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PHENOTYPIC AND GENOTYPIC DETECTION OF CARBAPENEMASE-PRODUCING ESCHERICHIA COLI AND KLEBSIELLA PNEUMONIAE IN ACCRA, GHANA

BY

FELICIA POKUAAH DWOMOH

(10701284)

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DECLARATION

I, Felicia Pokuaah Dwomoh, hereby declare that the work presented in this thesis is wholly a record of my original research carried out at the Department of Medical Microbiology, University of Ghana Medical School, Korle-bu under the supervision of Prof. Eric Sampane-Donkor (UGMS) and Dr. Nicholas T. K. D. Dayie (UGMS), and that no part has been submitted for any purpose elsewhere; however, all sources of information have been specifically acknowledged using references.

Sign …………………… 20th July, 2022.

Felicia Pokuaah Dwomoh

(Student)

Sign……………………… 20th July, 2022.

Prof. Eric Sampane-Donkor

(Main Supervisor)

Sign…………………….. 20th July, 2022.

Dr. Nicholas T. K. D. Dayie

(Co-Supervisor)

DEDICATION

I dedicate this work to my parents (Mr. Joseph Dwomoh and Madam Dora Achiaa), my siblings, other members of my family, and my amazing friends. I cannot imagine my life without you. I love you all.

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First and foremost, I say a very big thank you to the Almighty God for how far He has brought me in life. My profound appreciation goes to my supervisors, Prof. Eric Sampane-Donkor and Dr. Nicholas T. K. Dzifa Dayie for taking some time off their busy schedules to supervise this work, and for their immense contribution, direction, and encouragement. I again want to thank Dr. Beverly Egyir, a senior research fellow, and Ms. Felicia Amoa Owusu all of Noguchi Memorial Institute of Medical Research also for their immense participation and contribution. My appreciation again goes to Dr. Appiah-Korang Labi and Mr. Fleischer C. N. Kotey for their profound contribution and direction.

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ABSTRACT

Background: *Escherichia coli* and *Klebsiella pneumoniae* belong to the Enterobacteriaceae family, and are pathogens of high clinical significance. They are prone to acquiring antibiotic resistance genes, including those encoding carbapenemases, which confer resistance to carbapenems (the current drugs of last resort against infections with multidrug-resistant [MDR] Enterobacteriaceae). However, little is known about carbapenemase-producing *E. coli* and *K. pneumoniae* in Ghana.

Aim: To investigate the occurrence of carbapenem resistance among MDR *E. coli* and *K. pneumoniae* isolated from clinical specimens in Accra using phenotypic and genotypic methods.

Methodology: The study was cross-sectional, involving 144 clinical MDR *E. coli* and *K. pneumoniae* isolates originating from 15 different sample types from the Central Microbiology Laboratory of the Korle Bu Teaching Hospital between the periods of December 2020 to March 2021. The isolates were re-cultured, subsequently identified using standard biochemical tests, and subjected to antibiotic susceptibility testing using the Kirby-Bauer method. Carbapenem resistance was determined based on imipenem, meropenem, and ertapenem zones of inhibition, as well as minimum inhibitory concentrations (MICs); carbapenemase production was confirmed phenotypically with the Modified Hodge test (MHT) and Modified Carbapenem Inactivation Method (mCIM) and genotypically with multiplex PCR targeting bla_{KPC}, bla_{IMP}, bla_{NDM}, bla_{VIM}, and $bla_{OXA\text{-}48}$.

Results: Of the 144 MDR isolates, 69.4% were *E. coli*, and 30.6% were *K. pneumoniae*. The distribution of antimicrobial resistance rates among them were ampicillin (97.2%), cefuroxime (93.1%), sulfamethoxazole-trimethoprim (86.8%), tetracycline (85.4%), cefotaxime and cefpodoxime (77.1% each), amoxicillin-clavulanate (75%), ceftriaxone (73.6%), ciprofloxacin (70.8%), levofloxacin (66.0%), cefepime (65.3%), ceftazidime (64.6%), gentamicin (48.6), piperacillin-tazobactam (40.3%), cefoxitin (14.6%), amikacin (13.9%), ertapenem and meropenem (5.6% each), and imipenem (2.8%). In total, 5.6% (8/144) of them were carbapenem-resistant (carbapenem MIC range = 0.094–32.0 µg/ml), with 75% (6/8) of these testing positive by the phenotypic tests and 62.5% (5/8) by the genotypic test (of which 80% [4/5] carried $\mathit{bla}_{\mathit{OXA\text{-}48}}$ and 20% (1/5) $\mathit{bla}_{\mathit{NDM}}$). The $\mathit{bla}_{\mathit{VIM}}$, $\mathit{bla}_{\mathit{IMP}}$, and $\mathit{bla}_{\mathit{KPC}}$ genes were not detected.

Conclusion: Although the rates of antibiotic resistance among the isolates were high, the prevalence of carbapenemase producers were low. There is evidence that $\mathit{bla}_{\mathit{OXA\text{-}48}}$ and $\mathit{bla}_{\mathit{NDM}}$ may be circulating in Ghana, warranting upscaling of antimicrobial resistance surveillance programmes and fortification of infection prevention and control programmes in the country.

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LIST OF ABBREVIATIONS

AK	Amikacin
AMC	Amoxicillin-clavulanate
AMP	Ampicillin
AST	Antibiotic Susceptibility Testing
AMR	Antimicrobial Resistance
BSI	Bloodstream infections
mCIM	modified Carbapenem Inactivation Method
CPE	Carbapenemase-Producing Enterobacteriaceae
FEP	Cefepime
CTX	Cefotaxime
FOX	Cefoxitin
CPD	Cefpodoxime
CAZ	Ceftazidime
CRO	Ceftriaxone
CXM	Cefuroxime
CIP	Ciprofloxacin
DNA	Deoxyribonucleic acid

E-test	Epsilometer test
ETP	Ertapenem
E. coli	*Escherichia coli*
ESBL	Extended Spectrum Beta-Lactamase
ExPEC	Extraintestinal pathogenic *E. coli*
FP	forward primer
CN	Gentamicin
GN	Gram-negative
IMI	Imipenem
IMP	Imipenem-resistant Pseudomonas
ICU	Intensive care unit
K. pneumoniae	*Klebsiella pneumoniae*
KPC	*Klebsiella pneumoniae* carbapenemase
KBTH	Korle Bu Teaching Hospital
LEV	Levofloxacin
LPS	Lipopolysaccharide
MEM	Meropenem
mRNA	messenger RNA
MBL	Metallo-β-Lactamases
MRSA	Methicillin-Resistant *Staphylococcus aureus*
µg/ml.	Micrograms per millilitre
MIC	Minimum Inhibitory Concentration
MHT	Modified Hodge Test

MHA	Mueller-Hinton agar
MDR	Multidrug-resistant
NHSN	National Healthcare Safety Network
NDM	New Delhi Metallo-β-Lactamases
NMEC	Neonatal meningitis *E. coli*
NDM	New-Delhi-Metallo-β-lactamases
NMC-A	*Non-metallocarbapenemase type A*
OXA-48	Oxacillinase – 48
OXA	Oxacillinase
PAIs	Pathogenicity islands
PBP	Penicillin-binding protein
PTZ	Piperacillin/Tazobactam
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RP	Reverse primer
SEPEC	Sepsis-causing *E. coli*
SXT	Sulfamethoxazole-trimethoprim
T	Tetracycline
tRNA	Transfer RNA
USD	United States Dollars
UTI	Urinary tract infections
UPEC	Uropathogenic *E. coli*
VIM	Verona Intergron-encoded Metallo-β-Lactamases

WHO ……………………………………………………………………World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background

The Enterobacteriaceae is a Gram-negative (GN) bacteria family whose members are found in the gastrointestinal tracts of humans and animals (Okoche *et al*., 2015). The majority of the members in this family are pathogenic, and mostly cause community-acquired and healthcare-related infections, some of which include meningitis, infections of the gastrointestinal and urinary tracts, and septicaemia (Nordmann *et al*., 2011; Lutgring & Limbago, 2016).

Two examples of pathogenic bacteria of the Enterobacteriaceae family are *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*), and these have been implicated in multidrug-resistant infections (Agyepong *et al*., 2018). Such resistance traits have a global distribution and threaten the efficacy of antibiotics, therapeutic agents used in treating bacterial infections (Labi *et al*., 2018). In the last few years, there has been an increase in the pervasiveness of multidrug resistance in the Enterobacteriaceae, primarily mediated by the acquisition of resistance trait-encoding genes, such as those that code for extended-spectrum beta-lactamase (ESBL) (Falgenhauer *et al*., 2019; Hassuna *et al*., 2020). Carbapenems, such as imipenem, meropenem, and ertapenem, have been used as the last resort in the treatment of such infections (Schwaber & Carmeli, 2008).

There has however, been an insurgence in infections with carbapenem-resistant Enterobacteriaceae (CRE), and this is of concern, not only because such bacteria could potentially transmit mobile genetic elements encoding the resistance traits to hitherto susceptible bacteria, but also the high death rates they are associated with (Gupta *et al*., 2011). For example, carbapenem-resistant bacterial infections frequently fail to respond to conventional antibiotic treatment and are known to kill about 50% of patients with septicaemia or bacteraemia (Bratu *et al*., 2005; Nordmann *et al*., 2011). Essentially, infections with CRE have posed a principal public health challenge, particularly, because of the limited therapeutic options available to combat them (Ho *et al*., 2015). The production of β-lactamases through carbapenem hydrolysis has become one of the most important emerging resistance traits that render almost all β-lactam antibiotics somewhat obsolete (Nordmann & Poirel, 2013). Carbapenem resistance can also occur due to porin deficiencies, which permits reduced entry of the ß-lactam drugs into the membranes of cells (Nordmann *et al*., 2012).

The major types of carbapenemases worldwide are Class A *Klebsiella pneumoniae* carbapenemases ($\textit{bla}_{\textit{KPC}}$), Class B Metallo-β-lactamases ($\textit{bla}_{\textit{NDM}}$, $\textit{bla}_{\textit{VIM}}$ and $\textit{bla}_{\textit{IMP}}$), and Class D OXA β-lactamases ($\textit{bla}_{\textit{OXA}}$), and mobile genetic elements (transposons and plasmids) that facilitate their transmission (Ho *et al*., 2015). Carbapenemase-encoding genes can be identified using molecular techniques while carbapenemase activity can be identified using phenotypic assays (Okoche *et al*., 2015). Although *K. pneumoniae* and *E. coli* are not inherently resistant to carbapenems, infections with carbapenem-resistant *K. pneumoniae* and *E. coli* surfaced in the 1990s, following which a global spread of CRE has been reported (Yigit *et al*., 2001). Recent antibiotic therapy, a prolonged hospital stay, the use of invasive devices, and immunosuppression have all been linked to infections caused by CRE in healthcare settings (Manenzhe *et al*., 2015). CRE has been identified by the US Centre for Disease Control and Prevention as a serious health challenge because such infections have increased dramatically over the last decade, with about 9000 of the nosocomially-transmitted ones attributed to *E. coli* and *K. pneumoniae* (CDC, 2013).

Given the high clinical significance of CRE and the threat they pose to public health globally, it is important to continually conduct surveillance of such infections to provide data to guide the design and implementation of public health interventions.

1.2 Problem Statement

Antimicrobial resistance (AMR) has been classified as a major threat to modern medicine by the World Health Organization (WHO), with carbapenemase-producing Enterobacteriaceae (CPE) identified as a growing global menace (WHO, 2015). About fifty thousand lives are lost annually in Europe and the US alone due to infections caused by resistant pathogens (WHO, 2014). It is projected that failure to deal with this menace adequately could lead to an annual death of 10 million people, a 2–3.5% reduction in gross domestic product, and an overall cost of 100 trillion United States Dollars (USD), by the year 2050 (O'Neill, 2014). This AMR burden is expected to be somewhat higher in sub-Saharan Africa with a decline in a gross domestic product of $2,895 billion, representing 20% of the region's total economic output (O'Neill, 2014). In 2017, the WHO cited CRE as among the highest critical category of the global priority list of pathogens, probably because carbapenems are considered a part of the limited last-line antimicrobials (WHO, 2017). The rapid spread of CRE and CPE amid the slow-paced discovery of newer antimicrobials have made CRE and CPE a significant public health problem around the world (Gupta *et al*., 2011). They remain a part of the most difficult-to-treat MDR infections globally (Mitgang *et al*., 2018), and their infections are associated with high morbidity and mortality rates ranging from 30% to 75% (Tischendorf *et al*., 2016) and increased healthcare costs ranging from 22,484 to 66,031 USD per patient in the USA (Bartsch *et al*., 2017).

Studies across different geographical areas have reported increasing prevalence of CPE with endemic places being the USA, India, and Greece, and an imminent worldwide CPE epidemic has been predicted (Nordmann *et al*., 2011). In Italy, there was a rise of 1.3% to 15.2% prevalence of carbapenem-resistant *K. pneumoniae* from 2006 to 2010, and Hungary recorded a rise from 0.0% to 5.5% during the same period (Savard *et al*., 2013). Additionally, data from the National Healthcare Safety Network (NHSN) showed that between the period 2000 and 2008, there was a significant rise in multidrug-resistant (MDR) *K. pneumoniae* and *Escherichia coli* (from 7.0% to 13%) in the United States (Savard *et al*., 2013). A systematic review by Manenzhe *et al*. (2015) in Africa highlighted increasing reports of carbapenemase-producing bacteria in hospital environs – from 2.3% to 67.7% in North Africa and 9% to 60% in sub-Saharan Africa. In Ghana, a study conducted by Owusu-Oduro (2016) reported the prevalence of CRE in urinary tract infection (UTI) patients to be 10.0%. Additionally, Hackman *et al*. (2017) in Accra also established the emergence of CRE of 7.2% among phenotypic ESBL isolates. To add to this, a study conducted by Codjoe *et al*. (2019) reported a carbapenem resistance prevalence of 2.9%, with even non-lactose fermenters such as *Pseudomonas aeruginosa* and *Acinetobacter baumanii* contributing significantly to this proportion.

The global health threat posed by CRE calls for active CRE surveillance in different parts of the world. The organisms most implicated in CRE infections are *K. pneumoniae* and *E. coli* (Agyepong *et al*., 2018; Eichenberger & Thaden, 2019). Thus surveillance of carbapenem-resistant *K. pneumoniae* and *E. coli* infections is an important strategy in combating the public health threat posed by CRE and other antimicrobial-resistant pathogens globally.

1.3 Rationale

Despite the high clinical significance of CRE infections and the immense public health threat they pose, very few studies have been conducted on these infections in Ghana and other parts of Africa. Most of the studies on carbapenem resistance have predominantly originated from Asia, Europe, and North America (Queenan & Bush, 2007; Grundmann *et al*., 2010; Nordmann *et al*., 2011). The first-ever study on the detection and characterization of carbapenem resistance genes in Ghana focused mostly on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* and the various carbapenemase enzymes associated with them (Codjoe, 2016). Owusu-Oduro (2016) and Hackman *et al*. (2017), who also contributed data on carbapenem-resistant isolates in Ghana, limited their detection to disc diffusion and minimum inhibitory concentration (MIC) determination with E-Test strips, with no molecular characterizations done. Codjoe *et al*. (2019) addressed this limitation and studied a wide range of Gram-negative bacteria, but just a few of these were *K. pneumoniae*. Besides these, detecting and distinguishing between carbapenemases is not routinely done by diagnostic laboratories in the country, a phenomenon that pertains to most resource-poor settings. Hence there are several gaps in knowledge on carbapenem resistance in *E. coli* and *K. pneumoniae* in the country, particularly, harboured carbapenemase genes. This information is crucial to effective antimicrobial therapy and infection prevention and control. To help fill the identified knowledge gaps, this study evaluated the occurrence of carbapenem resistance among multidrug-resistant *E. coli* and *K. pneumoniae* isolated from clinical specimens in Ghana.

1.4 Aim

This study aimed at investigating the occurrence of carbapenem resistance among multidrug-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolated from clinical specimens in Accra, Ghana.

1.5 Specific Objectives

The study had the following as its specific objectives:

- To determine the prevalence of carbapenem resistance among multidrug-resistant *E. coli* and *K. pneumoniae* isolates
- To determine the antimicrobial resistance pattern among the multidrug-resistant *E. coli* and *K. pneumoniae isolates*
- To evaluate the distribution of selected carbapenemase genes among carbapenem-resistant *E. coli* and *K. pneumoniae* isolates

CHAPTER TWO

LITERATURE REVIEW

2.1 Gram-Negative Bacilli, Enterobacteriaceae and Infections

The Enterobacteriaceae are a large family of Gram-negative bacilli, such as *Klebsiella* spp., *Escherichia coli*, and *Enterobacter* spp., that are mostly isolated from clinical samples. The family is of particular importance, as its members are commonly involved in causing community and healthcare-associated infections, which could be intestinal or extraintestinal (Duin & Doi, 2017). The major transmission route of the intestinal disease is the faecal-oral, which could either be by direct contact with animals or their environment, person-to-person contact, or by ingestion of contaminated food or water. The extra-intestinal infection can occur by endogenous pathway where bacteria translocate from the gut to other parts of the body mostly in immunocompromised hosts (Jenkins *et al*., 2014). Extra-intestinal infections, such as sepsis, UTI, pneumonia, intra-abdominal infections, osteomyelitis and meningitis are mostly due to *Escherichia coli and K. pneumoniae*, and these organisms are the leading cause of morbidity, mortality and rising medical costs (Russo & Johnson, 2003**)**.

Escherichia coli is a predominant bacteria that causes UTIs within this family, while *Klebsiella* spp. and *Enterobacter* spp. mainly cause pneumonia. Bloodstream infections, other intra-abdominal infections, and peritonitis have all been linked to the Enterobacteriaceae family. Moreover, *Salmonella* spp., which also belongs to this family causes foodborne illnesses and can lead to invasive infections in some patients (Paterson, 2006).

In Ghana, a study conducted by Opintan & Newman (2017) revealed that *Escherichia coli* (20.4%) and *Pseudomonas aeruginosa* (16.3%) were predominantly involved in causing bloodstream

Infections. Another study in Ghana revealed *E. coli* and *K. pneumoniae* to be predominantly involved in the majority of infections caused by Gram-negative bacteria (Agyepong *et al*., 2018). The study, which additionally compared the bacterial isolates to the clinical diagnosis demonstrated that these Gram-negative bacilli mostly caused UTI, sepsis, tuberculosis and wound infections (Agyepong *et al*., 2018).

2.1.1 *Escherichia coli*

Escherichia coli is one of the commonest members of the Enterobacteriaceae family which mostly inhabit the gastrointestinal tracts of both humans and animals. *E. coli* typically colonize the gut of newborns within a few hours after delivery and lives in a mutualistic association with the host for years (Kaper *et al*., 2004; Tenaillon *et al*., 2010; Allocati *et al*., 2013). This bacteria's niche is the human colon, where it is the most abundant facultative anaerobe in the microflora of the human intestine (Kaper *et al*., 2004). Some strains of *E. coli* have picked up virulence factors for them to survive in new niches and cause a wide range of diseases (Kaper *et al*., 2004; Croxen & Finlay, 2010). *E. coli* are classified into pathogenic types based on the type of virulence factors they carry as well as the clinical symptoms exhibited by the affected host (Allocati *et al*., 2013). *E. coli* are therefore involved in both intestinal diseases which mostly results in pathogenic diarrhoea and extra-intestinal diseases such as UTI (Tivendale *et al*., 2010; Navarro-Garcia & Elias, 2011; Dale & Woodford, 2015). In the past, Extraintestinal pathogenic *E. coli* (ExPEC) were classified into Neonatal meningitis *E. coli* (NMEC), Uropathogenic *E. coli* (UPEC), Avian pathogenic *E. coli*, and sepsis-causing *E. coli* (SEPEC) and members were found to have similar virulence factors despite the hosts they were isolated from (Tivendale *et al*., 2010; Dale & Woodford, 2015).

Currently, where multidrug-resistant isolates have become a global burden with less effective antimicrobial agents to tackle them, extraintestinal *E. coli* has turned to be an important public health concern, resulting in major financial consequences (Dale and Woodford, 2015). ExPEC has certain virulence factors that enable them to cause diseases once they are no longer in the host intestinal tract, and they mostly contain at least two of these virulence factors in their genomes (adhesins, iron acquisition systems, protectins, invasins, and toxins) (Basu *et al*., 2013; Dale and Woodford, 2015). UTIs are the commonest extra-intestinal infections caused by UPEC, with sepsis and meningitis extraintestinal *E. coli* infections gradually increasing in frequency (Kaper *et al*., 2004).

Pathogenic *E. coli* strains possess virulence factors (VFs) that are encoded on genetic elements and can move to different strains to create new combinations of virulence factors (Kaper *et al*., 2004; Croxen & Finlay, 2010). These huge clusters of virulence factors also termed pathogenicity islands (PAIs), cannot be found in non-pathogenic strains of *E. coli*. These PAIs are found on plasmids, or integrated into the chromosomes of the bacteria (Hacker & Kaper, 2000; Croxen & Finlay, 2010). Pathogenesis of pathogenic *E. coli* uses a multi-step approach, which includes colonization of host mucosal surface, dodging of host defence mechanisms, multiplication, and finally, damage to host cells. EIEC remains intracellular while the other pathogenic strains are extracellular (Kaper *et al*., 2004). Some of these virulence factors include adhesins, which help the bacteria to colonise new niches and cause disease, toxins that contribute to the spread of bacteria in tissues, increase cytotoxicity and insensitivity to damage by neutrophils, and iron acquisition systems which help the bacteria acquire iron from the host for their survival which in turn enhances their ability to cause and sustain infections (Hacker and Kaper, 2000; Kaper *et al*., 2004; Sharma *et al*., 2007; Navarro-Garcia & Elias, 2011).

2.1.2 *Klebsiella pneumoniae*

The genus *Klebsiella* is divided into five species, namely *K. pneumoniae*, *K. oxytoca*, *K. ornithinolytica*, *K. terrigena*, and *K. planticola* (Podschun *et al*., 2001). The most important species clinically are *K. pneumoniae* and *K. oxytoca* (Podschun and Ullmann, 1998; Podschun *et al*., 2001). *K. pneumoniae* is a non-motile Gram-negative bacillus that is widely distributed in the environment and also found as commensals in the gut and nasopharynx of mammals. The isolates from the environment are indistinguishable from those isolated clinically from humans concerning their virulence and biochemical reactions (Podschun *et al*., 2001). They were traditionally termed as opportunistic pathogens that cause nosocomial and community-acquired infections (Namikawa *et al*., 2019; Roe *et al*., 2019) with a high rate of morbidity and mortality (Highsmith and Jarvis, 2016)**.** The pathogenicity of *K. pneumoniae* is related to its varying virulence factors, which allow it to attack the immune system and cause infections, and its ability to easily acquire multiple resistance genes (Sharma *et al*., 2007; Gharrah *et al*., 2017). *K. pneumoniae* can cause infections in all ages but serious life-threatening infections are found in children, the elderly and immunocompromised individuals (Highsmith and Jarvis, 2016). It causes pneumonia, urinary tract infections, surgical site infections and bacteraemia (Moghadas *et al*., 2018; Namikawa *et al*., 2019).

Some of these virulence factors present in *K. pneumoniae* that enable it to cause infections are hypermucoviscosity, endotoxin synthesis, production of a capsule, adhesions, iron uptake and lipopolysaccharides formation (Sharma *et al*., 2007). The presence of the bacterial capsule contributes to its virulence by preventing or delaying phagocytosis and directly modifying the host immune response (Highsmith and Jarvis, 2016; Namikawa *et al*., 2019). As a result of the different roles the capsule plays, infections caused by *K. pneumoniae* are associated with severe clinical presentations (Namikawa *et al*., 2019). Iron is an essential factor for the growth of bacteria hence bacteria development and the spread of infection in the host also depends on the availability of iron (Podschun & Ullmann, 1998; Yu *et al*., 2007). In the host system, the level of free iron available is about 10^{-18} M which is too low for the normal functioning of bacteria. Most of these irons in the host are bound to proteins such as transferrin, haemoglobin, lactoferrin and ferritin hence the bacteria secure iron supply from the host by secreting siderophores which compete with the host for iron (Podschun & Ullmann, 1998).

Aerobactin as a siderophore produced by *K. pneumoniae* isolates enhances iron assimilation and mediates the virulence of hypervirulent *K. pneumoniae* (Yu *et al*., 2007; Namikawa *et al*., 2019). *K. pneumoniae* also secretes endotoxins that cause the production of cytokines, activate various complement components, alter the host cellular immune response, and lead to pathophysiologic changes in the host thereby resulting in organ dysfunction and sepsis (Highsmith and Jarvis, 2016; Namikawa *et al*., 2019). Another virulence factor, the lipopolysaccharide (LPS), protects the microorganism against the host humoral defences. Aside from phagocytosis, one of the first lines of the host defences is the bactericidal effects of serum, which contains antibodies to fight off infections. The presence of a full-length O-antigen on the LPS protects the *K. pneumoniae* isolate from the lytic effect of the complement system and confer virulence in contrast to strains with shortened or absence of O-antigen (Podschun & Ullmann, 1998; Highsmith and Jarvis, 2016; Khaertynov *et al*., 2018).

2.2 Treatment of Infections Caused by Gram-Negative Bacteria

In the twentieth century, healthcare systems and medical practices have improved drastically due to the discovery of antibiotics. This discovery has led to the treatment of serious life-threatening infections by carrying out complicated medical procedures with minimal risk of exposing the patient to severe infections (Labi *et al*., 2018). In the late 20th century, it was quite simple for the ordinary clinician engaged in clinical practice to choose therapy for significant infections caused by Enterobacteriaceae. Clinicians have successfully used empirical antimicrobial treatment to improve patient care and shorten hospital stays in many cases (Fraser *et al*., 2006). Gentamicin, cotrimoxazole, tetracycline, ampicillin, some cephalosporins and fluoroquinolones which are first-line treatments and affordable drugs with a broad range of activity have been used to treat Gram-negative bacterial infections since the 1990s (Khadri and Alzohairy 2009, Habte-Gabr 2010). Whether targeted or empirical, cephalosporins and fluoroquinolones were seen as reliable antibiotics of choice. Unfortunately, this reliability has been defied in the early 21st century as a result of these organisms becoming resistant to these antibiotics (Denton, 2007). The overuse of antibiotics has been a global challenge over the past years (Meyer *et al*., 2013; Van Boeckel *et al*., 2014), with about 50% of patients reported having received unnecessary antibiotics (Hecker *et al*., 2003). The misuse of antibiotics is a major contributor to the increase and dissemination of multidrug-resistant bacteria isolates which is currently a problem worldwide (Okeke *et al*., 2005; Iosifidis *et al*., 2008; WHO 2014; Holmes *et al*., 2016). Carbapenems have become the last resort antibiotics for many life-threatening bacterial infections, particularly in healthcare, but most antibiotics are ineffective against multidrug-resistant Gram-negative bacteria (Moyane *et al*., 2013).

2.3 Antibiotics and Resistance

Throughout history, humans and microorganisms have "engaged in a constant battle". Diseases such as bubonic plague, tuberculosis, malaria, and HIV/AIDS have affected a large proportion of the world's population, and have subsequently led to morbidity and mortality. The discovery and development of antibiotics in the beginning and around the mid-20th century has favoured humans in this fight. Infections caused by bacteria saw a drastic improvement in treatment with the discovery and use of penicillin in the early 1940s (Tenover, 2006). Soon after the discovery and clinical use of antibiotics, microorganisms started developing resistance to them. As antibiotics became more widely accepted and used, the level and complexity of antimicrobial resistance among microorganisms grew. Microorganisms have always devised intelligent antimicrobial resistance mechanisms (Krause, 1992). Antimicrobial resistance has emerged as a significant public health challenge over the years and has threatened the treatment of infections. This resistance has eventually affected social and economic development (Newman *et al.*, 2011). Antimicrobial resistance is a phenomenon that is naturally occurring and this situation has been worsened by the irrational use of antibiotics both in humans and animals (Labi and Ofori-Adjei, 2018). Some of the reasons why bacterial resistance has become a global concern are that, firstly, bacteria such as *K. pneumoniae* and *E. coli* are becoming predominant in causing nosocomial infections. Additionally, infections caused by these resistant bacteria have fewer therapeutic options and subsequently result in treatment failure especially with dire consequences in the critically ill.

Also, WHO has projected that by the year 2050, about ten million people will die annually from antimicrobial resistance globally if antimicrobial resistance is not adequately dealt with (Jim O'Neill, 2014). About fifty thousand lives are lost annually in Europe and the US alone due to infections whose etiological agents are drug-resistant (WHO, 2014), and this is alarming. Antimicrobial resistance in Gram-negative bacilli occurs through the expression of enzymes by these GNBs that inactivate these antibiotics and also through non-enzymatic factors. The problem of antimicrobial resistance in Gram-negative bacilli is a major challenge faced by clinicians in the intensive care unit daily (Ruppé *et al*., 2015).

2.3.1 Mechanism of Action of Antibiotics

Antibiotics that are used in the treatment of bacterial infections are categorised based on their modes of action. The modes of action of antibiotics are formulated partly based on the anatomy of the bacteria and the chemical compositions of the antibiotics. There are about four major modes of action of antibiotics. These are:

1. Antibiotics that inhibit bacterial cell wall synthesis. The Gram-positive bacterial cell wall is made up of a thick peptidoglycan layer representing about 60% or more of the cell wall coated with magnesium ribonucleate and intertwined with teichoic acid. The Gram-negative bacteria cell wall constitutes a thin layer of peptidoglycan with the majority of its components being Lipopolysaccharides and lipoproteins (Lorian, 1971). Therefore, the cell wall of a bacteria whether Gram positive or Gram negative has a peptidoglycan layer that provides rigid support as well as account for the shape of the organism. The peptidoglycan is made up of polymers of polysaccharides containing alternating units of N-acetylglucosamine and N-acetylmuramic acid that are joined together by glycosidic linkage (Walker, 1996). These reactions are mediated by several enzymes such as transpeptidase and carboxypeptidase (Neu, 1985). The binding of

antibiotics that targets the bacterial cell wall inactivates some of these enzymes such as transpeptidase and prevents the cross-linking of the amino peptides resulting in a new form of peptidoglycan chain which is weak and lacks tensile strength. As these weak chains develop in the growing bacteria cells wall, the cells gradually rupture due to osmotic lysis (Toinasz and Holtje, 1977; Yocum *et al*., 1980). Examples of antibiotics that act by inhibiting cell synthesis of cell walls are β-lactams such as penicillins, cephalosporins and carbapenems (Neu, 1992; McManus, 1997).

2. An antimicrobial agent that inhibits cell membrane synthesis. The selective permeability of the cytoplasmic membrane allows for the entry and exit of certain substances in and out of the cell. The cell membrane is involved in important cell functions like DNA replication and synthesis of cell wall hence any antimicrobial agent that will halt these functions will consequently harm the cell. An example of such an antibiotic is polymyxin, which is amphipathic (Lorian, 1971). As the polymyxin enters the cytoplasmic membrane, it inserts itself between the lipid and protein layers. As the lipid-soluble end is inserted into the lipid layer and the hydrophilic end into the protein layer, there is the disruption of the cytoplasmic membrane which causes an increase in cell membrane permeability and the leakage of cytoplasmic content thereby leading to cell death (Gottlieb and Shaw, 1967; Lorian, 1971; Storm *et al*., 1977).

3. Antimicrobials that inhibit protein synthesis. For all the essential processes in a cell to take place, they require specific enzymes which serve as catalysts. These enzymes are proteins in nature with the specific nucleotide sequence in the Deoxyribonucleic acid

(DNA) of the organism. During transcription, the information present in the DNA of the organism is transmitted to the messenger RNA (mRNA) to be further converted into functional proteins. In translation, the mRNA comes into contact with the transfer RNA (tRNA) which attaches the specific amino acids arranged on the ribosomes to form a particular polypeptide chain or protein (Walker, 1996). In bacteria, the ribosomal subunits present are the 30S and 50S that make up the 70S ribosomal mRNA complex that is involved in translation. The 30S subunit serves as the site where the messenger RNA is bound while the 50S subunit also serves as the site for the attachment of the amino acid building blocks as well as the site for holding the growing polypeptide chain. Antibiotics that inhibit protein synthesis take full advantage of the differences in the ribosomal subunit and selectively inhibits bacterial growth (Tenover, 2006). Some antibiotics that act using this mechanism are tetracycline, which acts by binding to the 30S ribosomal subunit and prevents the binding of the transfer RNA to the ribosome thereby preventing the synthesis of the polypeptide chain and chloramphenicol which also acts by blocking the binding of amino acids unto the growing polypeptide chain (Nathans & Lipman, 1961; Gottlieb & Shaw, 1967).

4. Those that inhibit nucleic acid synthesis. Some of these antibiotics exert their effects by inhibiting the synthesis of either DNA or ribonucleic acid (RNA) in the organism. A few of these antibiotics are in clinical use since some do not exhibit selective toxicity, thus they are not able to differentiate between the nucleic acid of the host and that of the bacteria. Rifampicin is an example of such an antibiotic that acts by binding to the enzyme responsible for DNA transcription (DNA dependent RNA polymerase) thereby

blocking the synthesis of RNA. Additionally, antibiotics such as nalidixic acid and ciprofloxacin also act by inhibiting DNA replication in the bacteria which in the long run halts cell division due to the disparity between continued protein synthesis and inhibited protein synthesis (Sanders, 1988; Walker, 1996). The site of inhibition is the DNA gyrase which is the enzyme responsible for the super-coiling of the chromosomal DNA so that the large chromosomal DNA can fit into a highly constrained space in the bacterial cell. Quinolones inhibit this DNA gyrase so that this special arrangement is not achieved, therefore affecting transcription (Walker, 1996).

2.3.2 Mechanisms of Antimicrobial Resistance

A microorganism may either acquire resistance genes carried on bacterial chromosomes or plasmids or be intrinsically resistant to an antimicrobial agent (McManus, 1997; Santajit and Indrawattana, 2016). Intrinsic or innate resistance is a genetic property mostly encoded in the Chromosomal DNA of the microorganism and all members of the genus exhibit such property. In such an instance, all the strains of that bacterial species will remain resistant to all the members of those antibiotic classes (McManus, 1997; Tenover, 2006;). Acquired resistance comes about when there is a change in the DNA of the bacteria resulting in the expression of a new phenotypic trait (McManus, 1997).

Mechanisms of resistance include the following: alteration or inactivation of the drug, changes in the drug binding sites/targets, and alterations in the bacterial cell permeability resulting in decreased intracellular drug accumulation (Santajit and Indrawattana, 2016).

1. Alteration or inactivation of the drug: the microorganisms can acquire genes that encode for enzymes that permanently alter and inactivate the antimicrobial agent before it can have any effect on the organism. Examples of such enzymes are; beta-lactamases and aminoglycoside-modifying enzymes (Tenover, 2006; Santajit and Indrawattana, 2016).
2. Changes in the drug binding sites or targets: Some bacteria prevent the recognition by the antimicrobial agents by modifying their binding or target sites. An example includes a change in penicillin-binding protein (PBP) 2b in pneumococci, which subsequently leads to penicillin resistance (Tenover, 2006). Also, the expression of the unique PBP2a in *Staphylococcus aureus* enables the bacteria to survive in the presence of high concentrations of β-lactam antibiotics (Sefton, 2002; Santajit and Indrawattana, 2016).
3. Alteration in bacteria cell permeability resulting in decreased intracellular drug accumulation: Microorganisms can limit access of antibiotics to their target site or it can actively remove the drug from the bacteria cells resulting in decreased uptake (McManus, 1997). Most Gram-negative organisms are intrinsically resistant to β-lactam antibiotics mainly because of the impermeable nature of their outer membrane. The degree of impermeability varies with each organism (McManus, 1997). Efflux pumps are membrane proteins that transfer antibiotics from bacteria's cells to maintain a low intracellular concentration, resulting in antibiotic uptake being reduced (Giedraitienė *et al.*, 2011). Efflux pumps can be specific to a particular antibiotic but most of them can pump a wide range of unrelated antibiotics and this has contributed significantly to the development of Multidrug resistance (Giedraitienė *et al.*, 2011; Santajit and Indrawattana, 2016).

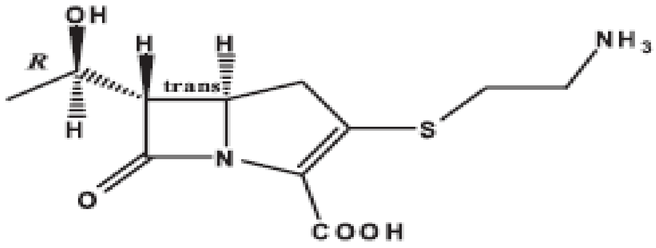
2.4 Carbapenems

Carbapenems are the most powerful beta-lactam antibiotics and have been strictly limited in treating severely ill patients. They exert a broad spectrum of activity against most bacteria including anaerobes (Bonfiglio *et al*., 2002). Due to this broad spectrum of activity, the clinical use of carbapenems has increased drastically in the last two decades since resistance to certain antibiotics (penicillins, cephalosporins, fluoroquinolones and aminoglycosides) have also increased (Schwaber *et al*., 2005; Zhanel *et al*., 2006).

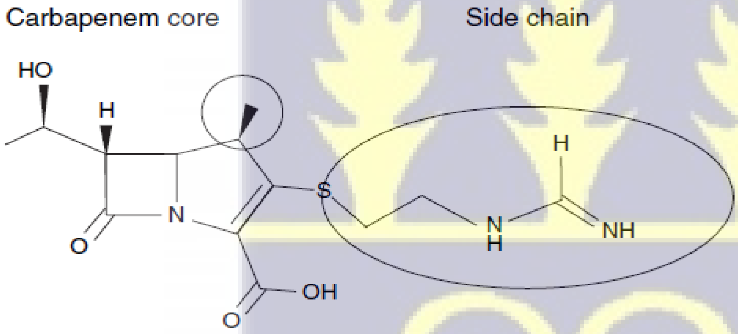
Carbapenems were developed in the 1980s as products of thienamycin. Initial members of the carbapenem class were imipenem and meropenem, which had a broad-spectrum activity against nearly all Enterobacteriaceae, including *Pseudomonas aeruginosa*, and sufficiently positioned for the treatment of infections (Perez and Van Duin, 2013). Cephalosporins were the first-line antibiotics used in the treatment of infections caused by Enterobacteriaceae, but resistance to these cephalosporins started to develop in the 1990s by Enterobacteriaceae acquiring extended-spectrum β-lactamases which inactivated these antibiotics. Invariably, as a result of the cephalosporin resistance, carbapenems that were still impermeable to these enzymes had to be used more frequently (Rahal *et al*., 1998). Carbapenems outperformed cephalosporins and fluoroquinolones in the treatment of infections caused by Enterobacteriaceae that produce these ESBLs (Endimiani *et al*., 2004; Paterson *et al*., 2004).

Carbapenems are similar in structure to penicillins. Their structure is made up of a β-lactam ring bonded to an unsaturated 5-membered ring. The double bond between C-2 and C-3 with the substitution of carbon for sulfur at C-1 distinguishes penicillins from carbapenems. The C-1 is important for the spectrum of activity, potency as well as stability of carbapenems against β-lactamases (El-Gamal *et al*., 2017). The carbapenems are distinguished from one another by the side chains attached to the two-ring structure.

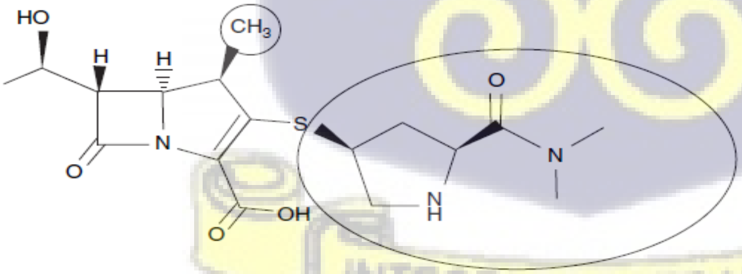
Thienamycin



Imipenem



Meropenem



Ertapenem

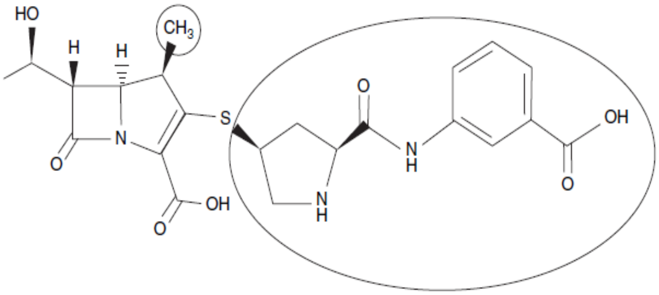


Figure 1: Chemical structures of Thienamycin, Penicillin, Imipenem, Meropenem and Ertapenem. Adapted from (Nicolau, 2008; Papp-Wallace *et al*., 2011).

2.4.1 Mechanism of action of Carbapenems

Carbapenem is a β-lactam antibiotic that act by binding to and deactivating penicillin-binding protein (PBPs) which are enzymes that are involved in the crosslinking and elongation of the peptidoglycan cell wall of bacteria (Nicolau, 2008). This leads to growth inhibition and cell wall destruction which consequently results in cell lysis and death of the bacteria, hence the activity of carbapenems tends to be bactericidal. Carbapenems interact with Gram-positive bacteria close to the cell’s surface. In Gram-negative bacteria, carbapenems enter through the porins (Papp-Wallace *et al*., 2011). After gaining entry into space which is between the surrounding membrane and the cell wall, the carbapenem acylates with critical PBPs. Unique features of carbapenem that make it more efficacious and able to exert a broad spectrum of activity are the ability to bind to multiple yet different penicillin-binding proteins (PBPs) and its resistance to a wide range of beta-lactamases (Nicolau, 2008; Papp-Wallace *et al*., 2011). In addition, carbapenem exhibits affinity to and binds their preferred PBPs and this uniqueness contributes to its inherent potency. Members of this class of antibiotics mostly have similar potency of antimicrobial activity because they largely exhibit similar affinities for PBPs.

Imipenem binds specifically to penicillin-binding protein 2, 1a and 1b, with only a weak affinity for PBP3. Also, meropenem has a high binding affinity for PBPs 2, 3, and 4. Ertapenem also shows a high binding affinity for PBPs 2 and 3 of *Escherichia coli*, even though it also binds to PBPS 1a, 1b, 4, and 5, just like imipenem. Doripenem, like meropenem, preferentially binds to PBP2 of *E. coli* and PBPs 2 and 3 of *Pseudomonas aeruginosa*, according to early studies (Davies *et al*., 2007).

Carbapenems have been classified into three groups. These are; Group 1: broad-spectrum carbapenems, which have a narrow spectrum of activity against non-fermentative Gram-negative bacilli (such as ertapenem). Group 2: broad-spectrum carbapenem which is more effective against non-fermentative Gram-negative bacilli (meropenem, imipenem and doripenem). Group 3: which is still underdeveloped but have clinical activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA) (Fukasawa *et al*., 1992; Nagano *et al*., 1999; Papp-Wallace *et al*., 2011; Michalska *et al*., 2013).

Most carbapenems have activities against clinically important Gram-negative bacteria, non-fermenters such as *Acinetobacter* spp. and anaerobes. Carbapenems provide the best spectrum of activity against Gram-negative bacteria unlike the other β-lactam antibiotics (Tellado and Wilson, 2005). They are also more stable to AmpC and ESBL β-lactamases (Jones *et al*., 2003; Colardyn F, 2005; DeRyke *et al*., 2005; Tellado and Wilson, 2005) but are susceptible to the inactivation by carbapenemases even though this resistant mechanism is less common but they are becoming widespread and global concern (Mushtaq *et al*., 2004; Fritsche *et al*., 2005).

2.5 Multidrug Resistance

In the past decade, infectious disease experts and intensivists have begun to face peculiar challenges in treating patients that are critically ill due to the selection and dissemination of multidrug-resistant isolates (Bassetti *et al*., 2019). Infections caused by these pathogens lead to high morbidity and mortality as well as prolonged hospital stay especially in developing countries (Agyepong *et al*., 2018). Resistance can develop through DNA mutations under selective antibiotic pressure (point mutations) or by horizontal gene transfer usually via mobile genetic elements. Antibiotic inactivating enzymes, such as extended-spectrum β-lactamases (ESBLs), and carbapenemases can be produced by these acquired resistance genes (Koulenti *et al*., 2019).

From a study conducted in Ethiopia, the researchers reported a high MDR of 86.5% among Gram-Negative bacilli from clinical specimens (Alemayehu *et al*., 2021). This study was in line with a study conducted in Ghana from a teaching hospital which showed an average multidrug resistance of 89.5% in Gram-negative bacilli (Agyepong *et al*., 2018). According to findings from a nationwide antimicrobial resistance surveillance study in Ghana, Opintan and his team saw an increased prevalence of multidrug resistance with most of the organisms being Gram-negative bacilli (*E. coli, P. aeruginosa, Citrobacter* spp., and *Klebsiella* spp.). It was also observed that 74% of the enterobacteria (*E. coli* and *Klebsiella* spp.) were ESBL producers with a greater than 85% resistance range to penicillins and third-generation cephalosporins (Opintan *et al*., 2015).

Carbapenems and cephamycins have the most consistent activity against ESBL-producing bacteria in vitro because they can withstand hydrolysis by ESBLs and porin penetration due to their general size and structure (Paterson, 2000; Paterson, 2006). Carbapenems are generally very useful as a therapy for multidrug-resistant organisms due to their susceptibility to most Enterobacteriaceae strains. According to Perez *et al*. (2013), the majority of the ESBL-producing Enterobacteriaceae are still susceptible to these drugs. However, enterobacteriaceae have also devised means to be resistant to these carbapenems.

2.6 Carbapenem Resistance and Mechanisms

Enterobacteriaceae resistance to carbapenems is mediated by three main mechanisms: enzymatic degradation through carbapenemase synthesis, efflux pump expression and decreased outer membrane permeability via porin mutations (Papp-Wallace *et al*., 2011; Eichenberger and Thaden, 2019) with carbapenemase production being the commonest. A combination of these resistant mechanisms can give rise to increased levels of resistance in isolates such as *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* (Papp-Wallace *et al*., 2011).

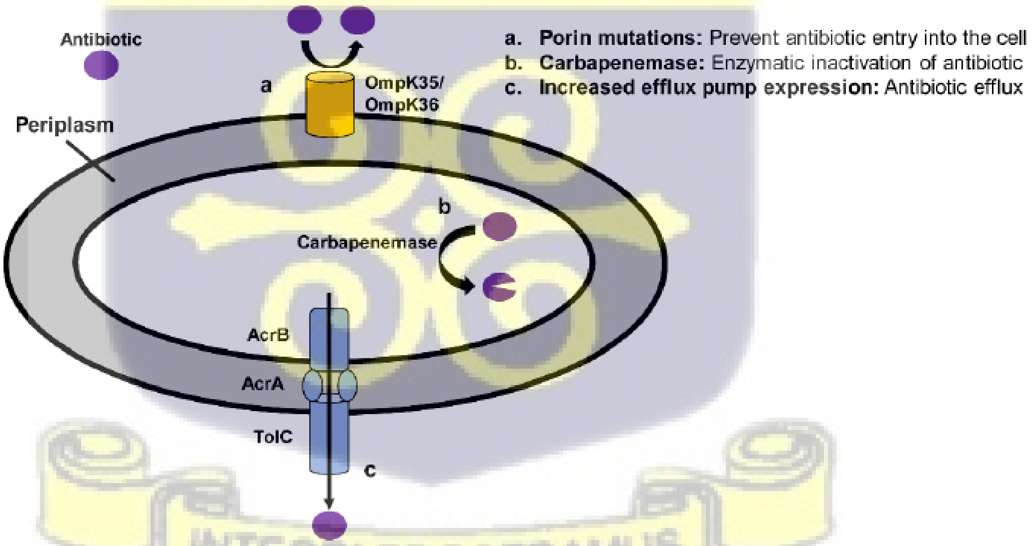


Figure 2: Mechanism of Enterobacteriaceae resistance to carbapenems.

Adapted from (Eichenberger and Thaden, 2019).

2.6.1 Carbapenemases

The Ambler classification system divides beta-lactamases into four categories, however, only classes A, B, and D are the three major classes of carbapenemases that confer carbapenem resistance in Enterobacteriaceae (Nordmann and Poirel, 2002).

Klebsiella pneumoniae carbapenemase family and the less frequently encountered *bla*$_{SME}$ and non-metallocarbapenemase type A (*bla*$_{NMC\text{-}A}$) enzymes belong to Class A and these enzymes may be found in *S. marcescens* and *E. cloacae*, respectively (Duin and Doi, 2017). All β-lactams are hydrolyzed by *bla*$_{KPC}$ enzymes but the activity of these enzymes is partially inhibited in-vitro by clavulanic acid, tazobactam, and boronic acid (Nordmann and Poirel, 2013).

Enzymes in the B class consist of Metallo-β-Lactamases (MBL) which include the Imipenem-resistant Pseudomonas (IMP)-type (*bla*$_{IMP}$) family of carbapenemases, New-Delhi-Metallo-β-lactamases (*bla*$_{NDM}$), as well as Verona Integron–encoded Metallo-β-lactamases (*bla*$_{VIM}$). These enzymes can hydrolyze a wide range of β-lactams in a zinc-dependent mode, but they can't hydrolyze monobactams like aztreonam (Duin and Doi, 2017). The activity of the Class B enzymes is not inhibited by any of the inhibitors currently in clinical use but is inhibited *in vitro* using compounds such as zinc chelators with EDTA (Nordmann and Poirel, 2013).

Class D carbapenemases comprise the oxacillinase (*bla*$_{OXA\text{-}48}$-like) β-lactamases which are produced by enterobacteriaceae. Separately, penicillins and carbapenems are weakly hydrolyzed by *bla*$_{OXA\text{-}48}$-like carbapenemases, but cephalosporins are not, making them more difficult to be detected (Poirel *et al*., 2012). These *bla*$_{OXA\text{-}48}$-like carbapenemases do not expressively hydrolyze cephalosporins but can hydrolyze aminopenicillins, ureidopenicillins, and carbapenems at low levels (Nordmann *et al*., 2011; P Nordmann *et al*., 2012). The activity of Class D enzymes is inhibited by sodium chloride (NaCl) *in vitro* (Nordmann *et al*., 2012).

When these carbapenemase enzymes coexist with other β-lactamases or porin permeability defects, high levels of carbapenem resistance develop (Duin and Doi, 2017)

2.6.2 Studies on Carbapenem Resistance

Carbapenem-resistant Enterobacteriaceae (CRE) have attracted a lot of research attention recently because of their high potential for rapid spread globally and outbreak episodes, such as occurred in the north-eastern United States (Jean *et al*., 2018).

A study conducted by Nagaraj *et al*. (2012) among 51 carbapenem-resistant *E. coli* and *K. pneumoniae* in a tertiary hospital in South India detected the presence of bla_{NDM} and bla_{VIM} carbapenemases. The clinical samples containing these isolates were collected from patients in various units of the hospital, including surgery, intensive care unit, and general medicine wards. Of the 36 *K. pneumoniae* isolates, 75% ($n = 27$) harboured bla_{NDM}, while 13.9% ($n = 5$) had bla_{VIM}. Additionally, out of the 15 *E. coli* isolates, 66.6% ($n = 10$) harboured bla_{NDM}, while 13.3% ($n = 2$) harboured bla_{VIM}, with the majority of these isolates detected in urine samples.

Again, in a systematic review and meta-analysis conducted by Rafailidis & Falagas (2014), the causative organism of CRE infections were predominantly *K. pneumoniae* and *E. coli*, with most of these pathogens being Metallo-β-lactamase carbapenemase producers, followed by bla_{KPC}. From these same studies, bacteremia was prevalent, and deaths associated with CRE were significantly higher than deaths associated with carbapenem-susceptible Enterobacteriaceae (CSE) infections.

In addition, a study was conducted in Hong Kong by Ho *et al*. (2015) on the characterization of carbapenem-resistant *E. coli* and *K.pneumoniae* obtained from a healthcare setting. In total, 92 carbapenem-resistant isolates, comprising 77.2% (*n* = 71) *K. pneumoniae* and 22.8% (*n* = 21) *E. coli* were analysed. Of these, the carbapenem resistance pattern among the study isolates were 98.9% (91/92) for ertapenem, 91.3% (84.92) for imipenem, and 95.7% (88/92) for meropenem. The PCR reported the presence of 3 bla_{NDM-1}, 3 bla_{IMP-4}, and 3 bla_{KPC-2} genes, and these were the isolates that tested positive for the Carba NP (phenotypic confirmatory tests); hence a correlation was found between the Carba NP and carbapenemase production.

Another study conducted by Park *et al*. (2019) in the Republic of Korea revealed that 69% of all carbapenemase-producing Enterobacteriaceae bloodstream infections (BSIs) were caused by *K. pneumoniae*, followed by *Enterobacter* species (10%) and *E. coli* (8%), and 66% of these developed *K. pneumoniae* carbapenemase (bla_{KPC}), followed by bla_{NDM}.

Furthermore, a study conducted in Shanghai, China on 880 faecal samples from children showed a 3.6% CRE carriage rate, and these strains were mostly found in *K. pneumoniae* (37.5%) and *E. coli* (37.5%). Additionally, except for polymyxin B and tigecycline, all CRE strains showed greater than 90% resistance to the most commonly used antibiotics, with the commonest carbapenemase gene in the GIT being bla_{NDM}, followed by bla_{KPC-2}, bla_{IMP-26}, and bla_{IMP-4} (Xu *et al*., 2020). Another study conducted in North California by Senchyna *et al*. (2019) showed that the majority of the carbapenem-resistant enterobacteriaceae infections were caused by *K. pneumoniae* and *E. coli*.

In Africa, in a study conducted on the emergence of carbapenem-resistant strains among urinary *K. pneumoniae* in Morocco (Bouamri *et al*., 2015), 85% of UTIs due to Enterobacteriaceae were *E. coli* and *K. pneumoniae*. Among the urinary ESBL-producing *K. pneumoniae* isolates, there was a 7% cross-resistance to carbapenems, which indicated an emergence of carbapenem-resistant isolates of *K. pneumoniae*, but no further tests for carbapenemase detection were performed.

Moreover, a study was conducted in Egypt by Kotb *et al*. (2020) on the epidemiology of carbapenem-resistant enterobacteriaceae, which reported 47.9% (1105/2306) of the enterobacteriaceae isolates to be CRE. Most of these isolates were *Klebsiella* spp. (53.7%) and *Enterobacter* spp. (43.5%), with 27.1% being *E. coli*. From the patient-level analysis, the CRE was predominantly isolated from blood and urine specimens. The study did not go further to characterize the CRE by genotypic methods.

Another study conducted in Nigeria on CRE by Oluwa-Okere *et al*. (2020) showed that among the 292 clinical isolates, 44.2% (*n* = 129) were resistant to third-generation cephalosporins, while 6.5% (*n* = 19) were carbapenem-resistant. The carbapenem-resistant isolates were 3 *Citrobacter freundii*, 6 *Enterobacter cloacae*, 8 *E. coli* and 2 *K. pneumoniae.* According to the phenotypic confirmatory tests (Carba NP) performed, 36.8% (7/19) showed carbapenemase activity, among which 5 $bla_{NDM\text{-}5}$ and 2 $bla_{OXA\text{-}181}$ were detected. These seven carbapenemase-producing Enterobacteriaceae were *E. coli* (2 $bla_{OXA\text{-}181}$), *Citrobacter freundii* (2 $bla_{NDM\text{-}5}$) and *Enterobacter cloacae* (3 $bla_{NDM\text{-}5}$).

In Ghana, a few studies have been conducted on carbapenem resistance. Owusu-Oduro (2016), for instance, conducted a study on the prevalence of carbapenemase-producing Enterobacteriaceae in persons with urinary tract infections using the Imipenem Epsilometer test (E-test). The study was conducted at MDS-Lancet laboratories Ghana limited at its head office at East Legon, Accra. In summary, 10% (22/220) of the isolates were found to be carbapenem-resistant, out of which 63.6% (*n* = 14) were *E. coli*, 27.3% (*n* = 6) were *K. pneumoniae*, and 4.5% (*n* = 1) each of *P. aeruginosa* and *Proteus mirabilis*. No additional confirmatory tests were performed on these carbapenem-resistant isolates.

A similar study was conducted at the same study site among phenotypic ESBL-producing Gram-negative bacteria by Hackman *et al*. (2017). According to the researchers, 1000 bacterial isolates were analysed, of which 600 turned out to be positive for phenotypic ESBL production using the combined disk synergy test. Out of the 600 isolates, 32.5% (*n* = 195) were *E. coli*, 22.5% (*n* = 135) were *K. pneumoniae*, 15% (*n* = 90) were *Providencia rettgeri*, and 7.5% (*n* = 45) each of *Citrobacter koseri*, *Acinetobacter baumanii*, *Pantoea species*, and *Proteus mirabilis*. The study recorded a carbapenem-resistant enterobacteriaceae prevalence of 7.2% (43/600) of which the majority were *E. coli* (34.9%) and *K. pneumoniae* (25.6%). The antimicrobial resistance patterns among the CRE were 100% each for penicillin, cephalosporins, amoxicillin-clavulanate, piperacillin-clavulanate, 83.7% for ciprofloxacin, 79.7% for gentamicin, 27.9% for amikacin, 18.6% for colistin and 11.6% for fosfomycin. Similar to the study conducted by Owusu-Oduro (2016), no further phenotypic or genotypic characterization tests were done on that CRE.

In another study conducted by Quansah *et al*. (2019) on β-lactam resistance among *Klebsiella* spp., the prevalence of meropenem, imipenem, and ertapenem resistance among the *K. pneumoniae* isolates were 29.7% (27/91), 14.3% (13/91), and 16.5% (15/91), respectively. Resistance of *K. pneumoniae* to other antibiotics was 100% for ampicillin and greater than 70% resistance for ceftriaxone, ceftazidime and cefotaxime. Three out of the ninety-one isolates harboured carbapenemase genes which were 3 *bla*$_{OXA-48}$ and 1 *bla*$_{NDM}$, with one of them harbouring two of the genes.

Also, another study conducted by Codjoe *et al*. (2019) on carbapenem-resistant Gram-negative bacilli from four selected hospitals showed an overall prevalence of 2.9% (111/3840). These facilities were the Korle-Bu Teaching Hospital (KBTH), Effia-Nkwanta Hospital (ENH), Ho Regional Hospital (HRH) and AngloGold Mines Hospital (AMH). The carbapenem-resistant isolates were made up of 51 *P. aeruginosa*, 31 *Acinetobacter spp*., 12 *E. coli*, 7 *Pseudomonas putida*, and 3 each of *K. pneumoniae* and *E. cloacae*, and 1 each of *Providencia stuartii*, *Cronobacter sakazakii*, *Shigella sonnei*, and *Sphingomonas paucimobilis*. Phenotypic confirmatory tests that determined the carbapenemase activities were found to be 18.9% (21/111) by the modified Hodge test and 2.7% (3/111) by boronic acid disc synergy test (BADST). No carbapenem-resistant isolate was positive by both phenotypic confirmatory tests. The only isolates that were positive for the BADST were the *E. cloacae* while the 21 isolates that were MHT positive comprised of 13 *Acinetobacter* spp., 6 *Pseudomonas aeruginosa*, and 2 *K. pneumoniae*. From the genotypic test, 23.4% (26/111) harboured carbapenemases of which 14.4% (16) were *bla*$_{NDM-1}$, 7.2% (8) were *bla*$_{VIM-1}$ and 1.8% (2) were *bla*$_{OXA-48}$. The distribution of carbapenemases among the resistant isolates were; *Acinetobacter* spp. (9 *bla*$_{NDM-1}$), *Pseudomonas aeruginosa* (2 *bla*$_{NDM-1}$ and 7 *bla*$_{VIM-1}$), *E. coli* (3 *bla*$_{NDM-1}$), *K. pneumoniae* (2 *bla*$_{OXA-48}$), *P. putida* (*bla*$_{VIM-1}$), *P. stuartii* (1 *bla*$_{NDM-1}$) and *S. sonnei* (1 *bla*$_{NDM-1}$). The two *K. pneumoniae* which tested positive for MHT were those detected by PCR to harbour *bla*$_{OXA-48}$ genes hence there was a correlation between the MHT positivity and OXA-48 detection.

2.7 Laboratory Detection of Carbapenemases

Antibiotic susceptibility testing results obtained by either disk diffusion method or automated systems are used to identify carbapenemase producers in clinical infections. (Miriagou *et al*., 2010). CRE diagnosis must be accurate and timely to determine appropriate treatment and infection control measures (Bouamri *et al*., 2015). Several techniques have been established to identify the presence of carbapenemases in Enterobacteriaceae (Iovleva and Doi, 2017) and they are grouped into phenotypic and genotypic tests (Bouamri *et al*., 2015). Phenotypic tests are further grouped into those that are directed at detecting elevated MIC to specific carbapenems (MIC testing for ertapenem, imipenem and meropenem) and those that are directed at detecting carbapenem hydrolysis either directly (Carbapenem Inactivation Method) or indirectly (modified Hodge test, CarbaNP test) (Bouamri *et al*., 2015). Molecular techniques are still the gold standard for identifying and distinguishing carbapenemases. Most are based on PCR, and if necessary, sequencing can be used to confirm the identity of a carbapenemase rather than just its group (Nordmann *et al*., 2012).

2.7.1 Phenotypic Tests

2.7.1.1 Modified Hodge Test (MHT)

In this test procedure, 1 in 10 dilutions of 0.5 McFarland suspension of *E. coli* ATCC 25922 is prepared and seeded on Mueller-Hinton agar (MHA) plate using a sterile cotton swab. A 10 µg carbapenem disk is then placed in the centre of the MHA plate, and each of the test isolates streaked from the carbapenem disk to the periphery of the plate. The agar plates are then incubated in ambient air (35 °C ± 2 °C) overnight for 16–24 hours. After incubation, if a carbapenemase is produced by the test isolate, the carbapenem on the agar plate is hydrolyzed, allowing the susceptible *E coli* in the background to grow in toward the disk, creating a cloverleaf-like appearance (Lee *et al*., 2001; Anderson *et al*., 2007; Iovleva and Doi, 2017; Codjoe *et al*., 2019). This assay is unable to distinguish between the different types of carbapenemase. Most importantly, increased amounts of AmpC β-lactamases and the presence of certain ESBLs have produced false-positive results. MHT produces false-negative results mainly with enterobacteriaceae with Metallo-β-Lactamases (MBL) genes and hence is unreliable in detecting *K. pneumoniae* producing bla_{NDM} (Tzouvelekis *et al*., 2012).

A study was conducted in France on 30 carbapenem-resistant Enterobacteriaceae genotypically characterized and was observed that 22 of them showed positivity for carbapenemase (bla_{KPC} = 7, bla_{VIM} = 6, bla_{NDM-1}=3, and bla_{OXA-48} = 6). Modified Hodge tests using meropenem, ertapenem, imipenem, and doripenem each successfully identified 21 of 22 carbapenemase producers (95%). The unidentified strain was a bla_{NDM} producer making MHT a less sensitive test for the identification of MBLs (Birgy *et al*., 2012). Another study conducted in South India on carbapenem-resistant *E. coli* and *K. pneumoniae* from a tertiary hospital using phenotypic tests revealed that the presence of Metallo-β-Lactamases (bla_{NDM} and bla_{VIM}) was poorly detected by MHT (Nagaraj *et al*., 2012).

In Ghana, a study by Codjoe *et al*. (2019) revealed that Modified Hodge Test positivity among the isolates was 18.9% among the carbapenem-resistant isolates. From the same study, a correlation was observed between MHT and PCR assay positivity for the bla_{OXA-48} gene but undetected for bla_{VIM-1}, bla_{KPC-1} and bla_{IMP-1}.

2.7.1.2 Carbapenem Inactivation Method (mCIM)

In this method, the main principle is incubating a carbapenem with a bacterial suspension to detect enzymatic hydrolysis. A 10 µg meropenem disk is incubated in a suspension of the test bacteria with tryptic soy broth in ambient air for 2–4 hours. After incubation, the meropenem disk is removed from the suspension and placed on an MHA plate already seeded with 0.5% suspension of *E. coli* ATCC 25922. The MHA is then inverted and incubated in ambient air (35 °C ± 2 °C) for 18-24 hours after which the zone sizes are measured. If carbapenemase is produced, the meropenem in the disk would have been broken down during the initial incubation process hence there will be no inhibition or limited growth of the *E coli*, making it appear as if the susceptible *E coli* is resistant. A clear inhibition zone is formed around the meropenem disc if no carbapenemase is produced. This method has been validated against Enterobacteriaceae with sensitivity and specificity greater than 99% among strains producing bla_{KPC}, $bla_{OXA\text{-}48}$, bla_{NDM}, bla_{VIM}, and bla_{IMP} carbapenemases (Skov and Skov, 2012; Iovleva and Doi, 2017; CLSI, 2018).

From a study conducted in China, mCIM was highly recommended as against Carba NP and MALDI-TOF/MS due to its simplicity, low cost, equipment and skills employed knowing they all have sensitivities and specificities around 99% (Zhong *et al*., 2020). A study conducted in Canada using enterobacterales isolates from different continents across the globe with different carbapenemases showed that mCIM had an overall sensitivity of 97.7% and a specificity of 96.8% for detecting different carbapenemases (Chan *et al*., 2020). Furthermore, from a study conducted in North California from 2013 to 2016 on carbapenem-resistant Enterobacteriaceae using mCIM, 62 CRE isolates were identified by Antimicrobial Susceptibility Testing (AST). Out of the 62 isolates, 24 of them were positive for a single carbapenemase gene. The study recorded a 100% (24/24) positivity for mCIM for the detection of carbapenemase activity (Senchyna *et al*., 2019).

Across the African sub-continent, there is a scarcity of data on the use of mCIM as an appropriate phenotypic tool for detecting carbapenemase activity in Enterobacteriaceae. A study from Ethiopia found that the prevalence of CRE from clinical specimens using mCIM was 9% out of 111 Gram-negative bacilli with 13 of the isolates resistant to meropenem by disc diffusion (Alemayehu *et al*., 2021).

Currently, there is no published study in Ghana that has employed the use of mCIM to detect carbapenemase activity and this study seems to bridge that gap by employing mCIM as one of its phenotypic tests comparing results to the PCR assay.

2.7.2 Genotypic Tests

Carbapenemase genes can be detected using either a conventional or a real-time polymerase chain reaction. Multiplex PCR assays to identify the most common carbapenemase genes have been developed and can be used in situations where they are available (Iovleva and Doi, 2017). The genotypic tests employ amplification and detection of specific carbapenemase genes (bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and bla_{OXA-48}) by polymerase chain reaction (PCR), and it remains the gold standard (highly specific and sensitive) for gene detection (Bouamri *et al*., 2015). The PCR can be either a single technique or multiplex and can give results within a few hours with higher sensitivity and specificity (Nordmann *et al*., 2012).

From a study conducted on carbapenem-resistant *E. coli* and *K. pneumoniae* isolates in Hong Kong, nine (9.8 %) of them were carbapenemase producers and these were made up of six *K. pneumoniae* (2 bla_{IMP-4}, 3 bla_{KPC-2} and 1 bla_{NDM-1}) and three *E. coli* (1 bla_{IMP-4} and 2 bla_{NDM-1})

isolates (Ho *et al*., 2015). Another study conducted in South India from a tertiary hospital detected bla_{NDM} and bla_{VIM} genes only. The prevalence of bla_{NDM} was in the study was found to be 75% (27/36) for the *K. pneumoniae* and 66% (10/15) for *E. coli* (Nagaraj *et al*., 2012).

Again, from the study conducted by Senchyna *et al.* (2019), out of the 62 Enterobacteriaceae that tested positive for carbapenem-resistant using AST, a single carbapenemase gene was detected in 24 of them which were bla_{OXA-48} like ($n = 6$), bla_{NDM} ($n = 5$), bla_{KPC} ($n = 5$), bla_{SME} ($n = 5$), bla_{IMP} ($n = 2$), and bla_{VIM} ($n = 1$).

Although molecular data on carbapenemase is limited in Africa, a review conducted by Mitgang *et al*. (2018) showed that the most frequent genotype groups distributed in the continent are bla_{OXA}, bla_{NDM} and bla_{VIM} among *E. coli* and *K. pneumoniae*.

From the study conducted by Codjoe *et al*. (2019) in Ghana, the most prevalent carbapenemase group were bla_{NDM-1} followed by bla_{VIM-1} and bla_{OXA-48} mostly isolated from *Acinetobacter* species and *P. aeruginosa*. Additionally, another study in Ghana also reported on bla_{OXA} and bla_{NDM} only in *K. pneumoniae* isolates (Quansah *et al*., 2019). These studies are in line with the study conducted in Africa by Mitgang *et al*., (2018) on the most frequent genotypes in the continent.

Furthermore, there is an increasing report of multidrug-resistant *E. coli* and *K. pneumoniae* in Ghana (Obeng-Nkrumah *et al*., 2013; Feglo, P. K and Adu-Sarkodie, 2016; Quansah *et al*., 2019). A recent study in Ghana reported on carbapenemase distribution in only *Klebsiella* spp. from selected health facilities (Quansah *et al*., 2019) even though there is also an MDR rise in *E. coli*. Although both *E. coli* and *K. pneumoniae* are involved in both healthcare and community-associated infections, little is known about the carbapenemase groups of these isolates in Ghana and this study seems to address that.

2.8 Treatment of Carbapenem-Resistant Enterobacteriaceae Infections

There are limited therapeutic options available to treat infections caused by CRE. Polymyxins which act on the cell membrane of the organism thereby producing a bactericidal effect is available for use. The commonest is colistin (Polymyxin E) and Polymyxin B which are mostly used in treating human infections. Due to the risks (nephrotoxicity) associated with the administration of polymyxin, other novel agents are preferred (Bassetti *et al*., 2019). Aminoglycosides such as gentamicin, amikacin and tobramycin are also used particularly when there is polymyxin resistance (Gonzalez-Padilla *et al*., 2015; Bassetti *et al*., 2019). Tigecycline, fosfomycin and carbapenems are all used either as a monotherapy or combination therapy in the treatment of infections caused by CRE (Rafailidis and Falagas, 2014). There have been suggestions that inappropriate treatment of CRE infections can be reduced by combination therapy as a result of the synergistic effect they exert (Petrosillo *et al*., 2013).

2.9 Risk factors for CRE

Various factors predispose an individual to infections caused by multidrug-resistant organisms including ESBL and CRE producers. These resistant organisms can lead to life-threatening infections and the risk factors that have been reported to be implicated include receipt of mechanical ventilation, immune suppression, previous exposure to antimicrobials, advanced age, admission to intensive care unit (ICU), organ transplantation and longer hospital stay (Codjoe, 2016).

A study conducted by Asare *et al*. (2009) in a neonatal ICU in a tertiary hospital in Ghana revealed that the major contributing factor to high ICU infections was associated with non-compliance of health professionals in the practice of hand hygiene protocols.

One of the prominent risk factors for the acquisition of CRE is exposure to antibiotics. Specific antibiotic classes normally implicated as risk factors for CRE are carbapenems, cephalosporins, fluoroquinolones, aminoglycosides and β-lactam/β-lactamase inhibitors (Gupta *et al*., 2011). This is because additional resistance determinants that can lead to cross-resistance to other antibiotics are carried by the same plasmid that confers resistance to CRE (Gupta *et al*., 2011; Brink, 2012). Research by Jeon *et al*. (2008) showed that imipenem and metronidazole were the antibiotics associated with the acquisition of carbapenem-resistant *E. coli*. They explained that metronidazole use disrupts the anaerobic flora of the intestinal tract which is involved in preventing the enteric colonization of exogenous bacteria, thus conferring “colonization resistance”. The risk of acquisition of CRE infections is higher in developing countries due to the high proportion of irrational use of antibiotics (Codjoe, 2016).

CHAPTER THREE

METHODOLOGY

3.1 Study Area

The study was carried out at the Korle Bu Teaching Hospital (KBTH). This facility is a 2000-bed capacity referral hospital situated in Accra, the capital city of Ghana. It attained a teaching hospital status in 1962 and is ranked as the third-largest hospital in Africa. The hospital is made up of 17 clinical and diagnostic departments or units including Child Health, Medicine, Pathology, Obstetrics and Gynaecology, Radiology and Laboratory just to mention but a few. Daily, the hospital sees about 1,500 patients and has about 250 patient admissions. KBTH is one of the few hospitals in Africa where sophisticated laboratory investigations are carried out (https://kbth.gov.gh). The Central Microbiology Laboratory of KBTH, where the study isolates were collected, processes more than 40,000 clinical cultures annually (Obeng-Nkrumah *et al.*, 2013), including samples such as blood for culture, urine, cerebrospinal fluids, stool, skin scraping, wounds, sputum and other fluids (pleural and abscess).

3.2 Study Design and Sampling

The study was cross-sectional, involving 144 clinical, non-duplicate multidrug-resistant *E. coli* and *K. pneumoniae* isolates that were randomly selected from wound, urine, sputum, blood, and miscellaneous (pus, pleural fluid, aspirate, ear, eye and high vaginal swab) samples, and processed by the Central Microbiology Laboratory of the Korle-Bu Teaching Hospital from December 2020 to March 2021. In this study, multidrug resistance was defined as isolates resistant to at least one agent from three or more antibiotic classes (Agyepong *et al*., 2018). Patients' information on clinical diagnosis and demographics were obtained from the patients' records using the clinical data collection form in Appendix I. These data collection forms were completed and accompanied each bacterial isolate that was collected from the Central Microbiology Department, KBTH**.** The isolates were collected into tryptic soy broth (Oxoid, Basingstoke Hampshire, UK) containing 10% glycerol, and stored under -20 °C until further analysis.

3.2.1 Sample Size

The sample size calculation was done using the formula:

$n = Z^2 \times p\,(1\text{-}p) / d^2$ (Daniel, 1999).

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

p = expected prevalence of CRE (7.2% – the prevalence reported by Hackman *et al*. (2017) with regard to CRE in Ghana)

d = allowable error for the study (0.05).

This provided a minimum sample size of 103.

3.2.2 Eligibility Criteria and Sampling

3.2.2.1 Inclusion Criteria

Patients whose clinical specimens were confirmed by the Central Microbiology Laboratory of the Korle Bu Teaching Hospital to contain multidrug-resistant *E. coli* or *K. pneumoniae* from all sample sites were included in the study.

3.2.2.2 Exclusion Criteria

Positive cultures other than *E. coli* and *K. pneumoniae* multidrug-resistant isolates were not included in this study.

3.3 Bacterial Identification

Stored clinical isolates were retrieved from the freezer (-20 °C), allowed to thaw, vortexed, and recultured onto MacConkey agar and blood agar plates (Beckton, Dickinson and Company, USA). These agar plates were then incubated aerobically at 37 °C for 18–24 hours. These isolates were worked on at the Department of Medical Microbiology at the University of Ghana. The culture media were examined for growth and bacteria were identified based on colonial morphology, Gram staining, and appropriate conventional biochemical tests (indole, oxidase, citrate, urease, triple sugar iron, and motility). The MDR status of Enterobacteriaceae that were *E. coli* and *K. pneumoniae* were further confirmed by performing antibiotic susceptibility testing (AST) using the Kirby-Bauer disc diffusion method on Mueller-Hinton agar (MHA) plates (Oxoid, Basingstoke

Hampshire, UK), and zones of inhibition compared to that of CLSI (2021) guidelines for interpretation.

3.4 Antibiotics Susceptibility Testing

The following antibiotics were used: meropenem (10 µg), ertapenem (10 µg), imipenem (10 µg), piperacillin-tazobactam (100/10 µg), ceftriaxone (30 µg), cefuroxime (30 µg), sulfamethoxazole-trimethoprim (1.25/23.75 µg), cefpodoxime (10 µg), gentamicin (10 µg), cefoxitin (30 µg), amoxiclav (20/10 µg), ampicillin (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), levofloxacin (5 µg), ciprofloxacin (5 µg), amikacin (30 µg), cefepime (30 µg) and tetracycline (30 µg) (Oxoid, Basingstoke Hampshire, UK; Mast Group, UK). The various zone sizes were compared to CLSI guidelines (CLSI, 2021) for their interpretation. Briefly, each test isolate was emulsified in sterile 0.85% saline (Fisher Scientific, UK) to create a suspension equivalent in turbidity to that of 0.5% McFarland standard, using a nephelometer (BD PhoenixSpec, USA). A sterile cotton swab was dipped into the suspension and subsequently seeded evenly across the entire surface of a Mueller Hinton agar (MHA) plate (Oxoid, Basingstoke Hampshire, UK) to obtain a semi-confluent growth after incubation. The plates were incubated at 37 °C for 18–24 hours, after which the zones of inhibition around the antibiotic discs were measured. Quality control was performed with *E. coli* ATCC 25299 strain and *K. pneumoniae* ATCC 700603.

3.5 Phenotypic Detection of Carbapenem Resistance

3.5.1 Kirby-Bauer Disc Diffusion Test

Carbapenem resistance among the MDR *K. pneumoniae* and *E. coli* isolates were determined by using these cut-offs for ertapenem (<22 mm, 10 µg), imipenem (<23 mm, 10 µg) and meropenem (<23 mm, 10 µg) disc after performing the antimicrobial susceptibility testing.

3.5.2 E-test Method

Confirmation of disc diffusion-based resistance profiles of carbapenems was performed using the E-test method. The test was performed on those isolates that were resistant to at least one of the carbapenems by the disc diffusion method. The E-test was used to determine the minimum inhibitory concentration (MIC) of antimicrobial agents against the multidrug-resistant *E. coli* and *K. pneumoniae* isolates. This test was made up of predefined gradients of imipenem, meropenem and ertapenem (Biomerieux SA, Marcy-l'Etoile, France) concentration on a plastic strip. The E-test method was performed by making a suspension of the *E. coli* and *K. pneumoniae* isolates using sterile 0.85% saline. These colony suspensions were each adjusted to 0.5 McFarland standard turbidity and inoculated onto MHA before the E-test strips were applied. The gradient of the antimicrobial agent from the strips was transferred immediately to the medium. An inhibition ellipse centred along the strip was formed after 16–18 hours of incubation at 35 °C ± 2 °C ambient air. At the point where the inhibition ellipse edge intersects the strip, the MIC was read directly from the scale in micrograms per millilitre (µg/ml). MICs using E-tests were performed with these breakpoints from CLSI (2021): ertapenem (≤ 0.5 µg/ml), imipenem (≤1 µg/ml) and meropenem

(≤1 µg/ml). Enterobacteriaceae that produce carbapenemases usually test intermediate or resistant to one or more carbapenems using the current breakpoints by CLSI (2021).

3.5.3 Confirmatory Tests for Carbapenem Resistance

Phenotypic carbapenemase confirmatory tests can be employed especially in situations where genotypic tests are not readily available to avoid delay in reporting potential carbapenemase producers. The phenotypic tests can be based on carbapenemase activity inhibition, detecting diffusible carbapenemases, and synergy between inhibitors and carbapenems. Some examples of phenotypic carbapenemase tests include the modified Hodge test (MHT), modified carbapenem inactivation method (mCIM), double-disc synergy tests, and CarbaNP test. Phenotypic confirmatory tests can be performed using one or two of these methods and for this study, both MHT and mCIM were chosen.

3.5.3.1 Modified Hodge Test (MHT)

This test detects diffusible carbapenemases. The test involves bacterial isolates streaked from the edge of a carbapenem disc that has been placed on an agar plate seeded with a carbapenem susceptible *E. coli* strain to the periphery of the plate. If carbapenemase is produced by the test isolate, the carbapenem on the agar plate is hydrolyzed, allowing the susceptible *E coli* in the background to grow in toward the disk, creating a cloverleaf-like appearance (Lee *et al.*, 2001; Anderson *et al.*, 2007; Iovleva and Doi, 2017).

Briefly, the test involved a 1 in 10 dilutions of 0.5 McFarland standard suspension of *Escherichia coli* ATCC 25922 seeded on MHA plates. Ertapenem discs (10 µg) were placed at the centre of these seeded plates, after which the test isolates (*K. pneumoniae* and *E. coli* cultured on blood agar for 18 to 24 hours) were streaked from the edge of the plate to the edge of the disc in a straight line. Afterwards, all the plates were incubated at 37 °C for 18 to 22 hours. Strains that showed cloverleaf shape around the ertapenem discs were considered positive for carbapenemase activity (Azimi *et al*., 2013). *K. pneumoniae* ATCC BAA-1705 and *E. coli* ATCC 25922 served as positive and negative control strains, respectively.

3.5.3.2 Modified Carbapenem Inactivation Method (mCIM)

The test involves incubating the test isolate with a carbapenem disc. After incubation, the carbapenem disc is placed on an agar plate already seeded with a carbapenem susceptible *E. coli* strain. After overnight incubation, the carbapenem in the disk is hydrolyzed if the test isolate is a carbapenemase producer, resulting in no or decreased growth of inhibition of the meropenem-susceptible *E. coli* ATCC 25922 (CLSI, 2021).

Briefly, a sterile bacteriological loop was used to pick 1 µL loopful each of isolates of *K. pneumoniae* and *E. coli* from an overnight blood agar and was separately emulsified in 2mL Tryptic Soy Broth (TSB) (Oxoid, Basingstoke Hampshire, UK) in test tubes. The inoculums were then vortexed and a 10 µg meropenem disc was added to each test tube. The TSB-meropenem disc suspension in the test tubes was incubated at 37 °C for four hours. Following the incubation for 4 hours, a 0.5 McFarland suspension of *E. coli* ATCC 25922 was prepared and inoculated onto MHA plates. The meropenem discs were removed from each TSB-meropenem disc suspension using a

10 µL loop and then placed on the MHA plate previously seeded with *E. coli* ATCC 25922 strain. These plates were then incubated at 37 °C for 18–24 hours. Following incubation, zones of inhibition were measured, and zone diameters between 6–15 mm were considered as carbapenemase-positive or the presence of pinpoint colonies within 16–18 mm zones.

3.6 Molecular Characterization of Carbapenem Resistance

Molecular detection of carbapenem resistance genes was performed at the Bacteriology Department, Noguchi Memorial Institute for Medical Research. Crude DNA was obtained from isolates using the boiling method and used as a template for PCR. Each reaction mix (25µL) contained 12.5 uL of 2 x DreamTaq Green PCR Master Mix, 4.5 µL of primer mix 6 µL of molecular grade nuclease free water and 2 µL of DNA template. For detection of carbapenemase enzymes, 5 primers were chosen from the 3 classes of carbapenemase genes: Class A (KPC gene); Class B meta-llo-beta-lactamases (NDM, VIM, IMP); and Class D oxacillinases (OXA-48) (Khurana *et al*., 2017). *Klebsiella pneumoniae* ATCC BAA 1705 was used as positive control. Amplification was done at 94°C for 3 minutes as the initial step for denaturation, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 61.6°C for 30 seconds and extension at 72°C for 1 minute. Final elongation was at 72°C for 7 minutes. All PCR amplicons were analyzed by horizontal gel-electrophoresis in a 2% (weight/volume) (SeaKem®GTG®Agarose, Lonza) using Tris/Acetate/EDTA 50 x concentrate buffer. The agarose was stained with 5 uL of Gel-red (Bio-Rad, UK). About 5 µL of amplicons were put into the wells and run in a 1 x Tris-acetate EDTA (TAE) at 100 volts for 1 hour. A 100 bp molecular ladder (Fermentas, Germany), was used as a marker. The amplicons were visualized with the Ultra-violet Gel Illuminator (UVP BioDoc-It^2 Imager).

Details of the primer sequences that were used in the detection of the carbapenemase-producing isolates are presented in Table 1.

Table 1: Primers for the detection of isolates that produced carbapenemases

Carbepenemase Genes	Amplicon size (bp)	Primer Sequence (5' - 3')	References
bla$_{KPC}$	683	FP: GTATCGCCGTCTAGTTCTGC RP: 5'-GGTCGTGTTTCCCTTTAGCC	(Obeng-Nkrumah *et al.*, 2019)
bla$_{\mathrm{VIM}}$	390	FP: GATGGTGTTTGGTCGCATA RP: CGAATGCGCAGCACCAG	(Poirel *et al.*, 2011)
bla$_{\mathrm{IMP}}$	188	FP: GGAATAGAGTGGCTTAAYTCTC RP: CCAAACYACTASGTTATCT	(Obeng-Nkrumah *et al.*, 2019)
bla$_{OXA\text{-}48}$	438	FP: GCGTGGTTAAGGATGAACAC RP: CATCAAGTTCAACCCAACCG	(Poirel *et al.*, 2011)
bla$_{NDM}$	760	FP: GAAGCTGAGCACCGCATTAG RP: TGCGGGCCGTATGAGTGATT	(Obeng-Nkrumah *et al.*, 2019)

FP = forward primer; RP = reverse primer; *bla*$_{KPC}$ = *Klebsiella pneumoniae* carbapenemase; *bla*$_{\mathrm{VIM}}$ = Verona Intergron-encoded Metallo-β-Lactamases; *bla*$_{\mathrm{IMP}}$ = Imipenem-resistant Pseudomonas; *bla*$_{OXA\text{-}48}$= Oxacillinase – 48; *bla*$_{NDM}$ = New Delhi Metallo-β-Lactamases

3.7 Ethical Considerations

Ethical clearance was sought from the Ethical and Protocol Review Committee of the College of Health Sciences with Protocol Identification Number: **CHS-Et/M.7 – 5.2 / 2020-2021** and the Institutional Review Board of KBTH with protocol ID**: STC/IRB/000121/2020**. The participants' information were coded to ensure confidentiality. Patients' pieces of information that were collected onto the data collection form was kept safe in a locked filing cabinet and data was transcribed onto a password-protected computer whose access is restricted to the research team.

3.8 Data Analysis

Statistical Products and Services Solutions (SPSS), an Excel-based statistical tool, was used to enter and analyze the data. Descriptive analyses such as counts, frequencies, and percentages were computed. Proportions were compared using the Chi-square test. Prevalence was calculated by dividing the number of occurrences of carbapenem resistance *E. coli* and *K. pneumoniae* strains by the total number of multidrug-resistant *E. coli* and *K. pneumoniae* strains during the study period, and this was stratified based on gender. P-values ≤ 0.05 were interpreted as statistically significant.

CHAPTER FOUR

RESULTS

4.1 Demographics and characteristics of bacterial isolates

A total of one hundred and forty-four (144) *E. coli* and *K. pneumoniae* isolates were evaluated in this study. Among these, 69.4% (*n* = 100) were *Escherichia coli*, and 30.6% (*n* = 44) were *Klebsiella pneumoniae*. The study isolates were collected from fifteen (15) different sample types, with the majority of them isolated from urine (68.75%), followed by wound (9.0%), blood (4.9%), and other sources presented (Table 2). With regard to the gender distribution of the participants from whom the isolates were recovered, 59.0% (*n* = 85) were females and 41.0% (*n* = 59) were males (Table 2). As observed in Table 2, the participants were aged between 3 days and 89 years, and the overall mean age was 41.14 ± 24.5 years; the mean age for males was 49.10 ± 27.72 years, and that of females was 35.61 ± 20.41 years. Most of the isolates were from patients above the age of 60 years (25.7%), followed by the age range of 31 to 40 years (20.1%), with the least number of samples collected from those within the ages of 11 to 20 years (5.6%).

Table 2: Distribution of bacterial isolates by gender, age, and sources of sample collection

DEMOGRAPHIC CHARACTERISTICS		BACTERIAL ISOLATES				
		E. coli		*K. pneumoniae*		
		Number	%	Number	%	Total
GENDER						
	Female	58	40.3	27	18.8	85(59.0)
	Male	42	29.2	17	11.8	59(41.0)
AGE						
	>60	29	20.1	8	5.6	37(25.7)
	0 – 10	12	8.3	9	6.3	21(14.6)
	11 – 20	4	2.8	4	2.8	8 (5.6)
	21 – 30	15	10.4	6	4.2	21(14.6)
	31 – 40	23	16.0	6	4.2	29(20.1)
	41 – 50	8	5.6	6	4.2	14(9.7)
	51 – 60	9	6.3	5	3.5	14(9.7)
TYPES OF SAMPLES						
	Abscess	1	0.7	0	0.0	1(0.7)
	Aspirate	3	2.1	1	0.7	4(2.8)
	Blood	4	2.8	3	2.1	7(4.9)
	Catheter tip	1	0.7	0	0.0	1(0.7)
	Cord	0	0.0	1	0.7	1(0.7)
	Ear	1	0.7	3	2.1	4(2.8)
	Endocervix	3	2.1	0	0.0	3(2.1)
	Vagina	5	3.5	1	0.7	6(4.2)
	Nose	1	0.7	0	0.0	1(0.7)
	Pleural fluid	0	0.0	1	0.7	1(0.7)
	Sputum	0	0.0	1	0.7	1(0.7)
	Throat	0	0.0	1	0.7	1(0.7)
	Urethral	1	0.7	0	0.0	1(0.7)
	Urine	70	48.6	29	20.1	99(68.8)
	Wound	10	6.9	3	2.1	13(9.0)

Males (Mean, SD) = 49.10, 27.72; Females (Mean, SD) = 35.61, 20.41

4.2 Resistance Patterns of the Bacterial Isolates

The bacterial isolates were tested against 19 different antibiotics belonging to 9 different antibiotic classes, namely penicillin, cephalosporin, cephamycin, β-lactam combination, tetracycline, carbapenems, aminoglycosides, quinolones, and folate pathway antagonists, as shown in Table 3 below. The study revealed a high proportion of resistance to ampicillin (97.2%) followed by cefuroxime (93.1%), sulfamethoxazole-trimethoprim (86.8%), tetracycline (85.4%), cefotaxime (77.1%), cefpodoxime (77.1%), amoxicillin-clavulanate (75%), ceftriaxone (73.6%), ciprofloxacin (70.8%), levofloxacin (66.0%), cefepime(65.3%), ceftazidime (64.6%), gentamicin (48.6), piperacillin-tazobactam (40.3%), cefoxitin (14.6%), amikacin (13.9%), ertapenem (5.6%), meropenem (5.6%) with the least resistance observed in imipenem (2.8%).

Eight isolates (5.6%) showed resistance to at least one of the three carbapenems used. Therefore the overall prevalence of carbapenem resistance among *E. coli* and *K. pneumoniae* according to the Kirby-Bauer disc diffusion method was 5.6%. Among the eight isolates, 50% (*n* = 4) were *E. coli*, and the other half were *K. pneumoniae*. All the eight carbapenem-resistant isolates showed 100% resistance to both meropenem and ertapenem; 50% of them were resistant to imipenem. Figure 3 shows detailed antibiotic resistance patterns (disc diffusion method) of the study isolates.

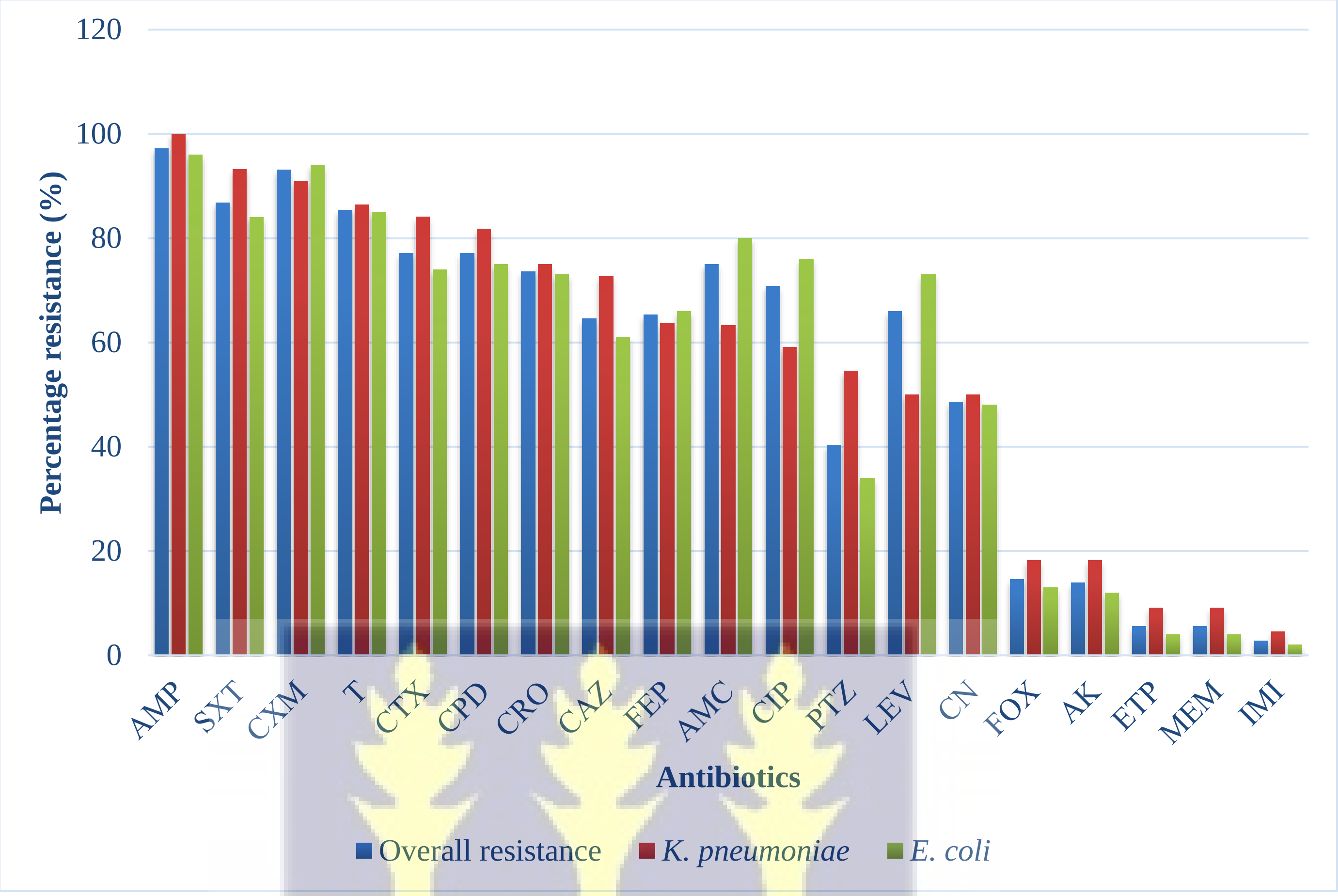


Figure 3: Antimicrobial resistance patterns of the study isolates.

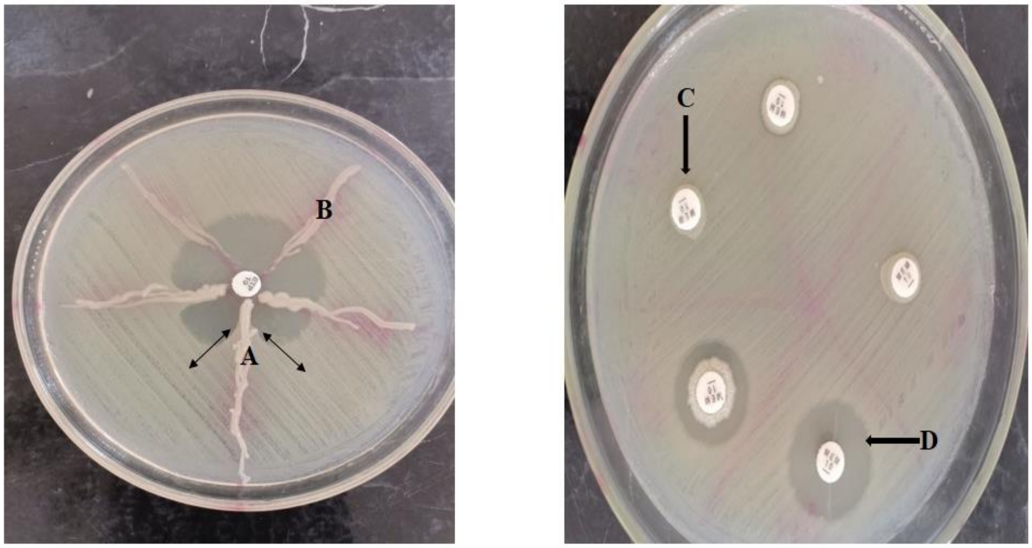
In the figure, MEM=Meropenem; IMI=Imipenem; ETP=Ertapenem, PTZ=Piperacillin/tazobactam; AMC=Amoxicillin/clavulanate; AMP=Ampicillin; CRO = Ceftriaxone; CXM=Cefuroxime; CPD = Cefpodoxime; FOX=Cefoxitin; CTX=Cefotaxime; FEP=Cefepime; CAZ=Ceftazidime; LEV=Levofloxacin; CIP=Ciprofloxacin; AK=Amikacin; CN=Gentamicin; T=Tetracycline; SXT= Sulfamethoxazole-trimethoprim

Table 3: Classification of antibiotics used in the study

CLASSES	ANTIBIOTICS (CODE)	CONCENTRATION (µg)
Carbapenems	Ertapenem (ETP)	10
	Imipenem (IMI)	10
	Meropenem (MEM)	10
Penicillin	Ampicillin (AMP)	10
β-Lactam combinations	Piperacillin/Tazobactam (PTZ)	100/10
	Amoxicillin clavulanate (AMC)	20/10
Cephalosporins	Cefepime (FEP)	30
	Ceftazidime (CAZ)	30
	Ceftriaxone (CRO)	30
	Cefotaxime (CTX)	30
	Cefpodoxime (CPD)	10
	Cefuroxime (CXM)	30
Cephamycin	Cefoxitin (FOX)	30
Quinolones	Levofloxacin (LEV)	5
	Ciprofloxacin (CIP)	5
Aminoglycoside	Amikacin (AK)	30
	Gentamicin (CN)	10
Tetracycline	Tetracycline (T)	30
Folate Pathway Antagonist	Sulfamethoxazole-trimethoprim (SXT)	1.25/23.75

4.3 Phenotypic Distribution of Carbapenemase-Producing *Escherichia coli* and *Klebsiella pneumoniae*

With regard to the phenotypic confirmation of carbapenem resistance, 75% (6/8) of the isolates were positive by the MHT and 75% (6/8) by the mCIM. Five out of the eight (62.5%) isolates exhibited carbapenemase activities by both methods. There was an equal distribution (3 *E. coli* and 3 *K. pneumoniae*) of positive tests by both MHT and mCIM among the resistant isolates. Therefore, there was a significant association between MHT and mCIM in the confirmation of carbapenem resistance ($p < 0.0001$). Figure 4 represents plates showing the interpretation of an MHT and mCIM while Table 4 shows the phenotypic confirmatory tests of the carbapenem-resistant isolates.



LEFT **RIGHT**

Figure 4: Plates showing Modified Hodge Test and modified Carbapenem Inactivation Method

Left: A = Positive Modified Hodge Test isolate with arrows showing the clover-leaf indentation; B= Negative Modified Hodge Test isolate

Right: C = Positive modified carbapenem Inactivation Method; D = Negative modified Carbapenem Inactivation Method.

Table 4: Phenotypic confirmation of carbapenem-resistant *E. coli* and *K. pneumoniae*

BACTERIA ISOLATES	PHENOTYPIC CARBAPENEMASE TESTS		BOTH
	MHT	mCIM	
Escherichia coli	3 (37.5)	3 (37.5)	2 (25.0)
Klebsiella pneumoniae	3 (37.5)	3(37.5)	3 (37.5)
Total	6 (75)	6 (75)	5 (62.5)

MHT, Modified Hodge Test; mCIM, Modified Carbapenem Inactivation Method; BOTH, both MHT and mCIM. Values are presented as n (%). $p < 0.0001$

4.4 Distribution of Carbapenemase-producing genes among *E. coli* and *K. pneumoniae* isolate

The overall prevalence of carbapenemase genes by PCR was 5/8 (62.5%). Out of these 62.5% carbapenemase genes, 80.0% (4/5) were $bla_{OXA\text{-}48}$ and 20.0% (1/5) were bla_{NDM} genes. *K. pneumoniae* carried the highest number of resistance genes (3 $bla_{\mathrm{OXA\text{-}48}}$ and 1bla_{NDM}) while one $bla_{OXA\text{-}48}$ gene was detected in one *E. coli* isolate. The study did not detect bla_{VIM}, bla_{KPC} and bla_{IMP} genes. There were no bacterial isolates that harboured multiple carbapenem resistance genes. Figures 5 and 6 below show the gel images of the carbapenemase genes detected.

The age groups whose infecting isolates harboured resistance genes were at the extremes of life 40% (2/5) from 0 to 10 years and 40% (2/5) above 60 years. More females (2.1%) carried resistance genes than males (1.4%) in this study. Table 5 shows the distribution of carbapenemases among the carbapenem-resistant isolates.

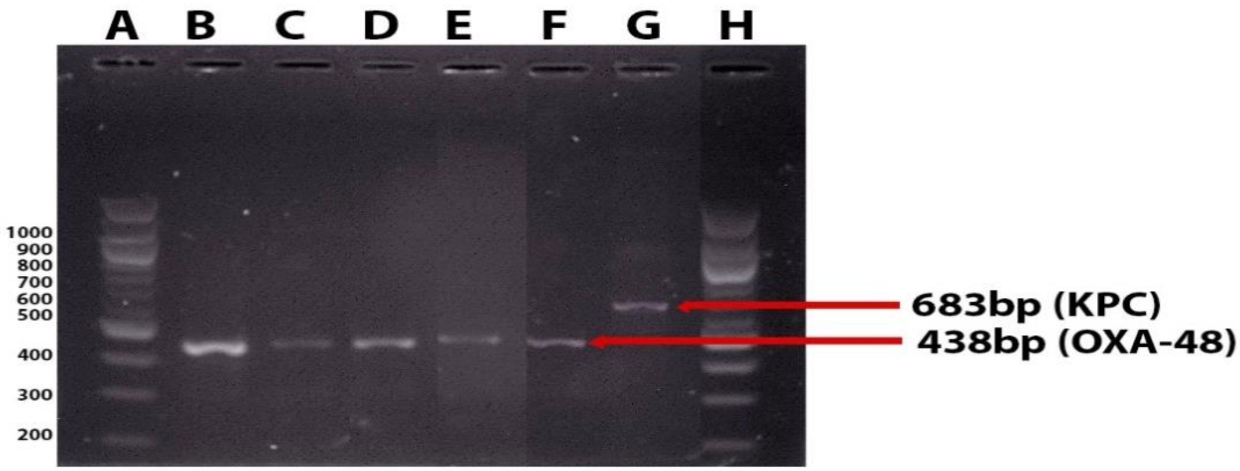


Figure 5: PCR screening of *E. coli* and *K. pneumoniae* isolates for the presence of *bla$_{KPC}$* and *bla$_{OXA-48}$* genes.

In the figure, A and H = Ladder; B = *bla$_{OXA-48}$* positive control; C to F = bacterial isolates; G = *bla$_{KPC}$* positive control

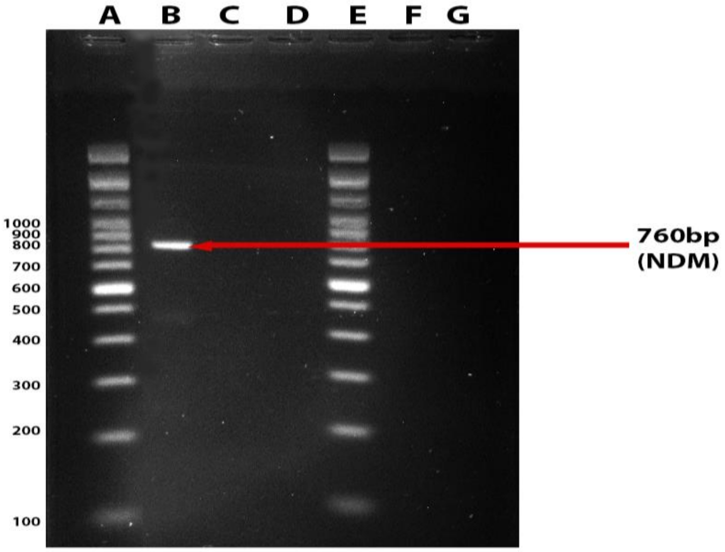


Figure 6: PCR Screening of *E. coli* and *K. pneumoniae* isolates for *bla*$_{NDM}$, *bla*$_{VIM}$, and *bla*$_{IMP}$ genes

In the figure, A and E = Ladder; B and C = Bacterial isolates, D = Negative control

Table 5: Distribution of carbapenemase genes among the carbapenem-resistant isolates

SAMPLE ID	ORGANISM	SEX	AGE (YEARS)	SAMPLE	*bla*NDM	*bla*OXA-48
001	***E. coli***	Female	65	Urine	-	POS
006	***K. pneumoniae***	Male	62	Aspirate	POS	-
061	***E. coli***	Male	28	Urine	-	-
069	***K. pneumoniae***	Female	1	Urine	-	POS
100	***K. pneumoniae***	Male	2 months	Blood	-	POS
102	***E. coli***	Female	39	Urine	-	-
103	***K. pneumoniae***	Female	41	Blood	-	POS
104	***E. coli***	Female	20	Blood	-	-

POS = positive; ESBL = Extended spectrum β-Lactamase, *bla*NDM = New Delhi Metallo-β-lactamase; *bla*OXA-48 = Oxacillinase-48; - = No gene detected

4.5 MICs of carbapenem-resistant isolates harbouring carbapenemase genes

In this study, carbapenem MICs ranged from 0.094 µg/mL to 32.0 µg/mL. Ertapenem MIC (2 resistant, 1 intermediate) was a better predictor of carbapenemase gene compared to those of imipenem (5 sensitives) and meropenem (5 sensitives). Ertapenem (disc and MIC) correlated with the presence of carbapenemase encoding genes. Overall, MIC values for the carbapenemase-encoding genes were lower as compared to values published by EUCAST and CLSI. Table 6 shows the MICs of the carbapenemase-producing isolates.

Table 6: Antibiogram of the PCR positive bacterial isolates

			CARBAPENEMS (Disc-µg)			CARBAPENEMS (MIC – µg/mL)		
SAMPLE ID	**ISOLATES**	**PCR**	**MEM**	**IMI**	**ETP**	**IP**	**MP**	**ETP**
001	*E. coli*	*bla*$_{\text{OXA-48}}$	R	R	R	0.75 (S)	0.5 (S)	3.0 (R)
006	*K. pneumoniae*	*bla*$_{\text{NDM}}$	R	S	R	1.0 (S)	1.0 (S)	2.0 (R)
061	*E.coli*	-	R	R	R	32 (R)	32 (R)	32 (R)
069	*K. pneumoniae*	*bla*$_{\text{OXA-48}}$	R	R	R	0.75 (S)	0.25 (S)	1.0 (I)
100	*K. pneumoniae*	*bla*$_{\text{OXA-48}}$	R	R	R	0.25 (S)	0.19 (S)	0.5 (S)
102	*E. coli*	-	R	S	R	0.25 (S)	0.094 (S)	0.38 (S)
103	*K. pneumoniae*	*bla*$_{\text{OXA-48}}$	R	S	R	0.75 (S)	0.5 (S)	0.38 (S)
104	*E. coli*	-	R	S	R	2.0 (R)	0.25 (S)	0.25 (S)

MEM= meropenem; IMI = imipenem; ETP = Ertapenem; R = Resistant; S= Sensitive; I = Intermediate; values of MICs are presented with their either susceptibility or resistant report as n (R/S/I)

4.6 Correlation of Phenotypic and Genotypic Carbapenem Resistance Tests

Of the eight isolates that were resistant to carbapenem by disc diffusion, 5 were positive for carbapenemase genes. All the four *K. pneumoniae* isolates that were resistant to carbapenem harboured resistance genes (3 *bla*$_{OXA\text{-}48}$, 1 *bla*$_{NDM}$). Both modified Hodge Test and modified carbapenem inactivation methods were excellent detectors of *bla*$_{OXA\text{-}48}$ genes but were not able to detect the presence of *bla*$_{\mathrm{NDM}}$. Therefore, there was a correlation between both MHT and mCIM positivity and the detection of *bla*$_{\mathrm{OXA\text{-}48}}$ genes. Out of the three *E. coli* isolates that were positive for MHT and mCIM, only 1 was a carrier of a resistance gene (1 *bla*$_{\mathrm{OXA\text{-}48}}$), and these are evident in Table 7.

Table 7: Summary of results on the Phenotypic and Genotypic Detection of Carbapenemase Resistance

ISOLATES	PHENOTYPE			GENOTYPE					
	DISC n=144	MHT n=8	mCIM n=8	PCR n=8	***bla***$_{OXA\text{-}48}$	***bla***$_{NDM}$	***bla***$_{KPC}$	***bla***$_{VIM}$	***bla***$_{IMP}$
K. pneumoniae	4 (2.8)	3 (37.5)	3 (37.5)	4 (50.0)	3 (37.5)	1 (12.5)	-	-	-
E. coli	4 (2.8)	3 (37.5)	3 (37.5)	1 (12.5)	1 (12.5)	-	-	-	-
Total	8 (5.6)	6 (75)	6 (75)	5 (62.5)	4 (50)	1 (12.5)	-	-	-

Values are represented as *n* (%); PCR = polymerase chain reaction; DISC = disc diffusion method; MHT = modified Hodge test; mCIM = modified carbapenem inactivation method; Common carbapenemase genes = *bla*$_{OXA\text{-}48}$, *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{IMP}$; - = No gene detected

CHAPTER FIVE

DISCUSSION

This study aimed to investigate the occurrence of carbapenem resistance among multidrug-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolated from clinical specimens in Accra, Ghana. One of its specific objectives was to determine the prevalence of carbapenem resistance among multidrug-resistant *E. coli* and *K. pneumoniae* isolates, and the prevalence was found to be 5.6%. This prevalence seems higher than that reported in the recent study conducted in Ghana by Codjoe *et al*. (2019) among Gram-negative bacilli – 2.9% – the majority of which were *Pseudomonas aeruginosa* and *Acinetobacter baumanii*. Studies conducted by Hackman *et al*. (2017) and Owusu-Oduro (2016) reported somewhat higher prevalence – 7.2% and 10% respectively – but Hackman *et al*. (2017) limited their study to CRE among phenotypic ESBL producers, and Owusu-Oduro's (2016) study was on Enterobacteriaceae involved in UTI infection. The disparities between the CRE prevalence observed in the current study compared to the three other studies cited may also be accounted for by the fact that the current study concentrated on multidrug-resistant *E. coli* and *K. pneumoniae*, and these were isolated from 15 different types of samples. Regardless of the differences in prevalence, that all of these studies are recent suggests that carbapenem resistance is gradually emerging in Ghana. It is noted that the prevalence recorded in the current study is consistent with those reported in some other African countries, such as Senegal (5.1%) (Camara *et al*., 2017), Nigeria (6.5%) (Olowo-okere *et al*., 2020), and South Africa (2.6–8.9%) (Ballot *et al*., 2019). Meanwhile, some other studies in Africa have reported a higher prevalence of CRE, such as 18.4% in Uganda (Okoche *et al*., 2015) and 54.1% in Egypt (Zaidah *et al*., 2017). Globally too, there have been varying reports on the prevalence of CRE – 9.1% in

Nepal (Gurung *et al*., 2020), 5.74% in Malaysia (Zaidah *et al*., 2017), and 4.9% in Argentina (Villar *et al*., 2013). These reports are indicative of a rise in carbapenem resistance globally, and urgent measures need to be put in place to curb this global menace.

Another objective of this study was to determine the antimicrobial resistance patterns of the multidrug-resistant *E. coli* and *K. pneumoniae isolates*. The MDR isolates demonstrated high resistance rates to most of the antimicrobials tested. For instance, *E. coli* demonstrated greater than 60% resistance towards penicillins and third-generation cephalosporins as follows: 96% resistance to ampicillin, 94% to cefuroxime, 85% to tetracycline, 84% resistance to sulfamethoxazole-trimethoprim, 80% to amoxicillin-clavulanate, 76% to ciprofloxacin, 75% to cefpodoxime, 74% to cefotaxime, 73% each of levofloxacin and ceftriaxone, 66% to cefepime and 61% to ceftazidime. The findings of this study are similar to another study conducted in Ghana by Feglo and Adu-Sarkodie (2016) on the antimicrobial resistance pattern of ESBL producing *E. coli* isolate in a tertiary hospital. From their study, *E. coli* showed a high level of resistance to ampicillin (91.7%), cotrimoxazole (89.7%), amoxicillin-clavulanate (67.2%), and cefpodoxime (64.7%) (Feglo and Adu-Sarkodie, 2016). Another study by Obeng-Nkrumah *et al*. (2016) in Ghana also reported on the antibiotic resistance pattern of *E. coli* bloodstream infections. Resistance was reported for ampicillin (97.7%), cotrimoxazole (92.4%), tetracycline (91.3%), amoxicillin-clavulanate (87.5%), cefuroxime (70.2%), ciprofloxacin (62.1%) and cefotaxime (56.2%) (Obeng-Nkrumah *et al*., 2016). *K. pneumoniae* also demonstrated greater than 60% to 100% resistance to ampicillin (100%), sulfamethoxazole-trimethoprim (93.2), cefuroxime (90.9%), tetracycline (86.4%), cefotaxime (84.1%), cefpodoxime (81.8%), ceftriaxone (75%), ceftazidime (72.7%), cefepime (63.6%), and amoxicillin-clavulanate (63.3%). A similar observation was reported among *K. pneumoniae* isolates from a recent study in Ghana (Quansah *et al*., 2019). Quansah and his team also reported 100% resistance to ampicillin, ceftriaxone (91.2%), cefuroxime (98..9%), cotrimoxazole (91.2%), tetracycline (84.6%), cefotaxime (76.9%) and ceftazidime (71.4%). Another study on *K. pneumoniae* from different types of hospital-acquired infections by Ranjbar *et al*. (2019) in Iran also observed a high level of resistance against ampicillin (100%), cefuroxime (100%), ceftazidime (96.52%), cotrimoxazole (77.39%), amoxicillin-clavulanate (95.65%) and tetracycline (53.91%) (Ranjbar *et al*., 2019). This high level of ampicillin resistance observed in *K. pneumoniae* might be attributed to the intrinsic resistance of *K. pneumoniae* to ampicillin because of the presence of SHV-1 beta-lactamase present on their chromosomes or transferable plasmids (Saravanan *et al*., 2018). Moreover, the overall resistance was 97.2% to 64.6% for ampicillin (97.2%), cefuroxime (93.1%), sulfamethoxazole-trimethoprim (86.8%), tetracycline (85.4%), cefotaxime (77.1%), cefpodoxime (77.1%), amoxicillin-clavulanate (75%), ceftriaxone (73.6%), ciprofloxacin (70.8%), levofloxacin (66.0%), Cefepime (65.3%) and ceftazidime (64.6%) in this study. Other studies in the country have reported a similar antibiotic resistance rate for both *E. coli* and *K. pneumoniae*. To illustrate, Obeng-Nkrumah *et al*. (2013) also reported greater than 80% resistance to commonly used antibiotics such as ampicillin, tetracycline, cotrimoxazole, cefuroxime, cefotaxime and ceftazidime among ESBL-producing Enterobacteriaceae which were predominantly *E. coli* and *K. pneumoniae.* Another study by Opintan and Newman (2017) also reported on a high level of resistance among *E. coli* against ampicillin (100%), sulfamethoxazole-trimethoprim (100%), tetracycline (100%), cefotaxime (88.9%), cefuroxime (87.5%) and ciprofloxacin (60%) from blood cultures. Agyepong *et al.*, (2019) reported resistance to ampicillin (94.4%), sulfamethoxazole-trimethoprim (84.5%), cefuroxime (79.0%) and cefotaxime (71.3%) among their study isolates which were predominantly *E. coli* and *K. pneumoniae*.

Interestingly, high resistance to commonly-used antibiotics such as penicillins and cephalosporins were recorded in this study; these drugs are part of the approved list of drugs for the treatment of bacterial infections in Ghana. It is, nonetheless, understandable that such rates were observed, as all the isolates investigated in the current study are known MDRs. Yet the cause for concern cannot be ruled out, as these high rates were recorded against several of the antimicrobials. This observation adds to the several lines of evidence that support the need to strengthen antimicrobial stewardship programmes in Ghana and elsewhere (Oliviera *et al*., 2015; Codjoe and Donkor, 2018; Donkor and Codjoe, 2019). Considering the resistance rates recorded against the carbapenems – ertapenem, meropenem, and imipenem – which in conjunction with the reports of Owusu-Oduro (2016), Hackman *et al*. (2017), Agyepong *et al*. (2018), Codjoe *et al*. (2019) and Quansah *et al*. (2019) cited above, demonstrate the emergence of carbapenem resistance in the country, it is surprising that carbapenems, which are antibiotics of last resort for treating serious bacterial infections, are not included in the Ministry of Health's Essential medicine list in Ghana (Ministry of Health, 2017). This study provides further evidence that warrants the inclusion of carbapenems in the essential medicine list in the country.

In this study, all the eight carbapenem-resistant isolates showed 100% resistance to both meropenem and ertapenem, with 50% resistance recorded in imipenem. This high resistance to meropenem was also reported by Codjoe (2016) in five different carbapenem-resistant isolates, including *K. pneumoniae*. Additionally, it has been reported that the disc diffusion test by ertapenem is a sensitive indicator of carbapenemase production (Hara *et al*., 2013), just as observed in this study. Understanding CPE antibiotic susceptibilities are critical for making therapy decisions (Park *et al*., 2019), and from the E-test results of the current study, ertapenem MIC seemed to be the best predictor of carbapenemase producers. Findings from this study agree with a study conducted by Nordmann & Poirel (2013), who also reported that because ertapenem's MIC values are typically greater than those of other carbapenems, it appears to be a promising choice for detecting most carbapenemase producers. However, Hara *et al*. (2013) also reported that CPE does not always show a minimum inhibitory concentration (MIC) value for carbapenems in the resistance range as seen in this study, making identification usually a problem. Hence detection of carbapenemase based on MIC reports alone may not be reliable, and other laboratory tests, such as molecular techniques, should be adopted for the precise identification of carbapenemase production. Additionally in this study, the prevalence of carbapenem resistance according to the phenotypic confirmatory tests was found to be 75% (6/8) by both the Modified Hodge Test and the modified Carbapenem Inactivation Method. The study by Codjoe *et al*. (2019) in Ghana showed a much lower prevalence of 18.9% by MHT. This low prevalence as compared to the current study may be due to most of their isolates being *Acinetobacter* spp., and *Pseudomonas* spp. as compared to the current study involving only *E. coli* and *K. pneumoniae*. A study conducted outside Africa also recorded a high prevalence of CRE by MHT; 43.3% *K. pneumoniae* and 27.7% *E. coli* from Iran (Gurung *et al*., 2020). Furthermore, out of the three isolates that tested positive for MHT in the *E. coli* isolates with 2/3 turned out to be false positives according to the PCR technique in this study. This occurrence might be due to other mechanisms of resistance that might mimic carbapenemase activities, such as porins loss co-expressing with ESBL and AmpC (Nordmann *et al*., 2011; Codjoe *et al*., 2019). Also, these false positives could be a result of screening for only commonly encountered carbapenemases in this study, and not the less-frequently encountered ones. In contrast to findings from this study, another study by Quansah *et al*. (2019), also in Ghana, showed that none of their carbapenem-resistant *Klebsiella* spp. tested positive for MHT making MHT a less sensitive test for the detection of carbapenemase activity meanwhile, from this study, MHT still proves to be a valuable test for detecting carbapenemase activity. There are currently no published data in Ghana concerning the use of mCIM for the detection of carbapenemase activity. This might be because mCIM is a new method recently published in the CLSI (2021) guidelines and the detection of carbapenemase by both phenotypic and genotypic methods are not incorporated into the routine microbiological practice in our various hospital laboratories. Comparing the 75% prevalence of CRE by mCIM to other countries in the African sub-region, a study conducted in Ethiopia revealed a lower prevalence of 9% (Alemayehu *et al*., 2021) and a high prevalence of 80.8% in Egypt (Kotb and Mowafy, 2019) in concordance with this study. The high prevalence recorded by Kotb and Mowafy (2019) was attributed to samples collected at different departments of tertiary hospitals such as ICU and surgical departments. These tertiary hospitals serve as referral centres for life-threatening cases, and hence the high prevalence of CRE.

Another objective of this study was to evaluate the occurrence and distribution of selected carbapenemase genes – $bla_{OXA\text{-}48}$, bla_{NDM}, bla_{KPC}, bla_{VIM}, and, bla_{IMP} – among the carbapenem-resistant *E. coli* and *K. pneumoniae* isolates. Among the eight carbapenem-resistant isolates, 50.0% harboured $bla_{OXA\text{-}48}$ and 12.5% bla_{NDM} genes. In contrast, Quansah *et al*. (2019), whose study was conducted in Ghana reported 2.16% and 0.72% for $bla_{OXA\text{-}48}$ and bla_{NDM} respectively. Meanwhile, just as reported from this study, the predominant carbapenemase genes were $bla_{OXA\text{-}48}$ in *K. pneumoniae*. In addition, Codjoe *et al*. (2019) also reported that 14.5% of their carbapenemase genes were bla_{NDM} which was similar to that of this study (12.5%). The only bla_{NDM} detected from the study was identified in *K. pneumoniae* which is in agreement with a study by Hara *et al*. (2013), who reported that the majority of Metallo beta-lactamase (MBL) producers are hospital-acquired and multidrug-resistant *K. pneumoniae*. Additionally, Codjoe *et al*. (2019), also reported on $bla_{OXA\text{-}}$

48, *bla*$_{NDM}$ and *bla*$_{VIM}$ in Ghana from their study where they characterized carbapenem-resistant Gram-negative bacteria. Though Codjoe and his team reported *bla*$_{NDM}$ as the predominant carbapenemase in their study, all the *bla*$_{NDM}$ genes were mainly from *Acinetobacter species* (9 *bla*$_{NDM\text{-}1}$) and then *Pseudomonas aeruginosa* (2- *bla*$_{NDM}$). The *E. coli* and *K. pneumoniae* from their study had 3 *bla*$_{NDM}$ and 2 *bla*$_{OXA\text{-}48}$ respectively. Unlike Codjoe *et al*. (2019), the *bla*$_{NDM}$ detected in this study was from *K. pneumoniae*. This shift might be due to ongoing interspecies transmission of resistant genes from *E. coli* to *K. pneumoniae* through transmissible plasmids that carry the *bla*$_{NDM}$ genes (Tzouvelekis *et al*., 2012). Also, findings from Codjoe *et al*. (2019) revealed that 23/26 (88.5%) of their carbapenemase producers were isolated from the KBTH but these isolates harboured only *bla*$_{NDM}$ and *bla*$_{VIM}$ genes. None of the isolates collected from KBTH harboured *bla*$_{OXA\text{-}48}$ genes. These findings are contrary to that of this study since all the *bla*$_{OXA\text{-}48}$ carbapenemase genes were from isolates collected from the KBTH. The discrepancies in the results could be attributed to the small numbers of *K. pneumoniae* isolates collected by Codjoe *et al*. (2019) as compared to only *E. coli* and *K. pneumoniae* isolates involved in this study. Hence this study, together with other studies in Ghana, seem to emphasize that the circulating carbapenemases among *E. coli* and *K. pneumoniae* in Ghana are predominantly *bla*$_{OXA\text{-}48}$ and *bla*$_{NDM}$. A study conducted in South Africa on CRE in patients with bacteraemia from a teaching hospital also reported that the majority (78%) of their isolates were *K. pneumoniae* with most of the Enterobacteriaceae harbouring *bla*$_{OXA\text{-}48}$ (52%) inconsistent with this study and *bla*$_{NDM}$ (34%) genes. Furthermore, a systematic review conducted in Africa further reiterated that frequently observed carbapenemases in the continent among *E. coli* and *K. pneumoniae* carbapenem-resistant isolates were *bla*$_{OXA\text{-}48}$, *bla*$_{NDM}$ and *bla*$_{VIM}$ (Mitgang *et al*., 2018). Unlike reports from Ghana and Africa, a study conducted on carbapenemase-producing *E. coli* and *K. pneumoniae* in Iran reported *bla*$_{VIM}$ (33.3%) as the predominant carbapenemase gene followed by *bla*$_{OXA-48}$ (14.4%) (Kazemian *et al*., 2019). However, Han *et al*. (2020) have reported predominantly *bla*$_{KPC-2}$ (51.6%), *bla*$_{NDM}$ (35.7%) and *bla*$_{OXA-48}$-like (7.3%) carbapenemases among CRE in China. Also, most of the carbapenemase genes from this study were isolated from *K. pneumoniae*. In agreement with this finding, Okoche *et al*. (2015), in their study involving the characterization of CRE from a national referral hospital in Uganda, also identified a similar pattern. This high level of carbapenemase genes reported in *K. pneumonia*e could be partly due to their ability to easily acquire multiple resistant genes (Sharma *et al*., 2007; Gharrah *et al*., 2017)**.**

In the current study, most of the carbapenemase gene-harbouring isolates were detected in urine samples of both the elderly and infants. This observation might be a result of the lack of personal hygiene and incomplete emptying of the bladder mostly observed in infants and hormonal changes in older (Foxman, 2014; Boye *et al*., 2016). Aside from the urine samples, carbapenemase genes were also predominant in blood specimens in this study. This observation is in agreement with a study conducted in Egypt, in which most of their carbapenem-resistant Enterobacteriaceae were from blood specimens (Kotb *et al*., 2020). Gram-negative bacilli tend to colonize the upper respiratory tract and skin of seriously ill and hospitalized patients, making these resistant bacterial isolates a common cause of bacteremia associated with central venous lines (Pena *et al*., 1998; Paterson and Yu, 1999). *K. pneumoniae* causes infections of all ages with serious life-threatening ones found in children, the elderly and immunocompromised individuals (Highsmith and Jarvis, 2016). In concordance with findings from Highsmith and Jarvis (2016), the majority of individuals whose samples harboured carbapenemase genes were children under 10 years and the elderly above 60 years. These findings in the elderly can be attributed to weakened immune function especially a decline in cell-mediated functions and a variety of chronic diseases associated with ageing which predisposes them to infections (Gardner, 1980; Yoshikawa, 2000). Also, the findings of this study as seen in the infants can be due to their underdeveloped immune system especially low levels of immunoglobulin M (IgM), which predisposes the infants to Gram-negative bacterial infections (Gardner, 1980).

Lastly, out of the eight bacterial isolates that were carbapenem-resistant, 37.5% were not identified by any of the primers that were used in the screening. This observation might be due to the limited number of carbapenemase genes that were targeted and the possibility of other resistance mechanisms, such as mutations or porin loss (Mushi *et al*., 2014; Ruppé *et al*., 2015). Although Codjoe *et al*. (2019) also reported that there were no corresponding results between their phenotypic-based methods and molecular assays, they stated that their two *K. pneumoniae* isolates that were positive for MHT were detected by PCR to harbour bla_{OXA-48}. In agreement with Codjoe *et al*.'s (2019) findings, three of the *K. pneumoniae* isolates that were MHT positive in this study also harboured bla_{OXA-48}, and these support the findings that MHT is a good detector of bla_{OXA-48} genes (Okoche *et al*., 2015). In contrast with reports from Iovleva and Doi (2017) and CLSI (2021), mCIM was unable to detect the presence of the only bla_{NDM} gene in this study. In this study, neither MHT nor mCIM were able to detect the presence of bla_{NDM} (Metallo-beta lactamase). Other studies (Birgy *et al*., 2012; Okoche *et al*., 2015) have also reported that MHT was unable to identify bla_{NDM} carriers. A study reported that MHT produces false-negative results mainly with Enterobacteriaceae with Metallo-β-Lactamases (MBL) genes and hence is unreliable in detecting *K. pneumoniae* bla_{NDM}-harbouring carbapenemases (Tzouvelekis *et al*., 2012). In contrast to reports by CLSI (2021), in which > 99% sensitivity and specificity are reported for the detection of bla_{NDM} among other commonly known resistance genes in Enterobacteriaceae by mCIM, this method however failed to detect the presence of *bla*$_{NDM}$ in this study. Therefore from this study, neither MHT nor mCIM supersedes the other in the detection of carbapenemase genes.

CHAPTER SIX

CONCLUSION, LIMITATIONS AND RECOMMENDATION

6.1 Conclusions

The following are conclusions drawn from the study:

The prevalence of carbapenem resistance/carbapenemase-encoding genes was relatively low (5.6%) among the study isolates, but suggest an emergence of carbapenem resistance in Ghana.

Also, carriage of both *bla*$_{OXA\text{-}48}$ and *bla*$_{NDM}$ genes were observed among the study isolates, with *bla*$_{OXA\text{-}48}$ predominantly found in *K. pneumoniae* isolates.

Moreover, the study isolates demonstrated high resistance (more than 70%) towards most of the antimicrobials tested, particularly ampicillin, cefuroxime, sulfamethoxazole-trimethoprim, tetracycline, cefotaxime, cefpodoxime, amoxicillin-clavulanate, ceftriaxone, and ciprofloxacin.

Furthermore, both MHT and mCIM are excellent detectors of *bla*$_{OXA\text{-}48}$ producing genes but not *bla*$_{NDM}$ genes. Children and the elderly have a relatively higher risk of CRE infections with limited therapeutic options available.

6.2 Recommendation

The following are recommended based on the findings from this research:

As this study confirms the emergence of carbapenem resistance in Ghana, there is a need for frequent surveillance studies to monitor these resistance genes and proper infection, prevention and control programmes in the healthcare system of the country.

The high resistance rates observed among the study isolates, particularly against ampicillin, cefuroxime, sulfamethoxazole-trimethoprim, tetracycline, cefotaxime, cefpodoxime, amoxicillin-clavulanate, ceftriaxone, and ciprofloxacin, warrant a review of the approved list of drugs for the treatment of bacterial infections in Ghana.

Also, additional studies are needed in this study area with a specific focus on uncommonly encountered carbapenemase genes and other mechanisms of antibiotic resistance other than the production of carbapenemase enzymes.

Finally, genomic sequencing is recommended where genetic relatedness of the carbapenemase genes can be compared to those of other geographical locations, to determine their lineage and understand their evolution.

6.3 Limitations

The study is limited by the fact that it targeted the presence of commonly known carbapenemase genes (*bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{IMP}$, and *bla*$_{OXA-48}$), and hence the presence of relatively less frequently encountered carbapenemase genes would have been missed.

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APPENDIX I: CLINICAL DATA COLLECTION FORM

PRINCIPAL INVESTIGATOR: FELICIA POKUAAH DWOMOH

PHENOTYPIC AND GENOTYPIC DETECTION OF CARBAPENEMASE-PRODUCING *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* AMONG MULTIDRUG-RESISTANT ENTEROBACTERIACEAE IN GHANA

PROTOCOL ID NUMBER: ***CHS-Et/M.7 – 5.2/2020-2021***

STUDY ID:
DATE:
DATA ABSTRACTOR:

DEMOGRAPHICS

MEDICAL RECORD NUMBER:

INITIALS OF SUBJECT:

DATE OF BIRTH:

AGE:

GENDER: (CHECK ONE)

☐ MALE ☐ FEMALE

RACE:

CLINICAL DATA

CLINICAL DIAGNOSIS:

INFECTION SITE:

MICROBIOLOGY LABORATORY DATA

LABORATORY ID:

SAMPLE TYPE:

ORGANISM ISOLATED:

ANTIMICROBIAL SUSCEPTIBILITY PATTERN:

PRINCIPAL INVESTIGATOR'S SIGNATURE______________________________

DATE: [___/___/___]