	<i>A. baumannii</i>	+	-	-	R	-
86	<i>P. aeruginosa</i>	+	-	-	-	H
87	<i>P. aeruginosa</i>	+	-	-	-	M
88	<i>P. putida</i>	+	-	-	-	-
90	<i>P. putida</i>	+	-	-	-	-
91	<i>En. cloacae</i>	+	-	-	-	R
92	<i>P. putida</i>	+	-	-	-	-
93	<i>E. coli</i>	+	-	-	-	R
95	<i>En. cloacae</i>	+	-	-	R	R
97	<i>E. coli</i>	+	-	-	-	-

Isolate Code	Name of isolate	A	B	C	Major porins	
					OprD ^a	OprF ^b
100*	<i>P. aeruginosa</i>	+	-	-	-	R
102*	<i>A. baumannii</i>	+	-	-	-	-
106	<i>A. baumannii</i>	+	-	-	R	-
108*	<i>A. baumannii</i>	+	-	-	R	-
109	<i>P. aeruginosa</i>	+	-	-	R	-
110	<i>P. aeruginosa</i>	+	-	-	R	H
2*	<i>E. coli</i>	-	+	-	R	-
13	<i>P. aeruginosa</i>	-	+	-	-	-
14	<i>P. aeruginosa</i>	-	+	-	-	+
15	<i>P. aeruginosa</i>	-	+	-	R	R
17	<i>P. aeruginosa</i>	-	+	-	-	-
18	<i>E. coli</i>	-	+	-	-	-
19*	<i>K. pneumoniae</i>	-	+	-	-	-
22	<i>A. baumannii</i>	-	+	-	-	-
27*	<i>A. baumannii</i>	-	+	-	-	-
28	<i>P. aeruginosa</i>	-	+	-	R	H
30	<i>A. baumannii</i>	-	+	-	-	-
31	<i>P. aeruginosa</i>	-	+	-	R	-
34	<i>A. baumannii</i>	-	+	-	-	-
50*	<i>A. baumannii</i>	-	+	-	-	-
52	<i>P. aeruginosa</i>	-	+	-	-	-
54	<i>P. aeruginosa</i>	-	+	-	-	-
55	<i>K. pneumoniae</i>	-	+	-	-	-
57	<i>P. aeruginosa</i>	-	+	-	-	-
58	<i>P. aeruginosa</i>	-	+	-	-	-
66	<i>P. aeruginosa</i>	-	+	-	-	-
69	<i>E. coli</i>	-	+	-	-	-
71	<i>P. aeruginosa</i>	-	+	-	R	-
73	<i>A. baumannii</i>	-	+	-	-	-
80	<i>P. aeruginosa</i>	-	+	-	-	-

Isolate Code	Name of isolate	A	B	C	Major porins	
					OprD ^a	OprF ^b
84*	<i>P. aeruginosa</i>	-	+	-	-	-
94	<i>A. baumannii</i>	-	+	-	-	-
96	<i>P. aeruginosa</i>	-	+	-	-	-
98*	<i>K. pneumoniae</i>	-	+	-	-	-
99	<i>P. aeruginosa</i>	-	+	-	M	H
101	<i>A. baumannii</i>	-	+	-	-	-
104	<i>En. cloacae</i>	-	+	-	R	-
107	<i>A. baumannii</i>	-	+	-	-	-
111	<i>P. aeruginosa</i>	-	+	-	R	H
3	<i>P. aeruginosa</i>	-	-	+	R	-
5*	<i>E. coli</i>	-	-	+	R	-
21	<i>P. aeruginosa</i>	-	-	+	-	R
35	<i>E. coli</i>	-	-	+	-	-
59	<i>P. aeruginosa</i>	-	-	+	M	M
67	<i>E. coli</i>	-	-	+	-	-
68	<i>E. coli</i>	-	-	+	H	-
70	<i>P. aeruginosa</i>	-	-	+	R	-
76*	<i>P. stuartii</i>	-	-	+	-	-
81	<i>P. aeruginosa</i>	-	-	+	-	H
83	<i>P. putida</i>	-	-	+	M	-
85	<i>E. coli</i>	-	-	+	H	-
89*	<i>S. sonnei</i>	-	-	+	-	-
103	<i>A. baumannii</i>	-	-	+	R	-
105*	<i>E. coli</i>	-	-	+	H	-

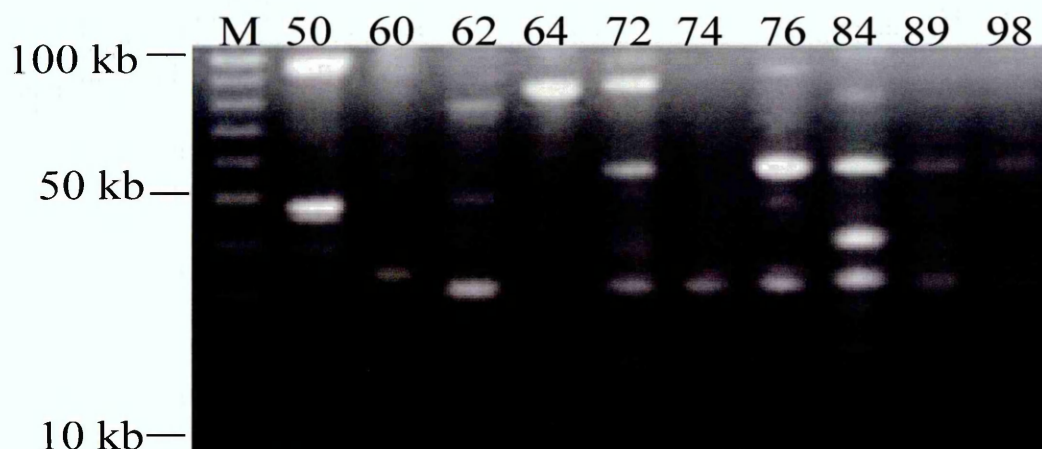
*Positive carbapenemase producers

^a Analysis mainly for *Pseudomonas* species only

^b Analysis for all CR isolates

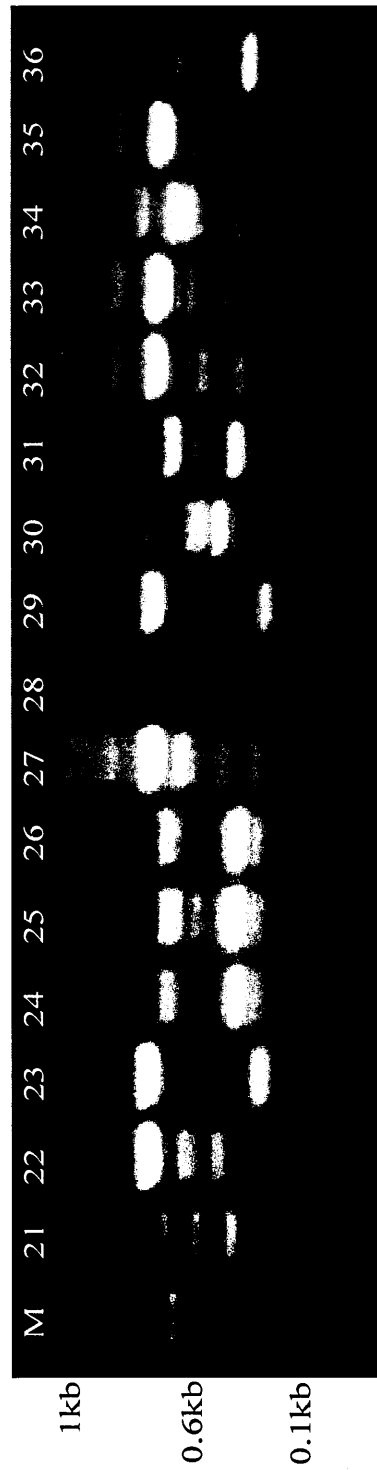
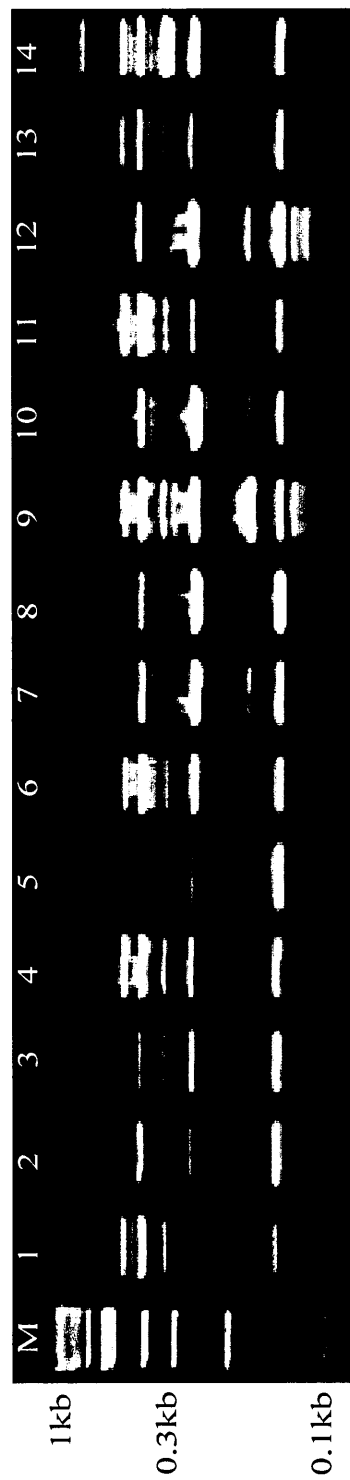
Note: A= 3 carbapenems (imipenem, meropenem, ertapenem) E-test levels were ≥ 32 $\mu\text{g/ml}$ (n= 63); B= 2 or 1 carbapenems (imipenem, meropenem, ertapenem) E-test levels were between ≥ 16 and ≤ 4 $\mu\text{g/ml}$ (n= 33); C= 1 or 2 carbapenems (imipenem, meropenem, ertapenem) E-test levels were < 4 $\mu\text{g/ml}$ (n= 15), += susceptible; - = negative; H= high level of protein; M= moderate level of protein; R= low/reduced level of protein

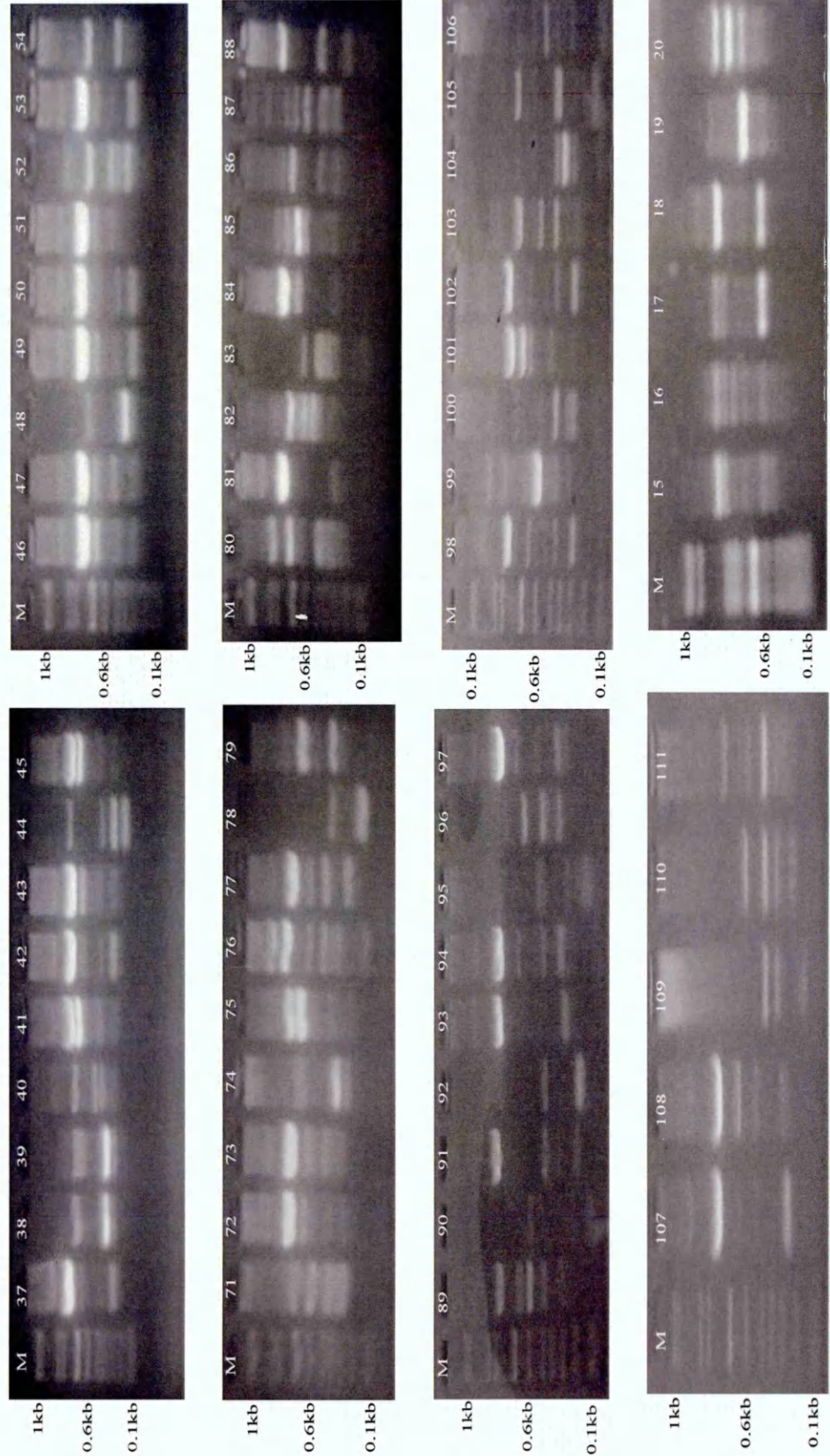
Appendix 5 Representative profiles of restriction enzyme digestion from cured carbapenemase producers



Note: M= DNA marker; Lanes, 50 through 98= plasmid-cured carbapenemase producers. In this gel, all the plasmid-cured carbapenemase producers are showing different band patterns

Appendix 6 Different band patterns obtained from carbapenem-resistant isolates on electrophoretic gels by ERIC-PCR technique





Note: M= DNA 1 kb marker, Lane numbers (1 through to 111 excluding 55 through to 70)= representative CR test samples listed in Appendix 1