UNIVERSITY OF CAPE COAST

DETECTION OF EXTENDED SPECTRUM BETA LACTAMASE PRODUCING ENTEROBACTERIACEAE FROM TUBERCULOSIS PATIENTS

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ELIZABETH AYOOLA

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Thesis submitted to the Department of Microbiology and Immunology, School of Medical Sciences, College of Health and Allied Sciences, University of Cape Coast, in partial fulfilment of the requirements for the award of Master of Philosophy degree in Infection and Immunity

SEPTEMBER, 2021

DECLARATION

Candidate's Declaration

I hereby declare that this thesis is the result of my own original research and that no part of it has been presented for another degree in this university or elsewhere.

Candidate's Signature …..... Date ……..

Elizabeth Ayoola

Supervisors' Declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on supervision of thesis laid down by the University of Cape Coast

Supervisor's Signature ……... Date ……

Name: Dr Akwasi Anyanful

Co-Supervisor's Signature …… Date ……

Name: Dr. Anthony Ablordey

ABSTRACT

Antimicrobial drug resistance is a global issue that affects health, economic, and social development. This study therefore set out to determine the prevalence of extended-spectrum beta-lactamases (ESBLs) in bacteria isolated from tuberculosis patients in three selected hospitals in Ghana. A total of 53 isolates consisting of 36 *Escherichia coli*, 6 *Klebsiella pneumoniae*, 3 *Enterobacter cloacae*, *3 Morganella morganii*, 1 *Citrobacter freundi*, 1 *Enterobacter asburiae*, 1 *Klebsiella varicola* ,1 *Proteus mirabilis* and 1 *Serraatia marcescens* were obtained from rectal samples and tested for their susceptibility to seventeen (17) commonly (due to easy accessibility and affordability) used antibiotics at the selected hospitals, ESBL production was determined by double disc synergy test and the ESBL genotypes were determined by PCR. A greater proportion 83.4% of isolates were resistant to the beta lactam antibiotics ranging from 5.6% for meropenem and ertapenem to 83.4% for amoxicillin/clavulanate when tested against *E. coli*. All the other isolates showed 100% resistance to amoxicillin/clavulanate. Also, five (5) isolates showed 100% resistance to trimethoprim-sulphamethoxazole. All the isolates were susceptible to imipenem and dorepenem. All of *K. pneumonia, E. cloacae* and *M. morganii* were resistant to amoxicillin/clavulanate. ESBL phenotypes were detected in *E. coli* and *E. cloacae*. *Bla*TEM genotype was present in *E. coli* and *K. pneumoniae. Bla*SHV and *Bla*OXA-1 genotypes were also detected in *K. pneumoniae* and *E. coli* respectively. The overall ESBL prevalence was 15.1%. From the results, imipenem and doripenem will be the recommended drugs of choice. However, the use of amoxicillin/clavulanate and trimethoprim-sulphamethoxazole should be regulated .

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DEDICATION

I dedicate this study to God Almighty who is the giver of life and to my lovely husband.

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CHAPTER ONE: INTRODUCTION

Introduction

Antimicrobial resistance (AMR) is a threat to the effective treatment of infection caused by microorganisms**.** Health facilities treating mycobacterial diseases such as tuberculosis (TB), buruli ulcer or leprosy may be high-risk settings for the development and spread of antimicrobial resistance. This may be due to exposing heavy antibiotics use to a high density patient population and the possible occurrence of cross infections (Struelens, 1998). The study aimed to determine the antimicrobial resistant effect that may result from the long-term antimicrobial exposure in patients treated for TB by investigating the prevalence and genetic diversity of antimicrobial resistant Gram-negative microorganisms, including Extended Spectrum β-lactamases (ESBL). Immunocompromised patients such as TB patients are known to be at higher risk of AMR due to their frequent exposure to broad-spectrum cephalosporins and monobactams agents (Fridkin, 2001)

Background

Antibiotics are one of the most successful drugs in human history (Aminov, 2010). Antibiotics are agents that are used to combat microbes. They are either cytostatic or cytotoxic to the micro-organisms, allowing the body's immune system, to eliminate them (Zaman *et al.*, 2017). Antibiotics especially the beta-lactams often act by inhibiting the synthesis of a bacterium cell wall by specifically inhibiting the synthesis of peptidoglycan layer of the cell wall. Most of these antibiotics may use porins to cross the cell walls of bacteria and inhibit the synthesis of proteins and peptidoglycan (James *et al.*, 2009). Some antibiotics such as the tetracyclines block bacterial protein synthesis by

interfering with the processes at the 30S subunit or 50S subunit of the 70S bacterial ribosome. They primarily attack (i) the formation of the 30S initiation complex which is made up of mRNA, 30S ribosomal subunit, and formylmethionyl-transfer RNA), (ii) the formation of the 70S ribosome by the 30S initiation complex and the 50S ribosome, and (iii) the elongation process of assembling amino acids into a polypeptide. Other broad-spectrum antibiotics such as the rifamycins (eg. rifampicin) act by inhibiting the transcription of deoxyribonucleic acid (DNA) to ribonucleic acid (RNA) and the quinolones and fluoroquinolones such as ciprofloxacin inhibit DNA replication.

Antimicrobial resistance surveillance reported by WHO showed high resistance rates in bacteria pathogens. Five out of six WHO regions reported more than 50% resistance to third generation cephalosporins and fluoroquinolones in *Escherichia coli* and two out of six regions reported over 50% resistance to third generation cephalosporins and carbapenems respectively in *Klebsiella pneumoniae* (Essack *et al*., 2016). Antibiotic-resistant infections are a substantial health and economic burden to the health care system, as well as to patients and their families. They may occur in hospitals, due to the clustering of highly vulnerable patients and high rates of antibiotic use in this setting. Antibiotic-resistant infections add considerable costs to a nation's already overburdened health care system. When first and second-line antibiotic treatment options are limited or unavailable, health care professionals may be forced to use antibiotics that may pose adverse effects to the patient. Even when effective treatments exist, data show that in most cases patients with infections that are difficult to treat require significantly extended hospital stays and recuperations and experience a higher incidence of long-term disability

(Shaikh *et al.*, 2015 Despite the discovery and development of novel β-lactam antibiotics (Elander and biotechnology, 2003; Poole and infection, 2004), the abuse of these drugs has also resulted in the resistance of β-lactams by Gram-negative bacteria (Obeng-Nkrumah *et al.*, 2013; Shaikh *et al.*, 2015).

The extended periods of antibiotic use, overuse and misuse have rendered cephalosporins, fluoroquinolones, aminoglycosides, broad-spectrum penicillins ineffective (Kardos, 2017). Infections caused by bacterial species are relatively challenging to treat or control. This may be due to the expression of the intrinsic, acquired or adaptive resistant mechanisms that can occur through sporadic mutations or through the acquisition of foreign genetic material to resist different classes of antibiotics (Moore and Flaws, 2011). Gram-negative bacteria are particularly worrisome because they are becoming resistant to nearly all the antibiotic drug options available, creating situations reminiscent of the pre-antibiotic era (Ventola, 2015). The most serious Gram- negative infections occur in health care settings and are most commonly caused by Enterobacteriaceae (mostly *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter*). Multidrug-resistant (MDR) Gram-negative pathogens are also becoming increasingly prevalent in the community (Ventola, 2015).

Gram-negative bacteria confer resistance to antimicrobial agents via mechanisms such as chromosomal mutations leading to overproduction of intrinsic β-lactamases, hyper-expression of efflux pumps, permeability alterations, and target modifications. The dissemination of resistant genes including the spread of ESBL and carbapenemases by mobile genetic elements is becoming a cause for great concern (Bush and Bradford, 2016). Three

mechanisms involved in bacteria resistance to beta-lactam antibiotics include the ability to produce β-lactamase enzymes, utilization of β-lactam insensitive cell wall transpeptidase and the active expulsion of β-lactam molecules by efflux pumps (Isendahl *et al.*, 2012). Nevertheless, the variety of β-lactamase enzymes with their increasing substrate diversity and wide dissemination constitute the most important antimicrobial resistant mechanism in Gramnegative bacteria (Kumar, 2013). Generally, three broad groups of betalactamases are differentiated as class C cephalosporinases (AmpC), ESBLs and β-lactamases with carbapenemase activity (Girlich *et al.*, 2014). The ESBLs and carbapenemases with their increasing potential for associations with plasmidborne mobile genetic elements, constitute the most versatile cause of antimicrobial resistance among the Gram-negative bacteria (Nordmann *et al.*, 2012; Satlin *et al.*, 2014).

Immunocompromised patients have a reduced ability to fight infections and other diseases due to impaired or weakened immune system (Okafor, 2012). This may be caused by certain diseases or conditions such as anticancer drugs, radiation therapy and organ transplant. The inability to fight infection can also be as a result of illness, malnutrition and drugs (Fridkin, 2001). Immunocompromised patients may be vulnerable to opportunistic infections, in addition to normal infections that could affect everyone (O'Garra *et al.*, 2013). Patients with immune suppression particularly those receiving high-dose chemotherapy harbor many pathogens in their intestinal tract (Paczosa and Mecsas, 2016). The greater effect with the use of broad-spectrum antibiotics is the ability to disrupt a larger variety of normal human flora. Frequent exposure to antibiotics can change the body's normal microbial content by attacking

indiscriminately both the pathological and naturally occurring beneficial or harmless bacteria found in the intestines, lungs and bladder. The destruction of the body's normal bacterial flora disrupt immunity thereby leading to a relative overgrowth of microrganisms.

Immunocompromised patients such as TB patients are known to be at higher risk of AMR due to their frequent exposure to broad-spectrum cephalosporins and monobactams agents (Fridkin, 2001). Treatment of mycobacterial diseases such as tuberculosis (TB) entails long and intense antimicrobial therapy. TB patients are at risk of coinfection with other multidrug-resistant bacteria, such as those from Enterobacteriaceae family, because of antimicrobial selection pressure and nosocomial transmission during prolonged hospital admission (Gröschel *et al.*, 2016). These patients are usually placed on broad-spectrum antibiotics to treat microbial infections (McCarthy *et al.*, 2018). However, the recommended empirical regimens such as β-lactam are not active against ESBL and Carbapenemases Producing Enterobacteriaceae (CPE) thereby leading to increase in morbidity and mortality (Blaschke *et al.*, 2012). The emergence of ESBL and CPE in immunocompromised individuals have grave implications (Satlin *et al.*, 2014).

Generally, ESBL and CPE colonization occur in individuals who have frequent exposure to healthcare settings and in those with frequent antibiotic usage as well as immune suppression (Nakai *et al.*, 2016; Taglietti *et al.*, 2013; Tischendorf *et al.*, 2016). Other risk factors include age, place of hospitalization, underlying disease, invasive procedures or devices, previous hospitalization, intensity of care, proximity to an ESBL/CPE colonized patient

(Falagas *et al.*, 2007; Fouda *et al.*, 2016; Nakai *et al.*, 2016). Infections by ESBL and CPE are a challenge for physicians to treat because of the limited choice of therapeutic options available and due to the possibility of concomitant drug resistance of the isolate to other antimicrobials (Taglietti *et al.*, 2013).

Tuberculosis (TB) remains a major global health challenge with 10 million incident cases and 1.8 million fatalities in 2017 (Zaman *et al.*, 2017). *Mycobacterium tuberculosis* complex causes TB, it affects the lungs (pulmonary tuberculosis) and sometimes progresses to other parts of the body (extra-pulmonary tuberculosis). TB patients whose immune system are compromised are prone to secondary bacterial infections. Some of the bacterial capable of causing secondary infections were found to be resistant to many antibiotics such as cefazolin, ceftazidine, imipenem, and carbenicillin by producing enzymes such as ESBL which leads to little or no treatment options and may consequently lead to death (Gröschel *et al.*, 2016). Treatments available for tuberculosis include isoniazid, rifampicin and pyrazinamide during the first two months and isoniazid and rifampicin to complete six months of treatment (Coll, 2003). It usually entails long and intense antimicrobial therapy. Drug resistance of *M. tuberculosis* has been attributed to the structure of its mycolic acid containing cell wall that gives the bacteria a low permeability for antibiotics and other chemotherapeutic agents (Almeida Da Silva and Palomino, 2011; Jarlier and Nikaido, 1994). The role of efflux mechanisms has also been recognized as an important factor in the natural resistance of mycobacteria against antibiotics such as tetracycline, fluoroquinolones and aminoglycosides (Rossi *et al.*, 2006). Mycobacteria produce β-lactamases and are intrinsically resistant to β-lactam antibiotics (Flores *et al.*, 2005). In

countries where TB is endemic, nosocomial infections and multidrug resistant (MDR) organisms have the potential to cause fatalities due to the absence of routine screening and lack of effective antimicrobial agents for these high-risk organisms (Gröschel *et al.*, 2016). Antibiotic resistance is increasing worldwide and poses a fundamental, long-term threat to human health (Ferri *et al.*, 2017).Solutions tackling antibiotic resistance include the protection of our intestinal microbiota against antibiotics to prevent the acquisition, selection and overgrowth of MDR bacteria (Ruppé *et al.*, 2018).

Problem Statement

The threat of AMR is emerging worldwide (Ferri *et al.*, 2017). Health facilities treating mycobacterial diseases such as tuberculosis may be high-risk settings for the development and spread of AMR. The high antimicrobial exposure of TB patients both in terms of numbers of drugs as in the duration of therapy leads to compliance problems, high costs and toxicity (Macfarlane, 2014; Mimee *et al.*, 2016; Zhao, 2010).

Data on antimicrobial resistance in Ghana is scanty (Kpeli *et al.*, 2017). Prescription of β-lactam based drugs accounts for over 60% of all antibiotics prescribed for pregnant women and are regularly used for the treatment of most infections in Ghana (Mensah *et al.*, 2017). The abuse and misuse of β-lactam antibiotics including cephalosporins and carbapenems for treatment of serious infection pose significant risk of acquiring resistant strain of bacteria. Ghana is no exception to this development as carbapenems are being used to treat ESBL resistant bacteria when detected (Obeng-nkrumah *et al.*, 2013). The rapid spread of carbapenem resistant Enterobacteriaceae infections progressively narrows

the spectrum of therapeutic options available to patients thereby compromising effective treatment (Potter *et al.*, 2016; Sheu *et al.*, 2019).

There is a huge gap deficit in the quality of antimicrobial surveillance in Ghana compared to other countries largely due to the lack of appropriate logistics and laboratory equipment at the hospitals (Opintan *et al.*, 2015).The inadequate antimicrobial existence surveillance in Ghana could project possible future complications in carrying out important medical procedures such as caesarean sections and treatment of cancer in Ghana if not strengthened and important antibiotics may lose their effectiveness.

Aim

The aim of this study is to determine associations between the long-term antimicrobial exposure in patients treated for TB and the prevalence and genetic diversity of ESBL.

Specific objectives

1. Determine the prevalence of ESBL colonizing TB patients during different stages of antibiotic treatment using the Combined Disc method
2. Screen isolates for ESBL (bla_{CTX-M}, bla_{TEM} and bla_{SHV}) encoding genes by multiplex polymerase chain reaction to detect the specific types of ESBL.
3. Compare the genotypes of the isolates in the three hospitals in Ghana (Tamale hospital, Manya Krobo hospital and Tema hospital) to show the distribution of the various genotypes in the selected hospitals.

Significance of the Study

β-Lactam antibiotics are among the most often used antimicrobial agents and an increasing incidence of resistance to these drugs is a public health concern. In view of limited data, it is imperative to investigate the contribution of β-lactamases to antimicrobial resistance in Gram-negative bacteria among TB patients. The availability of a local epidemiological data will prove indispensable to patients' infection management whilst creating the necessary awareness on the clinical implications of β-lactamase producing organisms in Ghanaian hospitals and communities. The outcome of the study will improve antimicrobial administration and inform public health interventions including routine β-lactamase laboratory detection. It will also support hospital surveillance programs for drug-resistant bacteria.

Definition of terms

AMR: Antimicrobial resistance (AMR) is the ability of a microorganism (like bacteria, viruses, and some parasites) to stop an antimicrobial (such as antibiotics, antivirals and antimalarials) from working against it.

ESBL: Extended spectrum β-Lactamase that mediates resistance to extendedspectrum cephalosporins (ESCs), such as cefotaxime (CTX), ceftriaxone (CEF), ceftazidime (CAZ), and the monobactam aztreonam (ATM). These enzymes differ from their parent enzymes as they can hydrolyze broad-spectrum β-lactam antibiotic enzymes but cannot hydrolyze cephamycin and are been inhibited by clavulanic acid.

Carbapenemase: An enzyme that confers decreased susceptibility to any of the carbapenems (imipenem, meropenem, ertapenem and doripenem) as well as the second and third generation cephalosporins and cephamycins.

Organization of the study

This study is organized into five chapters. Chapter one provides a background to the whole study. Chapter two consists of the literature review and contains information on the microorganisms under study, resistances mechanisms, types of β-lactamases and laboratory detection of ESBL and Carbapenemases. Chapter three presents the research methodology of study design, study sites, bacterial isolates collection, identification of clinical isolates, phenotypic and genotypic detection of ESBL and Carbapenemase producing isolates as well as information on the data analysis. Chapter four consists of the results obtained and discussion of the key findings. Finally, chapter five consists of the summary, conclusion and recommendations for the study.

Summary

The administration of antimicrobials in the treatment and prevention of infectious disease has provoked an evolutionary response among microbes by producing resistance to the applied antibiotic. A graphic example of this is the widespread evolution of antibiotic resistance since the general introduction of penicillin during World War 2. While initially effective against a wide range of bacterial diseases, today, there are increasing numbers of pathogens that are not only resistant to penicillin and its derivatives but also to all other available antibiotics. This study aimed to determine the antimicrobial resistant effect that may result from the long-term antimicrobial exposure in patients treated for TB.

CHAPTER TWO: LITERATURE REVIEW

Introduction

In this chapter, the study reviewed the families of microorganisms being understudied, antimicrobial resistance and its causes, determination of antimicrobial activity, classification of antibiotics, thus, β-lactams and their subclasses as well as the classification of β-lactamases and their sub-classes. This chapter further evaluates the various phenotypic and genotypic methods used in the detection of ESBL, AmpC and carbapenemase genes.

Databases that were used in the conduction of the literature review includes PLOS, PubMed, Medline, Google Scholar, Web of Science and WHO.

Mesh headings such as "Detection of ESBL and Carbapenemase genes", "Prevalence of ESBL and Carbapenemase genes in Gram Negative Bacteria", "Molecular characterization of ESBL, AmpC and Carbapenemase genes in *Enterobacteriaceae* and *Pseudomonas aeruginosa*." among other relevant phrases were searched for in the databases.

The Family Enterobacteriaceae

The family Enterobacteriaceae are classified under the domain Bacteria, phylum Proteobacteria, class *Gammaproteobacteria* and order *Enterobacteriales* (Donnenberg, 2015). Members of the *Enterobacteriaceae* also called enteric microorganisms are a very large group of Gram- negative bacteria (GNR) found in the environment, water, soil and plants and they are causes of nosocomial infections (Fam *et al.*, 2015). Mostly they form part of the normal orientation of human intestines however some of them are human intestinal pathogens. *Escherichia, Shigella, Salmonella, Enterobacter,*

Klebsiella, *Serratia*, *Proteus*, among others all belong to the same family (Brooks *et al.*, 2012; Fam *et al.*, 2015). *Enterobacteriaceae* are rod-shaped facultative anaerobes that ferment glucose and other sugars are non-spore forming, can reduce nitrate to nitrite, and with the exception of *Plesiomonas spp* can produce the enzyme catalase. Most are motile by virtue oferitrichous (as opposed to polar) flagella (Donnenberg, 2015).

Enterobacteriaceae are associated with intestinal and extraintestinal infections such as bacteraemia, pneumonia, urinary tract infections, abdominal or pelvic infection, surgical site infections, meningitis and various abscesses including wound infections (Jenkins *et al.*, 2017). Among the Enterobacteriaceae, *Escherichia coli* and *Klebsiella pneumoniae* are the most frequently isolated in clinical samples and they cause urinary tract infections, septicaemia, pneumonia and gastrointestinal infections (Stanley *et al.*, 2018).

The Family Pseudomonadaceae

The family *Pseudomonadaceae* are a very large and important group of Gram-negative bacteria. Members of the group are widely distributed in substantial numbers as free-living saprophytes in soils, fresh water, marine environments, and many other natural materials, or in association with plants or animals as agents of diseases (Palleroni, 1981). Strains of *P. aeruginosa* have been found to display the characteristics of potential pathogens (Bucher, 1963). *Pseudomonas aeruginosa* is a non-fermenting, motile, Gram-negative bacilli belonging to the *Pseudomonadaceae* family (Novik *et al.*, 2015). It has a pearlescent appearance and grape-like or tortilla-like odour and a blue-green pigmentation. *P. aeruginosa* grows well at 25 ℃ to 37 ℃, and its ability to grow

at 42 ℃helps to distinguish it from many other *Pseudomonas* species. *P. aeruginosa* is a ubiquitous microorganism which has the ability to survive under a variety of environmental conditions (Wu *et al.*, 2015). It can also form hardy biofilms, both within the body and on the surfaces of medical instruments (Khatoon *et al.*, 2018).

P. aeruginosa is considered as an opportunistic microorganism that can cause disease infrequently in normal hosts but is a major cause of infection in patients with underlying or immunocompromising conditions and patients suffering from severe burns and cystic fibrosis (CF) (Planet, 2018; Wu *et al.*, 2015).

β-Lactamases and Their Classification

Members of the family *Enterobacteriacea*e and *Pseudomonadaceae* produce the β-lactamases which are capable of hydrolyzing the β-lactam rings of antibiotics such as penicillins, cephalosporins and carbapenemases. β-lactamases are members of an enzyme family (EC 3.5.2.6) that hydrolyze *β*-lactam antibiotics such as penicillins, monobactams, and carbapenems by attacking their *β*-lactam rings (Figure 1) (Öztürk *et al.*, 2015).

Often times, resistance against β-lactam antibiotics in Gram-negative bacteria occurs due to the expression of β-lactamases (Rice, 2012). Penicillinase was the first β-lactamase to be isolated and identified by Abraham and Chain in 1940 from *E. coli* even before penicillin entered clinical use (Abraham and Chain, 1940).

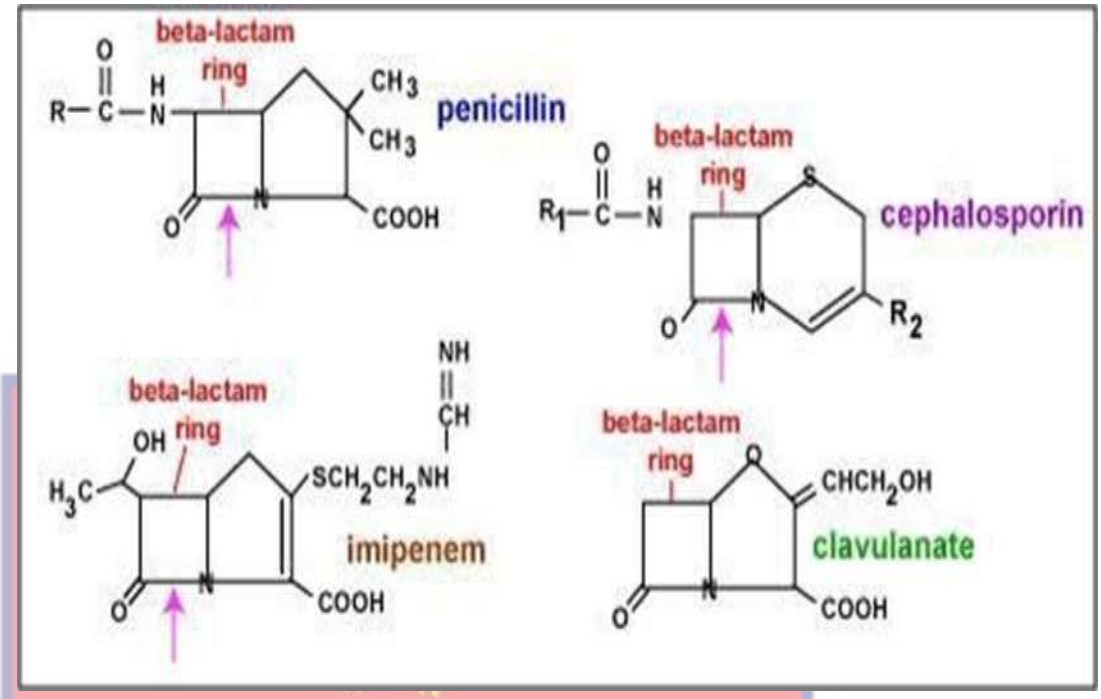

Figure 1: Site of action of β-lactamase enzymes

β-lactamases are classified by two different schemes; According to structural homology (Ambler's classification) (Ambler, 1980) and their functional property (Bush's and Jacoby's classification) (Bush *et al.*, 1995) (Table 1).

Classification of β-Lactamases

Table 1: β-lactamase classification schemes 1. **Ambler classification Scheme**

Class A	Penicillinases	TEMs, SHVs, PCI, CTX-Ms, SME-I, KPC-I
Class B	Metallo-β-lactamases (Zinc)	IMP-I, VIM-I, cer A
Class C	Cephalosporinases	AmpCs, CMY-2, ACT-I
Class D	Oxacillinases	OXA-I

2. Bush-Jacoby-Medeiros classification

Group 1	Cephalosporinases: hydrolyze extended spectrum cephalosporins, clavulanate-resistant.	AmpCs CYM-2, ACT-I MIR-I
Group 2	All are clavulanic acid susceptible	
2a	Penicillinases	PCI from *S. aureus*
2b	Broad spectrum penicillinases	TEM-1, SHV-1 CTX-M-2
2be	ESBLs	SHV-2, TEM-10, CTX-Ms TEMs, IRTs, TEM-30, TEM-31
2br	Inhibitor resistant	
2c	Carbenicillin-hydrolyzing	PSE-1
2d	Oxacillin-hydrolyzing	OXA-1, OXA-10
2e	Cephalosporinases inhibited by clavulanate	FEC-1
2f	Carbapenems	KPC-1, SME-1
Group 3	Metallo-β-lactamases hydrolyze Imipenem, inhibited by EDTA, Resistant to Clavunate	IMP-I, VIM-I, Cer A
Group 4	Miscellaneous	

(Pfaller and Segreti, 2006)

Based on the Ambler classification, β-lactamases are grouped into four (4) molecular classes, A, B, C and D due to conserved and difference in the amino acid pattern (Ambler, 1980; Ambler *et al.*, 1991). Whereas classes A (penicillinase), C (AmpC/ cephalosporinase) and D (oxacillinase) group of enzymes are serine active enzymes capable of hydrolyzing their substrates, class B group of enzymes are called Metallo-β-lactamases, they differ from the three (3) other groups by the fact that they possess in their active site metallic ions (zinc) (Bush and Jacoby, 2010). This group exhibit a broad-spectrum hydrolysis including all β-lactams except monobactam aztreonam.

According to the Bush-Jacoby-Medioros classification, these enzymes are grouped based on their functional property groups (hydrolytic property). This grouping is used in the medical setting to help clinicians and clinical microbiologists in correlating the properties of enzymes with the observed resistance for a clinical isolate (Bush *et al.*, 1995; Richmond and Sykes, 1973).

Acquired resistance in *P. aeruginosa* and Enterobacteriaceae is observed through the production of ESBLs and carbapenemases. Genes that code for all these three enzymes is often carried on plasmids facilitating rapid spread between bacterial species (Gupta, 2007).

Extended-Spectrum β -Lactamases (ESBLs)

ESBLs are plasmid-mediated enzymes that mediate resistance to extended spectrum cephalosporins (ESCs) including third (e.g ceftriaxone, ceftazidime, ceftiofur), fourth generation cephalosporins (e.g cefepime, cefpirome, cefquinome), penicillins and aztreonam. They are inhibited by βlactamase inhibitors, such as clavulanic acid, sulbactam and tazobactam. These

enzymes are produced from the genetic mutation from natural β-lactamases which are present in *Enterobacteriaceae* family (Kiiru *et al.*, 2012; Paterson and Bonomo, 2005). The enzymes evolved as an important mechanism of resistance in *Enterobacteriaceae* that constitute an increasing problem (Bonnet, 2004; Brolund and Sandegren, 2016; Mukherjee *et al.*, 2013). The first report of plasmid-encoded β-lactamases among *Enterobacteriaceae* capable of hydrolyzing ESC was published in 1983 (Knothe *et al.*, 1983). It has also been identified in *Pseudomonadaceae* (Paterson and Bonomo, 2005)..

β-Lactamase gene types and diversity

SHV-type ESBLs

SHV refers to Sulfhydryl variable. The SHV-type ESBL comprises of variants, majority of which are identified by substitution of serine for glycerine at position 238 and over 40 SHV type ESBLs has been identified. The first emergence of the SHV–ESBL was the SHV-2 type, however it differs from SHVI-1 by replacement of glycine with serine at position 238. Other SHV variants have substitution of lysine for glutamate at position 240 (Kliebe *et al.*, 1985). SHV-1 beta lactamase is encoded on the plasmid or chromosome and offers resistance to penicillins and first generation cephalosporins mostly produced by *K. pneumonae* (Paterson and Bonomo, 2005). The SHV-type ESBLs are frequently detected in a wide range of *Enterobacteriaceae* but have also been identified in the *Acinetobacter species* and *Pseudomonas aeruginosa* (Huang *et al.*, 2004). Studies show that the SHV family of beta-lactamases appear to come from *Klebsiella spp* SHV-1 universally found in *Klebsiella pneunoniae* (Shaikh *et al.*, 2015).

Temoniera (TEM)-type ESBLs

These type of ESBLs are derivatives of TEM-1 and TEM-2. TEM -1 was first reported from the patient named Temoniera in 1965 hence the name TEM (N. Datta and Kontomichalou, 1965). TEM-type enzymes which are more likely to resist the effects of beta-lactamase inhibitors have negligible hydrolytic activity against the extended-spectrum cephalosporins and are not considered ESBLs (Paterson and Bonomo, 2005). This is because TEM1 though can hydrolyze ampicillin at greater extent than oxacillin, carbenicillin or cephalothin and is inhibited by clavulanic acid, cannot hydrolyze extended spectrum cephalosporins such as ceftriaxone, cefotaxime, ceftazidime (Shahid *et al.*, 2009). TEM-2 also has the same hydrolytic profile as TEM-1, but possesses more active native promoter and different isoelectric point of 5.6 compared to 5.4 of TEM-1. TEM-13 on the other hand exhibits a similar hydrolytic profile as TEM-1 and TEM-2 hence is considered non-ESBL (Jacoby and Medeiros, 1991).

Currently, over 100 TEM-type β-lactamases have been described of which most of them are ESBLs (Paterson and Bonomo, 2005). Also mutants of TEM -lactamases are being recovered that maintain the ability to hydrolyze third-generation cephalosporins but which also demonstrate inhibitor resistance. These are referred to as complex mutants of TEM (CMT-1 to -4) (Poirel *et al.*, 2004).

CTX-M type ESBLs

These enzymes are so named because of their high hydrolytic activity against third-generation cephalosporin cefotaxime, and also cefepime, a

fourthgeneration cephalosporin with high efficiency but with minimal effect on ceftazidime (Paterson and Bonomo, 2005). CTX-M type ESBL was first recognized in 1989 and is classified as functional group 2 under the BushJacoby-Medeiros classification (Andriatahina *et al.*, 2010). However, this class of enzymes are thought to have been derived from the first transfer of chromosomal beta-lactamase from *Kluyvera species* to corresponding plasmids that are already spread among other members of *Enterobacteriaceae.*

The origin of the CTX-M enzymes is different from of that TEM and SHV ESBLs. Unlike SHV- and TEM-type ESBLs which were generated due to amino acid substitutions of their parent enzymes, CTX-M ESBLs were acquired as a result of horizontal gene transfer from other bacteria using mobile genetic elements such as conjugative plasmid transposons (Olson *et al.*, 2005; Shaikh *et al.*, 2015)**.**

Oxacillinase (OXA-type ESBLs)

These classes of β-lactamases are named due to their ability to hydrolyze oxacillin, a narrow-spectrum β-lactam antibiotic of the penicillin class. They mainly occur in *Pseudomonas spp.*, but have been detected in many other gram negative bacteria (Bradford, 2001). Oxacillinases are characterized by their ability to hydrolyze cloxacillin and oxacillin 50% more than benzyl penicillin but mostly do not hydrolyze extended spectrum cephalosporins to a significant degree hence are not ESBLs. OXA-10 weakly hydrolyze cefotaxime, ceftriaxone and aztreonam. Other OXA ESBLs derived from OXA-10 includes OXA-14, 16, 15, 18, 19, 28, 31 and 32 (Bedenic *et al.*, 2001)

Other ESBLS

A variety of other β-lactamases which are plasmid-mediated or integronassociated class A enzymes have been documented (Silva *et al.*, 2000). An example is PER gene which shares about 25% similarity with the TEM and SHV-type ESBLs (Girlich *et al.*, 2014). PER-1 β-lactamases hydrolyze penicillins and cephalosporins and is susceptible to clavulanic acid inhibitors and PER-1 beta-lactamase was first detected in strains of *P. aeruginosa* isolated in Turkey. The PER-2 which shares 86% homology to PER-1 was detected more frequently in South America (Bradford, 2001). Another rare ESBL type is the VEB-1 which was first found in isolate of *E. coli* from Vietnam (Kiremitçi *et al.*, 2011).

Other ESBLs types include VEB-1 & 2, GES, SFO and IBC. VEB-1 has greatest homology (38%) with PER-1 and PER-2 and confers higher level resistance to ceftazidime, cefotaxime and aztreonam which is reversed by clavulanic acid (Bedenic *et al.*, 2001). It was first isolated from a Vietnamese child hospitalized in France and plasmid mediated (Randegger and Hächler, 2001). Other VEB enzymes have been described in Kuwait and China. GES, SFO and IBC are examples of non-TEM, non –SHV ESBLs and have been found in a wide range of geographical locations (Ben *et al.*, 1990) .

Carbapenemases

Carbapenemases are diverse enzymes that differ in the ability to hydrolyze carbapenems and other β-lactams. Identification and detection of these enzymes is a very serious infection control issue because:

(i) They are often associated with extensive antibiotic resistance and

(ii) more-resistant organisms such as strains of *Pseudomonas* and *Acinetobacter* spp. that have acquired a carbapenemase can be vectors responsible for carbapenemase transmission to members of the family *Enterobacteriaceae* in which the resistance mechanism is not recognized (Thomson, 2010).

Carbapenem-hydrolyzing enzymes can be grouped into two based on molecular studies. The Serine enzymes possess a serine moiety at the active site whereas Metallo-β-Lactamases (MBLs) require divalent cations, usually zinc, as metal cofactors for enzyme activity. The serine carbapenemases belong to Class A or Class D enzymes. They usually result in carbapenem resistance in *Enterobacteriaceae* or *Acinetobacter spp*. (Walsh *et al.*, 2005).

Carbapenemases are falls under group 2f, 2d and 3 (Table 2)

Table 2: Substrate and inhibition profiles of the carbapenemases

Molecular Classes	Functional Group	Enzyme	Hydrolysis profile [a] Penicillins	Cephalosporins	Aztreonam	Carbapenems	Inhibitor profile [b] EDTA	Clavulanic acid	
A	2f	NMC	+	+	+	+	−	+			
		IMI		+		+	+	+	−	+	
		SME		+		+	+	+	−	+	
		KPC		+		+	+	+	−	+	
		GES		+		+	−	±	−	+	
B	3	IMP	+	+	−	+	+	−			
		VIM		+		+	−	+	+	−	
		GIM		+		+	−	+	+	−	
		SPM		+		+	−	+	+	−	

D	**2d**	**OXA**	+	+	−	±	−	±

(Wang *et al.*, 2010)

[a]+: Strong hydrolysis (generally, k_{cat} of >2/s), ±: Weak hydrolysis (generally, k_{cat} of 0.5-2/s), −: No measurable hydrolysis reported (generally, k_{cat} of <0.5 s^{-1}), [b]+: Reported inhibition, ±: Variable inhibition among β-lactamase family members, −: No inhibition reported, EDTA: Ethylenediaminetetraacetic acid, NMC: Not metalloenzyme carbapenemase, SME: *Serratia marcescens* enzyme, KPC: *K. pneumoniae* carbapenemases, GES: Guiana extended-spectrum, VIM: Verona integron-encoded metallo-β-lactamases, GIM: German imipenemase, SPM: Sao Paulo metallo-β-lactamases, or NDM enzymes. However, serine carbapenemases have also been reported, which include the KPC and GES variants (Wang *et al.*, 2010).

Class A Carbapenemases

Class A serine carbapenemases are members of functional Group 2f. They can hydrolyze a wide variety of β-lactams such as carbapenems, cephalosporins, penicillins, and aztreonam. However, they all are inhibited by clavulanate and tazobactam (Walsh *et al.*, 2005). Class A carbapenemase was first identified in *Enterobacter cloacae* more than 20 years ago (Nordmann and Poirel, 2014). Since then, other class A carbapenemases, including KPC (*Klebsiella pneumoniae* carbapenemase), IMI (imipenem-hydrolyzing βlactamase), SME (Serratia marcescens enzyme). Are all inhibited by clavulanate and tazobactam (Walsh *et al.*, 2005)). SFC (Serratia fonticola carbapenemase), NMC-A (non-metallo carbapenemase of class A) families and some GES (Guiana extended-spectrum β-lactamase) enzyme have been described (Jeon *et al.*, 2015). Mostly, IMI (IMI-1-3), SME (SME-1-3), SFC-1 and NmcA are chromosomally encoded whereas GES (GES- 1-20) and KPC (KPC-1-20) are plasmid encoded (Bedenić *et al.*, 2014).

Class B Metallo-β-Lactamases

This group of β-lactamases enzymes do not only hydrolyze carbapenems but are known by its resistant to commercially available β-lactamase inhibitors, but susceptibility to inhibition by Zn^{2+} chelators (Datta and Wattal, 2010). These enzymes are able to hydrolyze cephalosporins and penicillin but not aztreonam. MBLs require divalent zinc cations for substrate hydrolysis (Bush and Jacoby, 2010). This results in the distinctive trait of their inhibition by Ethylenediaminetetraacetic acid (EDTA), a chelator of Zn^{2+} and other divalent cations. The first MBLs for which an amino acid sequence was determined was the metallo-β-lactamase from *Bacillus cereus*, the prototypical metallo-

βlactamase for many years (Queenan and Bush, 2007). The presence of chromosomal MBLs is directly correlated with the prevalence of the producing species. There is however a great increase in the detection and spread of the acquired MBLs. The most common MBLs of the B class include the Verona integron-encoded MBLs (VIM), "active on imipenem" (IMP), "German imipenemase" (GIM), and "Seoul imipenemase" (SIM) enzymes. A new type of MBL, New Delhi Metallo-β-lactamase (NDM)-1 has been identified in India, United Kingdom and Pakistan (Mochon *et al.*, 2011). These are incorporated as gene cassettes present within a variety of integron structures. The transfer between bacteria is readily sped when these integrons become associated with plasmids or transposons (Queenan and Bush, 2007).

Class D Serine Carbapenemases

These group of β-lactamases can hydrolyze oxacillin or cloxacillin at a rate of >50% than for benzylpenicillin. Hence, these class of enzymes are known as OXA enzymes. They readily hydrolyze carbenicillin. The OXAenzymes now comprise the second largest family of β-lactamases (Zhanel *et al.*, 2007). The OXA carbapenemases have measurable hydrolytic activity against the penicillins, some cephalosporins, and imipenem (Bush and Jacoby, 2010; Walther-Rasmussen and Hoiby, 2006).

ESBL and Carbapenemase- producing Gram negative bacteria in *Mycobacterium tuberculosis* patients

Gram-negative bacteria such as *K. pneumoniae* and *P. aeruginosa* are among the common causes of nosocomial infections (Sedlakova *et al.*, 2014; Sonmezer *et al.*, 2016). However, the overdependence on β-lactam antibiotics in

the treatment of GNB infections have led to the production of β-lactamases (ESBL and carbapenemase)- producing bacterial strains. This results in prolonged hospital admission, limits treatment options and increased treatment costs (Leistner *et al.*, 2014).

Treatment of Tuberculosis includes isoniazid, rifampicin and pyrazinamide during the first two months and isoniazid and rifampicin to complete six months of treatment (Coll, 2003). It usually involves as long and intense antimicrobial therapy. TB patients are at risk of co-infection with other multidrug-resistant Gram-negative bacteria due to antibiotic selection pressure and nosocomial transmission during prolonged hospital admission (Gröschel *et al.*, 2016).

A previous study reported a prevalence of ESBL-producing *Enterobacteriaceae* carriage in MTB patients to be 28% among the Indian population (Jayapradha *et 2al.*, 2007). Also, Ravensbergen *et al*. (2018) reported on carbapenemase production in *P. aeruginosa* and *K. pneumoniae* in an Asylum seeker with Multidrug–Resistant Tuberculosis. A study carried in Aminu Kano Teaching Hospital in Nigeria revealed that the overall prevalence of ESBL production among tuberculosis patients is 37.1% (Yusuf *et al.*, 2011).

Laboratory detection of ESBLs

The Clinical Laboratory Standards Institute (CLSI) recommended both standardized screening and confirmatory tests for ESBL detection. The detection of ESBL according to CLSI is to use third generation cephalosporins for screening potential ESBL-producers and then observe the cephalosporin/clavulanic acid synergy to confirm ESBL producing bacteria

(CLSI, 2018).

Screening for ESBL Producing Bacteria using Disc Diffusion Method

The screening for ESBL producing bacteria as recommended by CLSI is done by the disc diffusion method (CLSI, 2018). In this test, either cefpodoxime (10 µg), ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg) or aztreonam (30 µg) is used as an indicator drug. Bacteria which produce zone of inhibition diameter of ≤ 21mm around cefpodoxime (10 µg), ≤ 17mm around ceftazidime (30 µg), ≤ 22mm cefotaxime (30 µg), ≤ 19mm around ceftriaxone (30 µg) or ≤ 17mm (30 µg) are suspected to produce ESBL and hence, need to be confirmed (CLSI, 2018).

Screening for ESBL producers by minimum inhibition concentration method

The Clinical Laboratory Standards Institute (CLSI) has recommended dilution methods for screening for ESBL production in *Enterobacteriaceae* and *P. aeruginosa*. Ceftazidime, aztreonam, cefotaxime and ceftriaxone could be used for screening ESBLs. The growth of bacteria at a screening concentration of ≥ 16 µg/ml for ceftazidime and aztreonam, ≥ 4µg/ml for cefotaxime and ceftriaxone are suspected to be ESBL producers and is an indication for the organism to be tested by a phenotypic confirmatory test (CLSI, 2018).

ESBL confirmatory Tests

Confirmatory test for ESBL depends on indicating synergy between the third-generation cephalosporin and clavulanic acid to which the isolate is initially found resistant in the screening test. The following tests can be used to confirm ESBL production: Double Disc Synergy Test (DDST), E-test and MIC broth dilution test (CLSI, 2018).

Double Disc Synergy Test (DDST)

In this test, the test inoculum (0.5 McFarland's turbidity) is spread onto Muller-Hinton Agar (MHA) by using s sterile cotton swab. A disc of Augmentin (30 µg ceftazidime and 30 µg cefotaxime + 10 µg of clavulanic acid) is placed on MHA; then discs of cefotaxime (30 µg) and ceftazidime (30 µg) are kept 20mm apart from the Augmentin disc. The plate is incubated at 37 ℃ overnight. An increase of ≥ 5 mm in the inhibition zones of either cephalosporin in combination with clavulanic acid compared to the cephalosporin alone is inferred as ESBL production (Cockerill, 2012).

MIC broth dilution test

In this method, the minimum inhibitory concentration (MIC) of a third generation cephalosporin alone and in combination with clavulanic acid is compared. A decrease in the MIC of the combination of 3 two-fold dilutions (8 times) indicates ESBL production (Joumana *et al.*, 2003).

E-test

In this method, ESBL strips that have cephalosporin gradient at one end and cephalosporin plus clavulanate gradient at the other are used. One side of the strip is calibrated with minimum inhibitory concentration (MIC) reading scale and the other side has two predefined antibiotic gradients. There are two strips used in this test and testing must be performed with both strips. One of the strips contains cefotaxime gradient (0.25 to 16 µg/ml) at one end and ceftazidime/clavulanic acid gradient (0.016 to 1 µg/ml plus 4 µg/ml of clavulanic acid) at the other end. The second strip contains ceftazidime gradient (0.5 to 32 µg/ml) at one end and ceftazidime/clavulanic acid gradient (0.064 to

4 µg/ ml plus 4 µg/ml clavulanic acid) at the other end. The presence of ESBL is confirmed by the appearance of phantom zone below the cefotaxime or deformation of the ceftazidime inhibition ellipse or when the clavulanic acid causes a more than or equal to three doubling concentration decreases (ratio of ≥8) in the MIC values of cefotaxime and ceftazidime (Sridhar Rao, 2015).

Other Methods

There are automated ESBL detection methods. These include Vitek ESBL cards, Microscan panels and the BD Phoenix automated microbiology systems (Peer *et al.*, 2008).

Molecular Characterization of ESBL Genes

One of the commonest molecular methods used is the polymerase chain reaction (PCR) followed by sequencing. Sequencing is needed to differentiate between different ESBL enzyme isolates (TEM 3, SHV2 etc) and non-ESBL isolates (TEM 1, TEM 2, or SHV1). The ability to differentiate between ESBLs and non-ESBLs makes this system the method of choice (Fluit *et al.*, 2001). Other molecular methods have been developed to characterize ESBLs. These include PCR with restriction fragment length polymorphism (RFLPs) (Arlet *et al.*, 1995; Chanawong *et al.*, 2000), PCR with single-strand conformational polymorphism (Chanawong *et al.*, 2000; M'Zali *et al.*, 1996), ligase chain reaction (Kim and Lee, 2000), restriction site insertion PCR (Chanawong *et al.*, 2001) and real-time PCR (C.C. Randegger and Hachler, 2001).

Laboratory Detection of Carbapenemases

Screening Test for Carbapenemase

The Clinical Laboratory Standards Institute (CLSI) recommended standardized test for Carbapenemase detection using the Modified Hodge Test (MHT). This is the only method of carbapenemase detection recommended by the CLSI (CLSI, 2018). Carbapenemase production is detected by the MHT when the test isolate produces the enzyme and allows growth of carbapenems susceptible strain towards a carbapenem disk.

Phenotypic detection using disc diffusion method

In this test, the test inoculum and (0.5 McFarland's turbidity) is spread onto Muller-Hinton Agar (MHA) by using s sterile cotton swab. Ertapenem (10 µg) or Meropenem (10 µg) and Imipenem (10 µg) are applied on the MullerHinton agar plates and incubated at 37^{o}C overnight. Following incubation, the

MHA plate are examined for enhanced growth around the test or quality control (QC) organism streak at the intersection of the streak and the zone of inhibition. Enhanced growth was positive for carbapenemase production no enhanced growth was interpreted as negative for carbapenemase production (CLSI, 2018).

Carbapenemase Confirmatory Tests in *Enterobacteriacae*

Test for Class A Carbapenemases

The inhibitory effect of boronic acids, usually 3-aminophenylboronic acid (APB) forms the basis of specific phenotypic detection of KPC producing strains and Class A carbapenemases. Meropenem (10 µg) and imipenem (10 µg) are indicator drugs used to test for Class A carbapenemases. Different cutoff

values of zone diameter differences between discs with a carbapenem plus boronic acid and the carbapenem are proposed to indicate the production of KPC (or another Class A carbapenemase) (≥4 to ≥7 mm). Another method is developed in which MICs of carbapenems are observed both in the absence and in the presence of boronic acid (0.3 g/L) by agar dilution. A reduction of carbapenem Minimum Inhibitory Concentration in the presence of APB by a three-fold or greater is proposed as the cut-off value for positive isolates (Doi *et al.*, 2008; Pasteran *et al.*, 2009; Tsakris *et al.*, 2009).

Test for Class B Carbapenemases (MBL) using Combined Disc Diffusion Test

Normal saline suspension of clinical isolate is adjusted to the McFarland 0.5standard and is used to inoculate MHA plates with zinc sulfate at a final concentration 70 µg/ml. Two discs of imipenem (10 µg), meropenem (10 µg) and ceftazidime (30 µg) are placed on the inoculated MHA plate. 750 µg of EDTA solution is added to one disc of imipenem (10 µg), meropenem (10 µg) and ceftazidime (30 µg). Then, the plates are incubated at 37 ℃ for 24 hours. The MBL-positive isolates are distinguished from the MBL negative isolates based on the criterion of greater than 7 mm increase in the inhibition zone for imipenem and/or meropenem or ≥ 4 mm increase in the inhibition zone for ceftazidime with the discs to which EDTA is added (Franklin *et al.*, 2006; Yong *et al.*, 2002).

Molecular Detection of Carbapenemase Genes

Simplex (Poirel *et al.*, 2011) and multiplex (Ellington *et al.*, 2007; Monteiro *et al.*, 2012) PCRs, real-time PCR (Bisiklis *et al.*, 2007), DNA hybrid

technique (Naas *et al.*, 2011), and sequencing techniques (Poirel *et al.*, 2011) have been developed for the detection of carbapenemase genes (bla_{KPC}) in research laboratories and reference centers.

Prevalence of tuberculosis Ghana

Globally, about one-third of humanity is infected with the TB bacterium although a greater proportion of the population shows no symptoms. About 10.4 million TB incidence and 1.7 million deaths from the disease were recorded in 2016. Africa is the second highest TB endemic continent in the world. The continent forms about 11% of the population of the world, however, it hosts about one-third of the global burden of TB incidence and 34% of related deaths. About three million individuals with TB re- main undiagnosed and untreated in Africa (Oppong *et al.*, 2015). In Ghana, the second national TB survey conducted revealed a national prevalence of 290 given a 100,000 population. The burden of TB in Ghana is unevenly distributed, and relatively prevalent among urban settlers owing to the challenges of contemporary urbanization such as high population density, poorly planned housing, and poor sanitation in developing countries which facilitate transmission of TB from person to person (Gyimah and Dako-gyeke, 2019).

ESBL-producing bacterial isolates have been reported across Africa, even in isolated remote communities (Mensah *et al.*, 2016). In Africa, outbreaks of infection with ESBL-producing enterobacteria have been reported in South Africa, Egypt, Tunisia, Morocco, Tanzania, and Nigeria. In Ghana, an overall prevalence of 49.3% of patients sampled from the Korlebu Teaching Hospital in Accra has been reported. The ESBL prevalence was significantly higher in

isolates from patients at extremes of ages: neonates and adult patients above 65 years of age (Obeng-nkrumah *et al.*, 2013).

Summary

This chapter reviewed the family *Enterobacteriaceae* and *Pseudomonadaceae*, β-lactamases and their classifications, ESBL and carbapenemase-producing Gram-negative bacteria in MTB patients and laboratory detection of the various β-lactamases.

β-lactamases are perhaps the most important resistant mechanisms as these enzymes mediate the inactivation of beta-lactams by hydrolyzing the amide bond of the β -lactam ring. Among the groups of β-lactamase enzymes, Extended Spectrum β-Lactamases (ESBLs) and Carbapenemases constitute the chief resistant mechanisms against β-lactam antibiotics.

Treatment of Tuberculosis involves the use of isoniazid, rifampicin and pyrazinamide during the first two months and isoniazid and rifampicin for the next four months to complete six months of treatment. It usually entails long and intense antimicrobial therapy. TB patients are at risk of co-infection with other multidrug-resistant Gram-negative bacteria due to antibiotic selection pressure and nosocomial transmission during prolonged hospital admission.

CHAPTER THREE: METHODOLOGY

Study design

A retrospective, analytical cross-sectional study was conducted on archived rectal samples from three health facilities in southern and northern Ghana, between December 2016 and November 2018.

Study site

The Tuberculosis (TB) chest clinic and trauma unit of Tamale Teaching Hospital, Tema General Hospital, and Manya Krobo Government Hospital were the sites where the rectal samples (archived) were collected. Together, these facilities serve residents from across the socioeconomic and rural-urban divides within their catchment areas.

Study Population

This study used archived rectal samples obtained from TB confirmed individuals, aged 6 years and above.

Ethical considerations

Ethical clearance was obtained from the review board of the Noguchi Memorial Institute for Medical Research and the selected healthcare facilities. Written informed consent was obtained from every study participant and in cases of minors (less than 18 years), consent was obtained from their parent, guardians or representatives. This study was carried out by following the Helsinki declaration for research involving human subjects, particularly, minors. Participants' confidentiality and anonymity were ensured from the beginning to the end of the study.

Sampling Procedure

A purposeful sampling strategy with maximum variation was employed. The study took into consideration the effect of socioeconomic status, gender and the different geographical areas on the outcome of the results. Investigation on the prevalence and genetic diversity of ESBL presentations with different duration of antibiotic therapy gave information on the outcome of the long-term antimicrobial exposure in these patients.

The minimum sample size of archived rectal samples retrieved for ESBL production was computed using the formula below:

$$N = \frac{z^2 (P)(1-P)}{(d)^2}$$

where Z= Z statistic for a level of confidence [confidence level at 95% (1.96)]

P = % (), is the sample proportion of the prevalence of ESBL-producing Enterobacteriaceae and carbapenems in Ghana. d=Precision [margin of error at 5% (0.05)]

Sample size, N = $\frac{()^2 ()()}{()^2}$ =

Overall, a total sample size of 100 archived rectal samples from 32 patients was retrieved for use in this study.

Inclusion criteria

TB patients aged 6 years and above with laboratory-confirmed sputum smear were included in this study. TB patients provided with the six months antibiotic standard therapy, co-infected with HIV and undergoing treatment for 9 months were also included.

Exclusion criteria

Patients clinically suspected of having TB without sputum smear laboratory confirmation were excluded from this study. MDR-TB patients undergoing treatment for 12 months or longer duration were excluded from the study.

Data Collection Procedures

Sample Collection

Archived rectal samples (swabs) used in this study were collected by firstly selecting and training nurses from the tuberculosis chest clinic and trauma unit of the various facilities in rectal swabs collection. Rectal swabs from recruited TB patients were taken by the trained nurses using eSwab, with the help of the parent or guardian in the case of children at the day of diagnosis (before the start of treatment), at 3 months and 6 months after the start of treatment and at 3 months after finishing the treatment. The eSwab was suspended in a 1 mL liquid Amies medium (Copan). All samples were transported in an icebox to the Noguchi Memorial Institute for Medical Research for the culturing and isolation of ESBL. Cultivation of bacteria was done in a safety cabinet to prevent the spread of drug-resistant bacteria. Participants' socio-demographic characteristics were recorded. The outcome of diagnostic tests such as culture results for tuberculosis and treatment data such as antibiotics used were retrieved from the patient's folder.

Cultivation of isolates

A volume of 10 µL of the rectal suspension was streaked on Bromothymol blue Agar. Gram staining was done to rule out the Gram-positive

bacteria If Gram positive bacteria were detected, those isolates would have been excluded.

Bacterial isolates were purified by sub-culturing on Nutrient Agar. Pure cultures of isolates were identified using Matrix-Assisted Laser Desorption/IonizationTime of Flight (MALDI-TOF) mass spectrometry (MS) within 24h of sampling. Pure isolates were stored at -80 ℃ in Mueller Hinton Broth containing 20% glycerol.

Phenotypic ESBL Detection

ESBL Screening

Frozen isolates stored at -80°C were thawed at room temperature and subcultured on nutrient agar (Techno PharmChem, India) to obtain pure growth, for ESBL phenotypic and genotypic determination.

Briefly, all bacterial isolates were screened for the presence of ESBL according to guidelines for laboratory detection of ESBL from Clinical and Laboratory Standards Institute (CLSI, 2018).

Isolates were determined by inoculating Mueller-Hinton agar (Oxoid, UK) with overnight-cultured isolates with a turbidity of 0.5 McFarland standard using cefotaxime (30 µg) and ceftazidime (30 µg) antibiotic discs (Biomark Laboratories, India) and incubated at 37ºC for 18 hours in ambient air. A zone of inhibition less than 26mm and 21mm for cefotaxime and ceftazidime respectively was indicative of resistance of the isolate to the drug and a possible ESBL producer (CLSI, 2018). These isolates were then selected for confirmation of ESBL production.

ESBL Confirmation Testing

ESBL confirmation was done using the Combined Disc method. The Combined Disc test was performed at the laboratory on Mueller-Hinton agar plates (Oxoid,

UK) and using combined discs containing cefotaxime, cefotaxime (30 µg)/Clavulanic acid (CA) (10 µg), and ceftazidime, ceftazidime (30 µg)/ Clavulanic acid (10 µg) (Bio-Rad, France) (Sader *et al.*, 2015)

From a pure subculture of each isolate, 4 colonies were touched with a sterile inoculating wire loop and transferred into 3 mL of normal saline and adjusted with normal saline until 0.5 McFarland standard was achieved. A sterile cotton ended swab was dipped into the inoculum to seed the Muller-Hinton agar. The inoculum was swabbed on the Mueller–Hinton agar plate using a rotor retrospect to obtain a semi-confluent growth on the entire surface of the Mueller–Hinton agar (Oxoid, UK). The antibiotic disks were then applied firmly on the surface of the agar with sterile forceps and aerobically incubated at 37°C for 24 hours. *Escherichia coli* ATCC 25922 was used as the ESBL negative control and *Klebsiella pneumoniae* ATCC 700603 was used as the ESBL positive control. Isolates were considered positive for ESBL production if zone diameters increased by ≥ 5mm for either cefotaxime or ceftazidime, tested in combination with clavulanic acid versus its zone when tested alone, as indicated by the manufacturer or CLSI (CLSI, 2018).

Phenotypic Detection of Carbapenemase Genes

Carbapenemase Screening

Normal saline suspension of bacteria isolate was adjusted to the 0.5 M McFarland standard and was used to inoculate Mueller Hinton Agar (MHA)

plates. Meropenem, Doripenem, Ertapenem and Imipenem were placed on the inoculated Mueller Hinton Agar with the lawn of test organisms and incubated for 18 hours. Zones of inhibition were recorded after overnight incubation and compared with the CLSI guidelines to classify as potential carbapenemase producers (Hrabák *et al.*, 2014).

Carbapenemase Confirmatory Tests

Mast carbapenemase detection method which contained four antibiotic discs was used, namely;

- Disc A, which contained only a carbapenem (meropenem, 10 µg),
- Disc B which contained meropenem (10µg) with dipicolinic acid to inhibit Metallo Beta-Lactamases (MBL),
- Disc C, which contained meropenem (10µg) with a KPC inhibitor and
- Disc D, which contained meropenem (10µg) with an AmpC inhibitor w.

The plates were then incubated at 37°C for 24 hours. The Metallo BetaLactamases (MBL) positive isolates were distinguished from the MBL negative isolates based on the criterion of greater than 5 mm increase in zone diameter of disc B compared to disc A. A greater than 4mm increase in zone diameter of disc C compared to disc A denoted positive result for *Klebsiella pneumoniae* carbapenemase. A greater than 5 mm increase in zone diameter of disc C and disc D implied a positive result for a Porin-deficient AmpC producer.(Amjad *et al.*, 2011; Sader *et al.*, 2015)

Molecular Detection of ESBL and Carbapenemases

Lysate Preparation

Phenotypically documented ESBL and carbapenemase-producing Enterobacteriaceae were characterized by Polymerase Chain Reaction (PCR) technique to confirm the presence of gene families that encode ESBLs and Carbapenemases. Bacterial DNA extraction for use in PCR-based methods was carried out using the boiling method as described by (Espinosa *et al.*, 2013). Stored bacterial isolates were subcultured from frozen stock on nutrients agar to obtain pure colonies. Once purity had been ensured, 3-6 isolated colonies were suspended with a sterile loop into 200 uL of nuclease-free water in a screw cap tube, lid closed tightly and vortexed. Samples were then boiled for 10 minutes at 100^{o}C to allow for cell lysis and DNA extraction. All lysates were stored at -20^{o}C. The samples were then centrifuged at 14000 rpm for 10 mins at 4 ^{0}C and 100ul of the supernatant was transferred into a new sterile screw cap tube and stored at -20 ^{o}C. This served as the template for subsequent PCR reactions.

PCR reagent mix

For each of the ESBL resistance determined, gene primer was diluted to the stock solution of 100 µg/µL and stored at -20°C by adding equal volumes of nuclease-free PCR water. To make a working solution, the stock solution was diluted in ratio 1:10 with nuclease-free PCR water as stated by the manufacturer.

Detection of ESBL Resistant Genes by PCR Amplification

Multiplex PCR was done to detect each of the ESBL-resistance genes including those encoding resistance determinants. In all cases, positive and negative controls of appropriate values were run for quality control purposes. Positive controls consisted of bacterial DNA containing the gene (or genes) of

interest, while the negative control lacking the gene of interest or sterile dH_2O containing no DNA. Controls were run in parallel, each time tests were conducted for a given set of tests to be deemed viable.

Isolates were screened for SHV, TEM, CTX-M group 1, CTX-M group 2, CTXM group 9 and OXA-1. PCR was carried out using a thermal cycler (BioRad, USA) with a total volume of 50.0 µL containing 2.0 µL DNA template and 25 µL Emerald premix (2x) (New England Biolabs, UK). 7.51 µL primers mix (Sangon Biotech, China) and 15.5 µL dH_2O. The primer sequences and cycling conditions used for the different PCRs are shown in Table 3.

Table 3: Specific primers sequences used for PCR amplification of resistance genes

Resistant genes	Primer name	Primer Sequence (5' to 3')	Band size	Cycling conditions		
TEM,	MultiTSOT_for	FP:CATTTCCGTGTCGCCCTTATTC	800	Initial		
	MultiTSO-T_rev	RP:CGTTCATCCATAGTTGCCTGAC				
SHV	MultiTSO-S_for	FP:AGCCGCTTGAGCAAATTAAAC	713	denaturation at 94ºC for 5min,30		
	MultiTSO-S_rev	RP:ATCCCGCAGATAAATCACCAC			94°C	
OXA-1	MultiTSO-O_for	FP:GGCACCAGATTCAACTTTCAAG	564	cycles of 94ºC for 1min,		5 min
	MultiTSO-O_rev	RP:GACCCCAAGTTTCCTGTAAGTG				
CTX-M	MultiTSO-O_for	FP: TTCCAGAATAAGGAATCCC	688	57ºC for 1min and 72ºC for 1 min		
	MultiTSO-O_rev	RP: CGTTTCCGCTATTACAAAC				
CTX-M2	MultiTSO-O_for	FP:CGTTAACGGCACGATGAC	404	and final elongation 72ºC for 10min		
CTX-M9	MultiTSO-O_rev	RP:CGATATCGTTGGTGGTRCCAT				
	MultiTSO-O_for	FP:TCAAGCCTGCCGATCTGGT	561			
	MultiTSO-O_rev	RP: TGATTCTCGCCGCTGAAG				

Agarose Gel Electrophoresis

The results of PCR-based tests were determined by agarose gel electrophoresis using 2% agarose gel. Gels were prepared by dissolving 4g of agarose (BDH Prolabo®, Belgium) in 200 mL of 1x Tris Acetate EDTA (TAE) buffer. This solution was boiled until it was transparent and allowed to cool to ~50-55^{o}C with intermittent swirling. 5µL ethidium bromide were added, swirled and poured into gel tray with a comb. Once cooled (at 42^{o}C), the solution was poured into a casting tray with combs. The gel was then allowed to cool at room temperature for 30-45 minutes. Once prepared, gels were placed into an electrophoresis chamber containing 1X TAE buffer and 7 µL of each PCR product mixed with the loading dye was loaded into the wells. The first well was loaded with the quick load 100 base pair DNA ladder (New England Biolabs, UK). The two wells were loaded with the positive and negative control *Bla gene* (SHV, TEM, CTX-M, CTX-M2, CTX-M9, and OXA-1) under test. The remaining wells were filled with test samples. The electrophoresis was run at 100V for 35 minutes. The loaded gel was then washed under running water to remove excess staining prior to visualization and subsequent sequencing. In order to further distinguish blaCTX-M positive isolates, group-specific primers were used for amplification and sequencing (Dallenne *et al.*, 2010). The specific primers (MWG Ebensburg, Germany) sets used for amplification and sequencing are shown in Table 6.

Visualization of amplicons

After the electrophoresis, the resulting gel was visualized by trans-illuminator, photographed by a digital camera and transferred to computer data for labelling and storage (Brand/ Country)

The study was conducted following the developed standard operating procedures. In all tests the use of positive and negative controls were adhered to according to CLSI guidelines.

CHAPTER FOUR: RESULTS AND DISCUSSION

A number of analytical methods for quantitative determination of molecular epidemiology of Extended Spectrum beta-lactamase and Carbapenemase producing Enterobacteriacae have been evaluated using several parameters. The demographics of patients included in the study were analysed to determine the relation between the various demographic parameters. The prevalence of the resistance to the various antibiotics phenotypically and genotypically was determined. Reproducibility and accuracy of the techniques employed were tested and confirmed with controls in order to ensure quality results.

General Characteristics

A total of 53 isolates were obtained from the three different hospitals. Manya Krobo Hospital recorded the highest number of isolates (32 (60.4%)) followed by Tamale Teaching Hospital (16 (30.2%)) and then Tema Hospital which recorded the least (5 (6.4%)). *Escherichia coli* 36 (67.9%) formed the majority of the isolates obtained followed by *Klebsiella pneumonia* 6 (11.3%), *Enterobacter cloacae* 3 (5.7%), *Morganella morganii* 3 (5.7%), *Citrobacter freundi* 1 (1.9%), *Enterobacter asburiae* 1 (1.9%), *Klebsiella varicola* 1 (1.9%), *Proteus mirabilis* 1 (1.9%) and *Serraatia marcescens* 1 (1.9%).

Demographics of the participants

Age

The study included TB patients aged 14 years and above. The mean age of all the patients was 49.28 ± 17.31 years with the highest and lowest ages being 78 and 14 years, respectively. The average age of those infected with

Escherichia coli alone was 51.10 ± 18.49 years, *Klebsiella pneumoniae* alone was 39.00 ± 17.93 years, *Enterobacter cloacae* alone was 43.33 ± 11.72 years, *Morganella morganii* alone was 62.00 ± 5.20 years, *Enterobacter asburiae* alone was 33.00 years, *Klebsiella varicola* alone was 54.00 years, *Proteus mirabilis* alone was 45.00 years and *Serraatia marcescens* alone was 52.00 years. There was a significant difference ($p = 0.004$) between age and the isolate recorded (Table 4). This suggests that the microorganisms detected from the isolates are affected by the age of the patients.

Gender

Most of the isolates used for this study were obtained from males 36 (67.9%) and 17 (32.1%) isolates were obtained from females. Of the number of *Escherichia coli* that was isolated, 23 (63.9%) were from males and 13 (36.1%) were from females, *Klebsiella pneumoniae* were isolated from 4 (66.7%) males and 2 (33.3%) females, *Enterobacter cloacae* and *Morganella morganii* were both isolated from 3 (100%) males each, *Citrobacter freundi*, *Enterobacter asburiae*, and *Proteus mirabilis* were isolated from 1 (100%) male each, *Klebsiella varicola* and *Serraatia marcescens* were isolated from 1 (100%) female each. There was no significance difference ($p = 0.363$) between gender and the isolates (Table 4). This therefore implies that gender of patients does not determine the type of isolate detected

Facility

The majority of isolates obtained from Manya Krobo General Hospital (MKGH) were *Escherichia coli* 23 (71.9%) followed by *Klebsiella pneumoniae* and *Morganella morganii* 3 (9.4%) each, and then *Citrobacter freundi*, *Proteus*

mirabilis and *Serraatia marcescens* 1 (3.1%) each. A total of 10 (62.5%) *Escherichia coli* were obtained from Temale Teaching Hospital (TTH) followed by *Klebsiella pneumoniae* 3 (18.8%), *Enterobacter cloacae* 2 (12.5%) then *Enterobacter asburiae* 1 (6.2%). Tema General Hospital (TGH) recorded a total of 3 (8.3%) *Escherichia coli*, *Enterobacter cloacae* 1 (33.3%) and *Klebsiella varicola* 1 (100%) (Table 4). The relationship between the facilities and isolates obtained was not statistically significance (p = 0.129). The type of isolates detected are therefore not affected by the facilities they were obtained from.

Treatment stage

The isolates obtained from TB patients were at various treatments stages. Out of the total number of isolates obtained, 19 (35.8%) were from TB patients at the second stage of treatment, followed by patients at the first stage of treatment 18 (34.0%) and patients at the third stage of treatment 16 (30.2%). *Escherichia coli* were isolated from 13 (36.1%), 12 (33.3%) and 11 (30.6%) patients at the first, second and third treatment stages respectively. *Klebsiella pneumoniae* were isolated from 4 (66.7%) and 2 (33.3%) patients at the second and third treatment stages respectively, *Enterobacter cloacae* were obtained from 3 (100%) patients at the first stage of treatment, *Morganella morganii* were isolated from 2 (66.7%) patients at the first stage of treatment and 1 (33.3%) patient at the third stage of treatment, *Citrobacter freundi*, *Klebsiella varicola* and *Serraatia marcescens* were isolated from 1 (100%) patient each at the second stage of treatment, *Enterobacter asburiae* and *Proteus mirabilis* were isolated from 1 (100%) patient each at the third stage of treatment. There was no significant (p = 0.156) relationship between isolates and treatment stages

(Table 4). This suggests that the treatment stage of TB patients has no impact on the type of isolate detected.

Table 4: General demographics of patients classified by enterobacteria

Demographics	***E. coli*** **(N = 36)**	***K. pneumonia*** **(N = 6)**	***E. cloacae*** **(N = 3)**	***M. morganii*** **(N = 3)**	***C. freundi*** **(N = 1)**	***E. asburiae*** **(N = 1)**	***K. varicola*** **(N = 1)**	***P. mirabilis*** **(N = 1)**	***S. marcescens*** **(N = 1)**	**P value**
Age	51.10±18.49	39.00±17.93	43.33±11.72	62.00±5.20		33.00	54.00	45.00	52.00	0.004
Gender										
Male	23 (63.9%)	4 (66.7%)	3 (100%)	3 (100%)	1(100%)	1(100%)		1(100%)		
Female	13 (36.1%)	2 (33.3%)	-				1(100%)		1 (100%)	0.363
Facility										
MKGH	23 (63.9%)	3 (50%)	-	3 (100%)	1(100%)	-	-	1(100%)	1 (100%)	
TTH	10 (27.8%)	3 (50%)	2 (66.7%)	-	-	1(100%)	-	-	-	
TGH	3 (8.3%)	-	1 (33.3%)	-	-	-	1(100%)	-	-	0.129
Treatment stage										
First stage	13 (36.1%)		3 (100%)	2 (66.7%)						
Second stage	12 (33.3%)	4 (66.7%)			1(100%)		1(100%)		1 (100%)	
Third stage	11 (30.6%)	2 (33.3%)		1 (33.3%)		1(100%)		1(100%)		0.156

Continuous data were presented as mean ± SD, categorical data presented as proportion. Continuous data were compared to each other using One-way ANOVA whilst categorical data compared to each other using Chi-square analysis.

Antibiotic susceptibility profiles of the isolates

A total of 53 Enterobacteria were tested against 17 different antibiotics which includes thirteen (13) beta lactams namely meropenem, ertapenem, imipenem, dorepenem, cefepime, cefoxitin, cefuroxime, cefotaxime, ceftazidime/avibactam, ceftazidime, trimethoprim-sulphamethoxazole, amoxicillin/clavulanate, nalidixic acid and four (4) non-beta lactams such as ciprofloxacin, gentamicin, augmentin and tetracycline. A greater proportion of isolates were resistant to the beta lactam antibiotics ranging from 5.6% for meropenem and ertapenem to 83.4% for amoxicillin/clavulanate when tested against *E. coli*. All the other isolates showed 100% resistance to amoxicillin/clavulanate. Also, five (5) isolates showed 100% resistance to Trimethoprim-sulphamethoxazole. These findings agree with the low susceptibility of micro-organisms to amoxicillin/clavulanate and trimethoprimsulphamethoxazole reported in literature. The resistance of micro-organisms to these antibiotics may be because they are the first-choice antibiotic treatment for most infections (Oteo *et al.*, 2008). A high proportion (100%) of *K. pneumoniae*, *E. cloacae* and *M. morganii* were resistant to amoxicillin/clavulanate. The proportion of isolates resistant to non-beta lactams tested ranged from 27.8% for ciprofloxacin to 83.4% tetracycline (Table 5). There was no significant relationship between the antibiotic susceptibility of the beta lactams and the micro-organisms except for imipenem (p = 0.030) and cefoxitin (p = 0.001). This suggests that the antibiotic susceptibility of the micro-organisms is not determined by the type of antibiotic except for

imipenem and cefoxitin. From the results, with the beta lactams imipenem and dorepenem will be the recommended drugs of choice since none of the isolates were resistant to them . This will be followed by meropenem and ertapenem which only a few *E. coli* isolates were resistant whilst the other isolates showed no resistance. A detailed report of all the drugs against all microbes is shown in Table 5.

Table 5: Proportion of Enterobacteria isolates stratified by resistance to some selected antibiotics

Antibiotics	Resistance pattern	*E. coli* (N = 36)	*K. pneumonia* (N = 6)	*E. cloacae* (N = 3)	*M. morganii* (N = 3)	*C. freundi* (N = 1)	*E. asburiae* (N = 1)	*K. varicola* (N = 1)	*P. mirabilis* (N = 1)	*S. marcescens* (N = 1)	P value
Beta-lactams			-					-	-		
Meropenem	R	2 (5.6%)		-	-	-	-			-	
	I	6 (16.7%)	2 (33.3%)	2 (66.7%)	-	1(100%)	1(100%)	1(100%)	-	1 (100%)	
	S	28(77.8%)	4 (66.7%)	1 (33.3%)	3 (100%)	-	-	-	1(100%)	-	
											0.344
Ertapenem	R	2 (5.6%)	-	-	-	-	-	-	-	-	
	I	1 (2.8%)	-	-	-	-	-	-	-	-	
	S	33(91.7%)	6 (1000%)	3 (100%)	3 (100%)	1(100%)	1(100%)	1(100%)	1(100%)	1 (100%)	
										-	1.000
Imipenem	R	-	-	-	-	-	-	-	-	-	
	I	-	-	-	1 (33.3%)	-	-	-	-	-	
	S	36 (100%)	6 (1000%)	3 (100%)	2 (66.7%)	1(100%)	1(100%)	1(100%)	1(100%)	1 (100%)	
											0.030
Dorepenem	R	-	-	-	-	-	-	-	-	-	
	I	1 (2.8%)	-	-	1 (33.3%)	-	-	-	-	-	
	S	35(97.2%)	6 (1000%)	3 (100%)	2 (66.7%)	1(100%)	1(100%)	1(100%)	1(100%)	1 (100%)	
											0.447
Cefepime	R	3 (8.3%)	2 (33.3%)		2 (66.7%)	1(100%)	1(100%)				
	I	3 (8.3%)	-	-	-	-	-	-	1(100%)	-	
	S	30(83.4%)	4 (66.7%)	2 (66.7%)	1 (33.3%)	-	-	1(100%)	-	1(100%)	
											0.161
Cefoxitin	R	3 (8.3%)	-	3 (100%)	-	1(100%)	1(100%)	-	-	1(100%)	

	I	1 (2.8%)	-	-	1 (33.3%)	-	-	-	-	-	
	S	32(88.9%)	6 (100%)	-	2 (66.7%)	-	-	1(100%)	1(100%)	-	
											0.001
Cefuroxime	R	9 (25.0%)	-	1(33.3%)	1(33.3%)	-	-	-	-	1(100%)	
	I	3 (8.3%)	-	1(33.3%)	-	-	-	-	-	-	
	S	24(66.7%)	6 (100%)	1(33.3%)	2 (66.7%)	1(100%)	1(100%)	1(100%)	1(100%)	-	
											0.767
Cefotaxime	R	7 (19.4%)	-	-	-	-	-	-	-	-	
	I	1 (2.8%)	-	1 (33.3%)	-	-	-	-	-	-	
	S	28(77.8%)	6 (100%)	2 (66.7%)	3 (100%)	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)	
											0.998
Ceftazidime/ avibactam	R	18(50.0%)	1 (16.7%)	3 (100%)	3 (100%)	1(100%)	1(100%)	-	-	1(100%)	
	I	10(27.8%)	2 (33.3%)	-	-	-	-	-	1(100%)	-	
	S	8 (22.2%)	3 (50.0%)	-	-	-	-	1(100%)	-	-	
											0.301

Ceftazidime	R	4 (11.1%)	-	-	-	-	-				
	I	1 (2.8%)	-	-	-	-	-	-	-	-	
	S	31(86.1%)	6 (100%)	2 (66.7%)	3 (100%)	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)	0.874
Trimethoprim-sulphamethoxazole	R	30(83.3%)	2 (33.3%)	1 (33.3%)	2 (66.7%)	1(100%)	-	-	-	-	
	I	-	-	-	-	-	-	-	-	-	
	S	6 (16.7%)	4 (66.7%)	2 (66.7%)	1 (33.3%)	-	1(100%)	1(100%)	1(100%)	1(100%)	0.023
Amoxicillin/ clavulanate	R	30(83.4%)	6 (100%)	3 (100%)	3 (100%)	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)	
	I	3 (8.3%)	-	-	-	-	-	-	-	-	
	S	3 (8.3%)	-	-	-	-	-	-	-	-	0.509
Nalidixic acid	R	15(41.7%)	-	1 (33.3%)	-	-	-	-	-	-	
	I	-	-	-	-	-	-	-	-	-	
	S	21(58.3%)	6 (100%)	2 (66.7%)	3 (100%)	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)	0.403
Ciprofloxacin	R	10(27.8%)	-	-	-	-	-	-	-	-	
	I	1 (2.8%)	-	1 (33.3%)	-	-	-	-	-	-	
	S	25(69.4%)	6 (100%)	2 (66.7%)	3 (100%)	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)	0.991
Gentamycin	R	-	-	-	-	-	-	-	-	-	

	I	4 (11.1%)	1(16.7%)	-	-	-				
	S	32(88.9%)	5(83.3%)	2(66.7%)	3 (100%)	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)	
										0.955
Augmentin	R	28(77.8%)	6 (100%)	3 (100%)	3 (100%)	-	-	-	-	-	
	I	-	-	-	-	1(100%)	-	1(100%)	-	1(100%)	
	S	8 (22.2%)	-	-	-	-	1(100%)	-	1(100%)	-	
										0.001
Tetracycline	R	30(83.4%)	3 (50.0%)	2 (66.7%)	2 (66.7%)	1(100%)	1(100%)	-	1(100%)	1(100%)	
	I	3 (8.3%)	2 (33.3%)	1 (33.3%)	-	-	-	-	-	-	
	S	3 (8.3%)	1 (16.7%)	-	1 (33.3%)	-	-	1(100%)	-	-	
										0.427

Key: R means Resistant, I means Intermediate susceptibility and S means Susceptible

Socio-demographic characteristic of *E. coli, K. pneumoniae, E. cloacae* and *M. morganii*

The antibacterial resistance patterns for four (4) common isolates that showed significant resistance against some of the antibiotics tested (imipenem, cefoxitin, trimethoprim-sulphamethoxazole and augmentin) were examined. The adjusted P values for the significant resistance were determined to ascertain where the difference occurred specifically.

It was determined that significant resistance pattern occurred in *E. coli* ($p = 0.015$) and *E. cloacae* ($p = 0.000$) against cefoxitin and in *E. coli* ($p = 0.000$) against trimethoprim-sulphamethoxazole. There was also a significant intermediate antibacterial resistance in *M. morganii* against imipenem and cefoxitin at $p = 0.000$ and $p = 0.006$ respectively (Table 6).

Table 6: Proportion of *E. coli*, *K. pneumoniae*, *E. cloacae* and *M. morganii* isolates stratified by resistance to antibiotics with their adjusted P values (aP value) Antibiotics Resistance *E. coli* *K. pneumonia* *E. cloacae* *M. morganii* P value aP aP aP aP

	pattern	(N = 36)	(N = 6)	(N = 3)	(N = 3)		value E	value K	value EC	value M
Trimethoprim-sulphamethoxazole										
Imipenem	R	-	-	-	-		-	-	-	-
	I	-	-	-	1 (33.3%)		0.142	0.718	0.805	0.000
	S	36 (100%)	6 (1000%)	3 (100%)	2 (66.7%)		0.142	0.718	0.805	0.000
						0.030				
Cefoxitin	R	3 (8.3%)	-	3 (100%)	-		0.015	0.239	0.000	0.420
	I	1 (2.8%)	-	-	1 (33.3%)		0.580	0.606	-	0.006
	S	32 (88.9%)	6 (100%)	-	2 (66.7%)		0.012	0.183	-	0.580
						0.001				
	R	30 (83.3%)	2 (33.3%)	1 (33.3%)	2 (66.7%)		0.000	0.054	0.186	0.962
	I	-	-	-	-		-	-	-	-
	S	6 (16.7%)	4 (66.7%)	2 (66.7%)	1 (33.3%)		0.000	0.054	0.186	0.962

						0.023				
Augmentin	R	28 (77.8%)	6 (100%)	3 (100%)	3 (100%)		0.701	0.183	0.361	0.361
	I	-	-	-	-		0.142	-	0.805	0.805
	S	8 (22.2%)	-	-	-		0.364	-	0.390	0.390
						0.001				

Adjusted P values for E represent values for *E. coli*, K is *K. pneumoniae*, EC is *E. cloacae* and M is *M. morganii.* R means Resistant, I means Intermediate susceptibility and S means Susceptible

Socio-demographic characteristics of participants in relation to ESBL phenotype

The socio-demographic characteristics of the participants in relation to the production of ESBL was ascertained. Of the total of 53 isolates, ESBL prevalence was 8 which represents 15.1%. The average age of the patients infected with ESBL was 53.00 ± 21.19 whereas that of non-ESBL infected patients was 48.73 ± 16.91. The difference between the groups was not statistically significant (p = 0.533). A high proportion of the patients infected with ESBL were females 5 (62.5%) whereas a greater proportion of the nonESBL infected patients were males 33 (73.3%). The relationship between gender and ESBL phenotype was significant (p = 0.045). There was however no significant (p = 0.575) relationship between the treatment stage and ESBL phenotype (Table 7). Most of the ESBL producing Enterobacteriaceae were obtained from Manya Krobo General Hospital (62.5%). There was no significant relationship between the sample collection site and ESBL genotype (p = 0.182). This suggests that the sample collection site does not influence the ESBL genotype of isolates.

Table 7: Socio-demographic characteristics of participants in relation to ESBL phenotype

Variable	ESBL (N = 8)	Non-ESBL (N = 45)	Total (N = 53)	P value
Age	53.00±21.19	48.73±16.91		0.533
Gender				
Male	3 (37.5%)	33 (73.3%)	36(67.9%)	
Female	5 (62.5%)	12 (26.7%)	17(32.1%)	
				0.045
Treatment stage First stage	4 (50.0%)	14 (31.1%)	18(34.0%)	

Second stage	2 (25%)	17 (37.8%)	19(35.8%)	
Third stage	2 (25%)	14 (31.1%)	16(30.2%)	
				0.575
Facility				
MKGH	5 (62.5%)	27 (60.0%)	32(60.4%)	
TTH	1 (12.5%)	15 (33.3%)	16(30.2%)	
TGH	2 (25.0%)	3 (6.7%)	5(9.4%)	
				0.182

Continuous data were presented as mean ± SD, categorical data presented as proportion. Continuous data were compared to each other using One-way ANOVA whilst categorical data compared to each other using Chi-square analysis.

ESBL production of the isolates as a phenotypic predictor of antimicrobial resistance

The antimicrobial resistance patterns of ESBL positive isolates and the nonESBL phenotypes were compared to ascertain whether or not ESBL production has an influence on the prevalence of antimicrobial resistance. Most of the ESBL isolates showed significant resistance against cefuroxime, cefotaxime, ceftazidime/avibactam, ceftazidime, Trimethoprim-sulphamethoxazole, nalidixic acid, ciprofloxacin and gentamycin at $p < 0.05$ (Table 8).

Meropenem	R	1(12.5%)	1(2.2%)		
	I	1(12.5%)	13(28.9%)	14(26.4%)	
	S	6(75.0%)	31(68.9%)	37(69.8%)	
					0.269
Ertapenem	R	1(12.5%)	1(2.2%)	2(3.8%)	
	I	1(12.5%)	-	1(1.9%)	
	S	6(75.0%)	44(97.8%)	50(94.3%)	
					0.019

Table 8: Antibiotic resistance in relation to ESBL phenotype

Antibiotics		ESBL (N = 8)	Non-ESBL (N = 53)	Total	P value
Imipenem	R	-	-	-	
	I	-	1(2.2%)	1(1.9%)	
	S	8(100%)	44(97.8%)	52(98.1%)	
					0.670
Dorepenem	R	-	-	-	
	I	1(12.5%)	1(2.2%)	2(3.8%)	
	S	7(87.5%)	44(97.8%)	51(96.2%)	
					0.160
Cefepime	R	4(50%)	7(15.6%)	11(20.8%)	
	I	-	4(8.9%)	4(7.5%)	
	S	4(50%)	34(75.6%)	38(71.7%)	
					0.034
Cefoxitin	R	2(25%)	7(15.6%)	9(17.0%)	
	I	-	2(4.4%)	2(3.8%)	
	S	6(75.0%)	31(68.9%)	42(79.2%)	
					0.693
Cefuroxime	R	8(100%)	4(8.9%)	12(22.6%)	
	I	-	4(8.9)	4(7.5%)	
	S	-	37(82.2%)	37(69.8%)	
					0.000
Cefotaxime	R	8(100%)	-	-	
	I	-	1(2.2%)	1 (1.9%)	
	S	-	44(97.8%)	44(83.0%)	
					0.000
Ceftazidime/ avibactam	R	4(50.0%)	-	4(7.5%)	
	I	1(12.5%)	1(2.2%)	2(3.8%)	
	S	3 (37.5%)	44 (97.8%)	47(88.7%)	
					0.000
Ceftazidime	R	8 (100%)	20(44.4%)	28(52.8%)	
	I	-	13(28.9%)	13(24.5%)	
	S	-	12(26.7%)	12(22.6%)	
					0.015
Trimethoprimsulphamethoxazole	R	8(100%)	28 (62.2%)	36(67.9%)	
	I	-	-	-	
	S	-	17 (37.8%)	17(32.1%)	

				0.035
Amoxicillin/ clavulanate	R 8(100%)	38 (84.4%)	46(86.8%)	
	I -	3(6.7%)	3(5.7%)	
	S -	4(8.9%)	4(7.5%)	
				0.488
Nalidixic acid	R 8(100%)	8(17.8%)	16(30.2%)	
	I -	-	-	
	S -	37(82.2%)	37(69.8%)	
				0.000
Ciprofloxacin	R 8(100%)	3(6.7%)	11(20.8%)	
	I -	1(2.2%)	1(1.9%)	
	S -	41(91.1%)	41(77.4%)	
				0.000
Gentamycin	R 3(37.5%)	3(6.7%)	6(11.3%)	
	I -	-	-	
	S 5(62.5%)	42(93.3%)	47(88.7%)	
				0.011
Augmentin	R 8(100%)	34(75.6%)	42(79.2%)	
	I -	1(2.2%)	1(1.9%)	
	S -	10(22.2%)	10(18.9%)	
				0.291
Tetracycline	R 8(100%)	33(73.4%)	41(77.4%)	
	I -	6(13.3%)	6(11.3%)	
	S -	6(13.3%)	6(11.3%)	
				0.252

Categorical data presented as proportion and compared to each other using Chi-square analysis.

Proportion of enterobacteria isolates stratified by ESBL phenotype and Genotype

ESBLs were phenotypically detected in low proportions in all the clinical isolates tested. ESBL production was detected in *E. coli* 7 (87.5%) and *E. cloacae* 1 (12.5%). ESBL genotypes were absent in almost all the isolates except for *Bla*TEM which was present in *E. coli* and *K. pneumoniae*. Also,

*Bla*SHV and *Bla*OXA-1 were present in *K. pneumoniae* and *E. coli* respectively. *Bla*CTX-M and *Bla*CTX-M9 were both detected in *E. coli*. Three (3) of the isolates had multiple genes, thus *Bla*OXA-1 and *Bla*TEM, and *Bla*OXA-1 and *Bla*CTX-M. The percentages of the genotypes that expressed *Bla*TEM, *Bla*SHV, *Bla*OXA-1, *Bla*CTX-M and *Bla*CTX-M9 were 37.7%, 1.9%, 9.4%, 7.5% and 2.8% respectively (Table 9).

Table 9: Proportion of Enterobacteria isolates stratified by ESBL phenotype and genotype

Isolates	*E. coli* (N = 36)	*K. pneumonia* (N = 6)	*E. cloacae* (N = 3)
ESBL phenotype	7(87.5%)	0 (0.0%)	1(12.5%)
ESBL genotype			
TEM	17 (47.2%)	3 (50.0%)	0 (0.0%)
SHV	0 (0.0%)	1 (16.7%)	0 (0.0%)
OXA-1	5 (13.9%)	0 (0.0%)	0 (0.0%)
CTX-M	4 (11.1%)	0 (0.0%)	0 (0.0%)
CTX-M2	0 (0.0%)	0 (0.0%)	0 (0.0%)
CTX-M9	1 (2.8%)	0 (0.0%)	0 (0.0%)

Proportion of enterobacteria isolates stratified by ESBL phenotype and genotype in the selected hospitals

Low proportions of ESBLs were phenotypically detected in low proportions in all the clinical isolates tested from the selected hospitals. ESBL production was detected in isolates from Manya Krobo Hospital 5 (62.5%), Temale Teaching Hospital 1 (12.5%) and Tema General Hospital 2 (25%). ESBL genotypes were absent in almost all the isolates from the selected hospitals except for *Bla*TEM which was present in Manya Krobo Hospital 12 (60.0%), Temale Teaching Hospital 7 (35.0%) and Tema General Hospital 1 (5.0%). Also, *Bla*SHV was present in clinical isolates from Manya Krobo Hospital. *Bla*OXA-1 was detected in isolates from both Manya Krobo Hospital and Temale Teaching Hospital. *Bla*CTX-M and *Bla*CTX-M9 were both

detected in isolates from Manya Krobo Hospital (Table 10).

Table 10: Proportion of Enterobacteria isolates stratified by ESBL phenotype and genotype in the selected hospitals

Isolates	Manya Krobo Hospital	Temale Teaching Hospital	Tema General Hospital
ESBL phenotype	5 (62.5%)	1 (12.5%)	2(25.0%)
ESBL genotype			
TEM	12 (60.0%)	7 (35.0%)	1 (5.0%)
SHV	0 (0.0 %)	1 (100%)	0 (0.0%)
OXA-1	3 (60.0%)	2 (40.0%)	0 (0.0%)
CTX-M	4 (100%)	0 (0.0%)	0 (0.0%)
CTX-M2	0 (0.0%)	0 (0.0%)	0 (0.0%)
CTX-M9	1 (100%)	0 (0.0%)	0 (0.0%)

Discussion

Antibiotic resistance surveillance has a major role among all strategies to manage the problem of antibiotic resistance. Since their first description in the mid-1970s, ESBLs have been isolated worldwide and form a major contributor of drug resistance in many genera of Entererobacteriaceae (Jayapradha *et al.*, 2007). Amongst antibiotics, ß-lactams are the safest and the most widely used antibiotics to date. The extended spectrum ß-lactams are commonly used empirically for the treatment of Gram-negative sepsis. But the emergence of ESBL producing organisms has posed a serious threat for their continuing use. Mortality, morbidity and cost of treatment have considerably risen because of these resistant isolates (Al-mussawi, 2018).

This study showed a prevalence of ESBLs among the isolates obtained from the archived samples from the selected health facilities. Greater portion of the samples were obtained from Manya Krobo Government Hospital. Although susceptibility was seen in some antibiotics especially the Carbapenems (Meropenem, Ertapenem, Dorepenem and Imipenem), resistance was seen in some of the isolates against Trimethoprim-sulphamethoxazole (83.3%), Augmentin (77.8%) and tetratcycline (83.4%). These are drugs that have been reported as having high percentage resistance for a number of microorganisms for several years. The rate of resistance had been rising over the years, not only for clinical isolates but also for the normal intestinal flora of the healthy population (Newman *et al*, 2011). Although antibiotic resistance occurs in nature and is an inevitable consequence of prudent antibiotic use, overuse and misuse of antibiotics is the main determinant for the increases in antibiotic resistance (Bertrand and Hocquet, 2011). Also, anthropogenic factors such as farmers

employing antibiotics in animal production has been recorded in many developing countries including Ghana. Moreover, the prevalence of antibiotic resistance has been attributed to over-prescription of antibiotics, patients not finishing the entire antibiotic course, overuse of antibiotics in livestock and fish farming, poor infection control in health care settings, poor hygiene and sanitation and absence of new antibiotics being discovered. This prevalence was demonstrated in *E. coli,* showing (83.4%), *K. pneumoniae* (100%) *E. cloacae* (100%) and *M. morganii* (100%).

The increase in resistance to these antibiotics indicates that their abuse is also on the increase, hence control measures need to be put in place since they are major choices for the treatment of infections in most health facilities in Ghana. Comparison of the resistance pattern between the four (4) common organisms; *E. coli, K. pneumoniae, E. cloacae* and *M. morganii* showed a significant difference in the antimicrobial resistance. An adjusted P. value was calculated between the resistance, intermediate and sensitive patterns in order to ascertain where the specific significance occurred. It was observed that most of the significant resistance occurred in *Escherichia coli*. Due to the multifaceted nature of these organisms, serious infection prevention and control practices must be encouraged in the various health facilities.

A high proportion (35.8%) of isolates were obtained from samples from TB patients in the second stage of treatment. Of the patients who were infected with ESBL, 4 (50%) were in the first stage of treatment, 2 (25%) were in the second treatment stage and 2 (25%) were in the third stage of treatment. This may suggest that ESBL producing Enterobacteriaceae is more prevalent among

TB patients in the first stage of treatment, probably because most of the first line treatments used are no more effective.

ESBLs were phenotypically detected in *Escherichia coli* (87.5%) and *Enterobacter cloacae* (12.5%). This higher prevalence in *E. coli* could be due to the rectal samples obtained since this organism is the commonest cause of diarrhoea. In a genotypic detection, TEM, SHV and CTX-M were identified in 37.7% of the isolates identified. ESBL was more prevalent at Manya Krobo General Hospital (62.5%) as compared to other sampling sites. The percentages of the genotypes that expressed *Bla*TEM, *Bla*SHV and *Bla*OXA-1 were 37.7%, 1.9% and 9.4% respectively. The overall prevalence of ESBL detected was 15.1 %. Comparing this value to previous works done in Ghana, it has been reported that the overall prevalence of ESBL at Korle-Bu Teaching Hospital in Accra was 49.3% (Obeng-nkrumah *et al.*, 2013) which suggested that ESBL-producing Enterobacteriaceae are significant causes of infections and antibiotic resistance at Korle-Bu Teaching Hospital. Comparing this prevalence with this study, there seem to be a low rate of drug resistance in those tertiary hospitals. But there is still a need for future surveillance studies because treatment of these multi-drug resistant organisms still remains a therapeutic challenge. The prevalence of ESBL call for immediate intervention strategies to prevent further spread.

Summary

This study set out to ascertain the prevalence of ESBL among TB patients in some selected hospitals in Ghana. ESBL phenotypes were detected in *E. coli* and *E. cloacae*. *Bla*TEM genotype was present in *E. coli* and *K. pneumoniae.*

*Bla*SHV and *Bla*OXA-1 genotypes were also detected in *K. pneumoniae* and *E. coli* respectively. The prevalence of ESBL was high in *E. coli* (87.5%). ESBL was more prevalent at Manya Krobo General Hospital (62.5%). The overall ESBL prevalence was 15.1%.

CHAPTER FIVE: SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

This study has brought the prevalence of ESBLs in Manya Krobo General Hospital, Tamale Teaching Hospital and Tema General Hospital to bare. This research has indicated the antimicrobial resistance pattern against commonly used antibiotics in the selected hospitals.

Conclusion

This research work identified a high rate of antimicrobial resistance amongst enterobacteria to antimicrobial drugs that are commonly prescribed at Manya Krobo Government Hospital, Tamale Teaching Hospital and Tema General Hospital. The occurrence of the resistance may be as a result of the production of ESBL by the isolates obtained. There was however no significant difference between prevalence of ESBL and the various facilities. The ESBL genes (*Bla*TEM, *Bla*SHV and *Bla*OXA-1) were all detected in the isolates. The overall prevalence of ESBL was 15.1%.

The high levels of antimicrobial resistance and the widespread prevalence of ESBL producing enterobacteria highlights the necessity to adopt immediate intervention strategies to prevent severe healthcare associated infections in the hospital and to continuously monitor ESBL spread in the community.

Recommendations

The following are recommended.

1. Health professionals should be trained and provided with refresher courses on the accurate, effective diagnosis and treatment of ESBLs.

2. Screening of ESBLs and carbapenem resistance should be incorporated in routine laboratory analysis.
3. Agricultural sector should also put measures in place to control the use of antibiotics by farmer.
4. People should avoid buying drugs that are not prescribed by a qualified medical practitioner.

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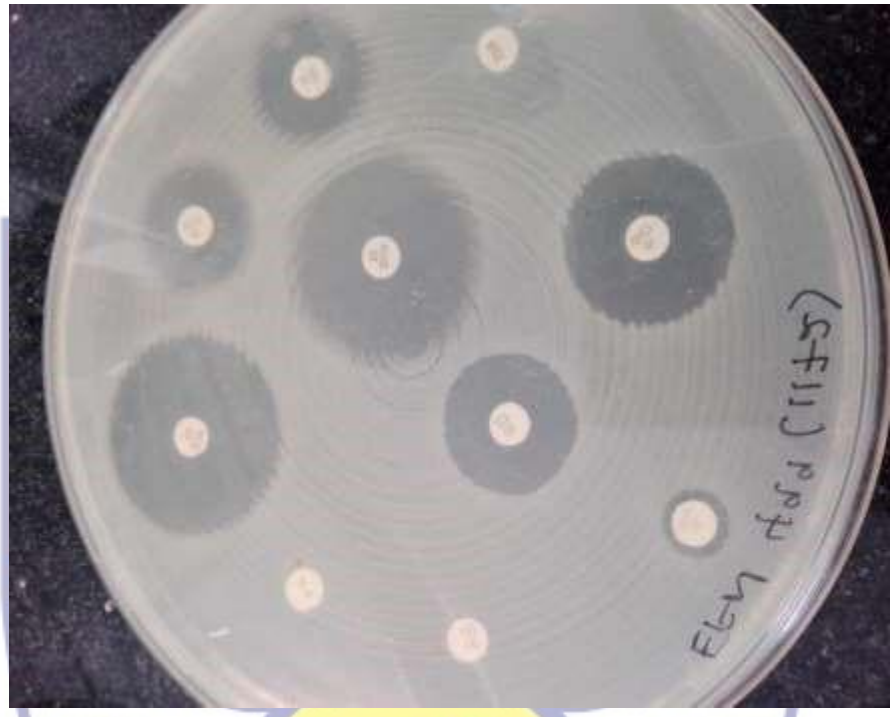
APPENDICES

Appendix 1: PFGE results for ESBL genes amplified



Appendix 2: Double Disc Synergy Test (DDST)