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COLLEGE OF HEALTH SCIENCES

EXTENDED SPECTRUM BETA-LACTAMASE IN CLINICAL ISOLATES OF *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* FROM THE TAMALE TEACHING HOSPITAL

BY

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INTEGRI PROCEDAMUS

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DECLARATION

This is to certify that this thesis is the result of research undertaken by Francis Kwame Morgan Tetteh towards the award of the Masters of Philosophy in Microbiology in the Department of Medical Microbiology, School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana. References to the works of other investigators have been duly acknowledged.

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ABSTRACT

BACKGROUND: Extended Spectrum Beta-Lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* are pathogens of significant public health interest to which new antibiotics therapies are urgently needed.

AIM: This study was designed to determine the prevalence of ESBLs in clinical isolates of *E. coli* and *K. pneumoniae* from patients attending the Tamale Teaching Hospital (TTH).

METHODOLOGY: The study was conducted from April through June, 2015. A total of 140 isolates of *E. coli* (83.6%; n=117) and *K. pneumoniae* (16.4%; n=23) were cultured from clinical specimens of consenting patients. Antimicrobial susceptibility were determined using the Kirby-Bauer disc diffusion method. Screening and confirmation of ESBL-producing phenotypes among the clinical isolates were performed according to the guidelines of the Clinical and Laboratory Standard Institute (CLSI, 2012). *Escherichia coli* and *K. pneumoniae* positive for ESBL phenotype were examined for the presence of TEM, SHV and CTX-M genes.

RESULTS: Sixty two (44.3%) of the 140 isolates phenotypically expressed ESBLs. Of these, 83.9% (n=52) were *E. coli* and 16.1% (n=10) were *K. pneumoniae* isolates. The proportion of ESBL-producing isolates were found to be relatively higher in adults (15-65 years) than in neonates (< 28 days) (p=0.14). Majority of the isolates showed high percentage resistance to ampicillin (96%) and tetracycline (89%), but relatively low for amikacin (36%). None of the isolates were resistant to meropenem. Extended Spectrum Beta-Lactamase (ESBL)-producers were multidrug resistant compared to non-ESBL-producers (23%, n=14/62 versus 18% n=14/78; p=0.573). Overall, 74.2% (n=46/62) of the ESBL genotypes expressed $Bla_{\text{CTX-M-1}}$ genes followed by 62.9% (n=39/62) Bla_{TEM}

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and 16.1% (n=10/62) *Bla*$_{SHV}$. Two (3.2%) isolates had both TEM and SHV genes, 29 (46.8%) harboured TEM and CTX-M-1, 2 (3.2%) had SHV and CTXM-1, while 4 (6.5%) harboured all three genes. None expressed genes for CTX-M 2 and CTX-M 9. In univariate comparisons, patients who reported their previous medication as having been prescribed by a Physician and those who reportedly completed their previous medication were more likely to be infected by ESBL organisms.

CONCLUSION: The study showed high ESBL positive *E. coli* and *K. pneumoniae*, mostly CTX-M-1 producers in Tamale Teaching Hospital. Routine laboratory ESBL detection is warranted.

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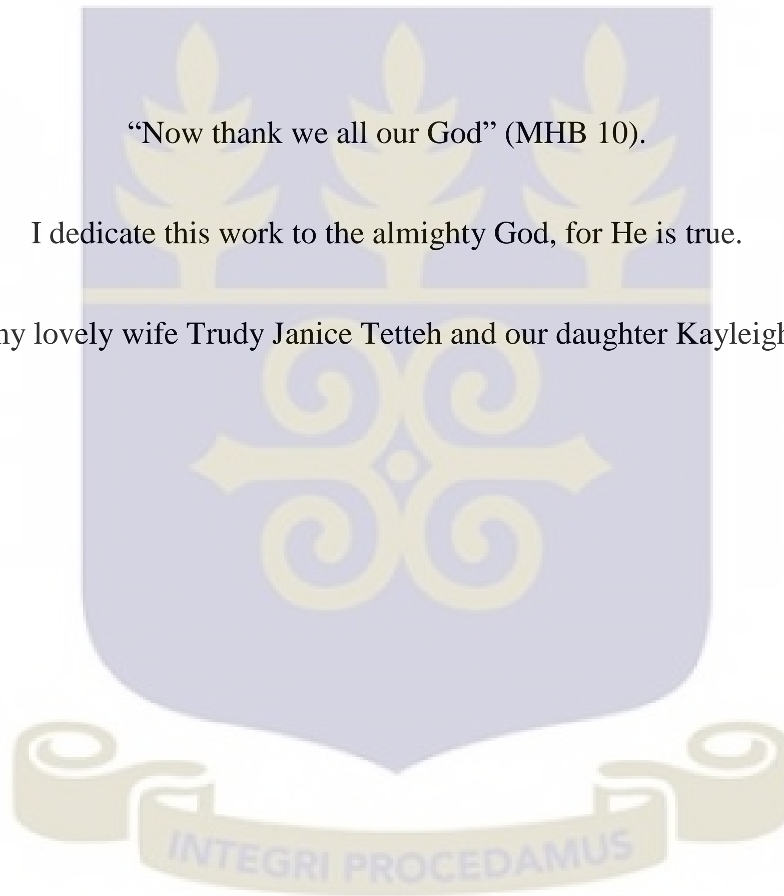
DEDICATION



"Now thank we all our God" (MHB 10).

I dedicate this work to the almighty God, for He is true.

Also to my lovely wife Trudy Janice Tetteh and our daughter Kayleigh Tetteh.

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"Gracious God, to Thee we raise, this our sacrifice of praise" (MHB 35). I ascribe all thanks and praise to the Almighty God for seeing me through the period of study. It was not easy but it was worth it.

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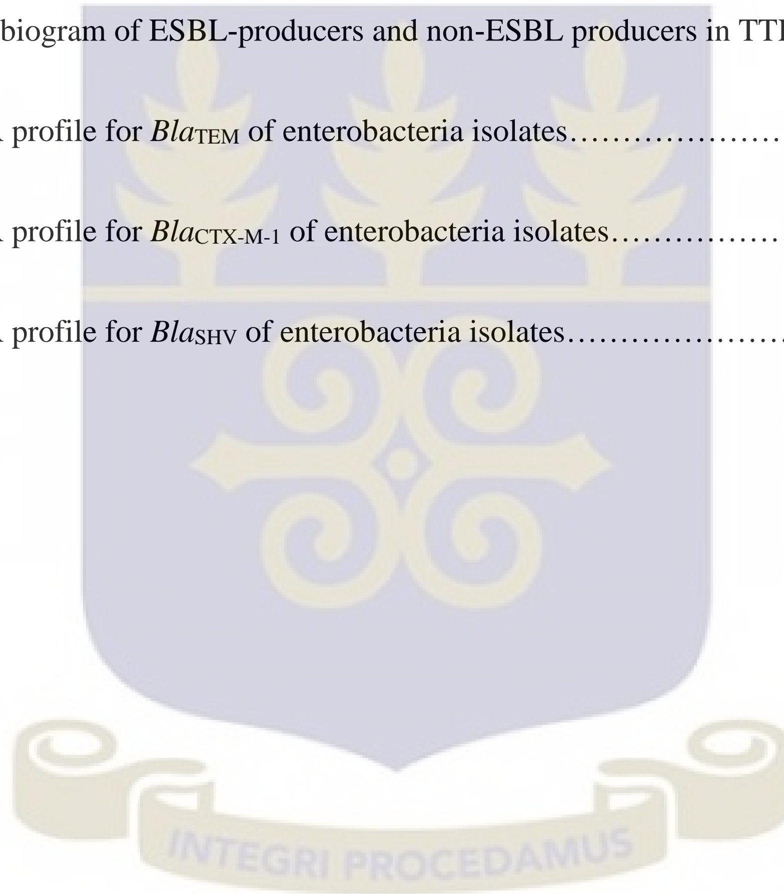

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LIST OF ABBREVIATIONS

AmpC – Ampillicin

API – Analytical Profile Index

AST – Antimicrobial Susceptibility Test

ATCC – American Type Culture Collection

BA – Blood Agar

Bla$_{CTX-M}$ – Beta Lactam Cefotaximase

Bla$_{TEM}$ - Beta Lactam Temoneira

Bla$_{SHV}$ - Beta Lactam Sufhydryl variable

CLSI – Clinical and Laboratory Standard Institute

cDNA - Complementary Deoxyribonucleic Acid

CTX-M - Cefotaximase

ESBL – Extended-Spectrum Beta Lactamase

HPA – Health Protection Agency

ID – Identification

KATH – Komfo Anokye Teaching Hospital

KBTH – Korle Bu Teaching Hospital

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MHA – Mueller Hinton Agar

MIC – Minimum Inhibition Concentration

µg - Microgram

µl - Microliter

NCTC- National Collection of Type Cultures

NMIMR – Noguchi Memorial Institute of Medical Research

OXA - Oxacillin

PBS – Phosphate Buffered Saline

SHV – Sufhydryl variable

SPSS - Statistical Package for the Social Scientists

TEM - Temoneira

TTH – Tamale Teaching Hospital

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CHAPTER ONE

INTRODUCTION

1.0 BACKGROUND

Beta-lactamases are bacterial enzymes that inactivates beta-lactam antibiotics by hydrolysis (Livermore, 2003). Among the groups of β-lactamase enzymes, Extended Spectrum β-Lactamases (ESBLs), Class C Cephalosporinases (AmpCs) and Carbapenemases constitute the chief resistant mechanisms against β-lactam antibiotics. Of these, ESBLs are the commonest source of resistance to the β-lactams (Jacoby and Munoz-Price, 2005). Extended Spectrum Beta-Lactamases have the ability to hydrolyze and cause resistance to Penicillins, Cephalosporins and Monobactams but not Cephamycins or Carbapenems (Pitout and Laupland, 2008).

The spread of bacterial resistance is mainly mediated by plasmids as they can be transferred between Gram negative bacteria by conjugation (Thomson and Smith, 2000). This transferability is responsible for outbreaks of resistance (Dbaibo, 1999; Sarma and Ahmed, 2010). Extended Spectrum Beta-Lactamases include the widespread plasmid-encoded enzyme families and their variants: Temoniera (TEM) (Shah *et al*., 2004), Sulfhydryl variable (SHV), and Oxacillinases (OXA) (Sarma and Ahmed, 2010). In recent years, many ESBLs of non-TEM, non-SHV and non-OXA types especially the cefotaximase (CTX-M) have been detected and reported worldwide (Sarma and Ahmed, 2010). The CTX-M type ESBL spreads rapidly and is now one of the most dominant types of ESBLs in many countries (Livermore *et al*., 2007; Lee *et al*., 2009).

Escherichia coli and *K. pneumoniae* remain the major ESBL-producing organisms isolated worldwide but the enzymes have also been identified in several other members of the

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Enterobacteriaceae and in some non-fermenting Gram negative bacteria (Jacoby and Munoz-Price, 2005) like *Pseudomonas* sp. and *Proteus mirabilis*.

Extended Spectrum Beta-Lactamase-producing enterobacteria have spread across the world but some Health Authorities are not aware of this problem especially in African countries (Pitout *et al*., 2004). In other African countries, the issue of routine detection remains a contentious issue owing to the huge financial demands involved (Pitout *et al*., 2004). Most clinical laboratories in Ghana do not routinely screen for ESBLs (Adu-Sarkodie, 2010). It is therefore imperative to heighten awareness among clinicians and policy-makers, and enhance testing by clinical laboratories, for the detection of ESBLs. This will improve patient management. It will also help determine the extent of the ESBL problem and inform appropriate interventions needed for the control of the spread of ESBL-producing bacteria (Pfaller and Segreti, 2006).

1.1 Problem Statement

Dissemination of ESBL-producers. An increasing number of antimicrobial classes are becoming ineffective against a significant number of ESBL-producing organisms. Across the United States and several areas in Europe, this phenomenon has been observed and is now being observed across countries in Africa (Paterson and Bonomo, 2005). Of greatest concern are reports from hospitals in Nigeria confirming high levels of ESBL-associated resistance to non-β-lactam antibiotics including ciprofloxacin (Kesah and Odugbemi, 2002; Aibinu *et al*., 2003).

Limited therapeutic options. The presence of Extended Spectrum β-Lactamases (ESBLs) in enterobacteria renders β-lactam antimicrobials ineffective, including extended spectrum cephalosporins, necessitating the wider usage of non-β-lactam agents including ciprofloxacin and

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amikacin in the treatment of serious infections caused by these pathogens. Nevertheless ESBL presence may be associated with a phenomenon of antimicrobial coresistance, to both β-lactam and non-β-lactam antibiotics (Luzzaro *et al*., 2006), significantly reducing therapeutic options available for treatment. Consequently, the duration of hospital stays, cost and mortality rates also increases by over 40% (Tumbarello *et al*., 2007).

Absence of ESBL data in Northern Ghana. Documented surveys exist on the resistance of *Enterobacteriaceae* in Korle-Bu Teaching Hospital (KBTH) (Newman *et al*., 2004) and Komfo-Anokye Teaching Hospital (KATH) (Feglo *et al*., 2013) to extended-spectrum cephalosporins and other non-β-lactam antibiotics. There are, however, no published reports on ESBL-producing isolates from the Tamale Teaching Hospital (TTH), which provides services to the three Northern regions and neighboring towns. Whereas many laboratories may not be fully aware of the importance of ESBL-producing enterobacteria, others may lack the ability to correctly identify and report these organisms. The absence of routine surveillance and laboratory detection of ESBLs in many clinical laboratories in Africa, and Ghana in particular, further compounds the ESBL-problem.

1.2 Justification

In view of absence of data from the Northern part of Ghana, it is imperative to investigate the contribution of ESBLs to antimicrobial resistance in *E. coli* and *K. pneumoniae* in Tamale Teaching Hospital. 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

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ESBL laboratory detection. It will also buttress hospital surveillance programs for drug resistant bacteria.

1.3 Hypothesis

High levels of ESBL exist among *E. coli* and *K. pneumoniae* of Tamale Teaching Hospital.

1.4 Aim of the Study

The overall objective of the study was to determine the prevalence of ESBLs in clinical isolates of *E. coli* and *K. pneumoniae* from patients attending Tamale Teaching Hospital.

1.5 Specific Objectives of the Study

1. To determine the prevalence of ESBL-producing *E. coli* and *K. pneumoniae* associated with clinical infections at the TTH.
2. To compare the antibiogram of ESBL-producing and non-ESBL-producing *E. coli* and *K. pneumoniae*.
3. To determine the presence of TEM, SHV and CTX-M genes among *E. coli* and *K. pneumoniae* isolates that phenotypically expressed ESBL.

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CHAPTER TWO

LITERATURE REVIEW

2.0 Epidemiological Prevalence of ESBL

Beta-lactam antibiotics are responsible for the inhibition of bacterial cell wall biosynthesis (Liras and Martin, 2006). This activity is achieved **by** two mechanisms targeting the inhibition of cell wall synthesis (Samaha-Kfoury and Araj, 2003). For the first action, antibiotics are incorporated into the bacterial cell wall, to inhibit the action of the transpeptidase enzymes responsible for the completion of the cell wall. The second action involves, antibiotics attachment to the penicillin binding proteins that normally suppress cell hydrolases, allowing the hydrolases free to act and lyse the bacterial cell wall (Samaha-Kfoury and Araj, 2003). In an attempt to survive, bacteria show resistance by bypassing these mechanisms of antimicrobial action, via production of β-lactam inactivating enzymes such as the beta-lactamases. Beta-lactam antibiotics are used all over the world, however, the distribution of the enzymes responsible for resistance to oxyimino-cephalosporins varies (Miro *et al*., 2005; Nordmann *et al*., 2009).

Beta-lactamases are bacterial enzymes that inactivate beta-lactam antibiotics by hydrolysis (Livermore and Woodford, 2006; Bush and Jacoby, 2010). Among the groups of beta-lactamase enzymes, Extended Spectrum β-Lactamases (ESBLs), Class C Cephalosporinases (AmpCs) and Carbapenemases constitute the main resistant mechanisms against beta-lactam antibiotics. Of these, ESBLs are the commonest source of resistance to the beta-lactams (Jacoby and Munoz-Price, 2005). Extended Spectrum Beta-Lactamases have the ability to hydrolyze and cause resistance to Penicillins, Cephalosporins and Monobactams but not Cephamycins or Carbapenems (Pitout and

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Laupland, 2008). Extended Spectrum Beta-Lactamases have been reported especially in *K. pneumoniae, K. oxytoca* and *E. coli*, (Apisarnthanarak *et al*., 2007; Coque *et al*., 2008). Extended Spectrum Beta-Lactamases have also been reported in *Citrobacter*, *Enterobacter, Proteus, Serratia* and other genera of enteric organisms (Chaudhary and Aggarwal, 2004; Blomberg *et al*., 2005; Apisarnthanarak *et al*., 2007; Canton *et al*., 2008; Coque *et al*., 2008) and non-enteric organisms such as *Acinetobacter baumanii* (Song *et al*., 2006) and *Pseudomonas aeruginosa* (Weldhagen *et al*., 2003).

Extended Spectrum Beta-Lactamase prevalence is higher in isolates from Intensive Care Unit (ICU) compared to isolates from other units or wards within the hospital settings (Jacoby and Munoz-Price, 2005; Rodriguez-Villalobos *et al*., 2011). The problem of ESBLs evolved in the 1960s (Nordmann and Guibert, 1998) from Western Europe, where Extended spectrum beta-lactam drugs were first used. Currently ESBL prevalence amongst clinical isolates within institutions and from country to country varies. Several studies have examined the incidence of ESBL in Europe, South America and Southeast Asia but only a few have studied isolates from Africa (Obeng-Nkrumah *et al*., 2013). Among the *Enterobacteriaceae* group, ESBL production ranges from 0 to 25% within institutions in the United States, with a national average of 3% (http://www.cdc.gov/ncidod/hip/SURVEILL/NNIS.HTM). The prevalence of ESBL varies greatly from country to country across Europe with relatively low occurrences of 3% and 1% in Sweden and the Netherlands respectively (Rodriguez-Villalobos *et al*., 2011), to as high as 42% in an intensive care unit in France (Branger *et al*., 1998). Extended Spectrum β-Lactamase production in Asia varies from 0.4% in Japan (Yagi *et al*., 2000), 4.8% in Korea (Pai *et al*., 1999) and 12% in Hong Kong (Ho *et al*., 2000). In the 1990s, ESBL types reported were chiefly TEM and SHV beta-lactamase families usually found in *Klebsiella pneumoniae* during nosocomial outbreaks (Coque *et*

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al., 2008), however, the CTX-M ESBL commonly found in *E. coli* isolates from community-acquired infections are increasingly being reported (Coque *et al*., 2008). This phenomenon has been attributed to dissemination of specific clones or clonal groups and epidemic plasmids in community and nosocomial settings. The most widespread ESBL reported in increasing order belong to the TEM (TEM-24, TEM-4, TEM-52); SHV (SHV-5, SHV-12) and CTX-M (CTX-M-9, CTX-M-3, CTX-M-14, CTX-M-15) families in Europe (Coque *et al*., 2008).

In Africa, there are reports of routine ESBL surveillance programmes in South Africa with several excellent reviews averaging low prevalence rates of less than 3% (Bell *et al*., 2002). Extended Spectrum Beta-Lactamases have been reported in Tunisia (Ben-Hamouda *et al*., 2004), Morocco (AitMhand *et al*., 2002), Egypt (Shannon *et al*., 1990) and Kenya (Kariuki *et al*., 2001). In West Africa, ESBLs have been reported in Nigeria (Kesah and Odugbemi, 2002; Aibinu *et al*., 2003), however, no surveillance and epidemiological data exist in these countries including Ghana (Obeng-Nkrumah *et al*., 2013). This increasing prevalence of ESBLs on the African continent has grave consequences on the already strained healthcare systems. Albeit treatment of infection with ESBL-producing bacteria remains difficult in high-income countries, the situation is formidable in low-income countries where expensive second-line drugs are unavailable and microbiological services are accessible only in few referral hospitals. It is therefore important that the emergence of such a phenomenon be carefully monitored and eventually contained.

2.1 Reports of Multidrug Resistant ESBL-Producing *Enterobacteriaceae*

In recent years, some studies have assessed the occurrence of ESBL induced multidrug resistance in clinical isolates of *Enterobacteriaceae*. In 2001, the result of a nationwide survey in Italy indicated that 6.3% of all clinical isolates harboured ESBL genes with an average antimicrobial coresistance

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of 25% to all non-β-lactam drugs tested (Spanu *et al*., 2002). On the contrary, a study in Israel involving a Jewish population in 2004 revealed that the overall prevalence of ESBL was 20% with an alarming high antimicrobial coresistance from a low of 40% to piperacillin-tazobactam to a high of 75% for gentamicin (Schwaber *et al*., 2004). This result was in agreement with a study in Tanzania, reporting extreme levels of coresistance of 90.9% to trimethoprim-sulphurmethoxazole and amikacin (Ndugulile *et al*., 2005) and also in Nigeria where a coresistance of 89% to streptomycin, 100% to gentamicin and trimethoprim-sulphurmethoxazole were reported (Aibinu *et al*., 2003).

There are no published reports on ESBL-producing isolates and the extent of antimicrobial coresistance conferred by these enzymes in Tamale Teaching Hospital (TTH). Therefore, there is a growing need for a study, particularly in tertiary and referral healthcare facilities like TTH, which may harbour many different microorganisms, to confirm the existence of ESBL-producing *Enterobateriaceae* associated with coresistance.

2.2 Contributory Factors to Multidrug Resistance Problem

A major contributing factor to possible occurrence of this phenomenon of coresistance is the use of sub-therapeutic antibiotics both prescribed and unprescribed (Emery and Weymouth, 1997). Of greater concern, is the apparent non-performance of routine laboratory screening for ESBL-producing *Enterobacteriaceae* to provide clinicians with reliable therapeutic options in many health communities (Tenover *et al*., 1999). Besides, there are not much data on comprehensive antibiotic susceptibility patterns for potentially useful beta-lactam and non-beta-lactam antibiotics in most countries in the sub-Saharan region. These could lead to inappropriate administration of antibiotics for empirical treatment, leading to the selection of species that may have naturally developed

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resistance to these drugs. These multiple-resistant Gram negative bacilli or rods may survive even on the finger tips, adding to their spread (Casewell and Desai, 1983). In addition to hand carriage, institutional and bacterial properties that contribute to both epidemic and endemic spread are not fully understood (Tullus *et al*., 1988), despite known risk factors such as length of hospital stay, severity of illness, time in the intensive care units (ICU) and gastric incubations (Pena *et al*., 1997). The consequences span from several treatment failures to outbreaks of multidrug resistance, which requires expensive control efforts. The problem of multidrug resistance are increasing and becoming more complex.

Some investigators have determined that animals and food vegetables might represent possible source of genes encoding ESBL to humans. There is evidence of ESBL-producing isolates in poultry (Weill *et al*., 2004), cattle (Shiraki *et al*., 2004), dogs and cats (Carattoli *et al*., 2005) and these might act as reservoirs for spread of resistant isolates of *Enterobacteriaceae*. Some workers, including Shiraki *et al*., (2004) have hypothesized that some types of ESBLs (CTX-M-2) emerged initially from cattle with subsequent transmission to humans.

As resistant plasmids are the major source of ESBL transmission (Harris *et al*., 2007), it has been postulated that ESBL-producing *Enterobacteriaceae* possess transferable elements that travel alongside the ESBL-containing plasmids, conferring resistance to other antimicrobial classes yielding multidrug resistance traits. It is possible that resistance factors including mutators and integrons may contribute to this possible emerging phenomenon of high antibiotic coresistance (Chopra *et al*., 2003; Gruteke *et al*., 2003).

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2.3 General Mechanisms of Antimicrobial Resistance

The general mechanisms of antimicrobial resistance include: altering the receptor for the drug; decreasing the amount of the drug that reaches the receptor by altering entry or increasing removal of the drug; destroying or inactivating the drug and developing resistant metabolic pathways. Bacteria can possess one or all of these resistant mechanisms simultaneously (Kern *et al*., 2008; Vollmer & Bertsche, 2008). A typical organism like *Pseudomonas aeruginosa* possesses an active efflux pump system that can reduce the intracellular accumulation of antibiotics and allow an enzyme with only limited hydrolytic capacity to inactivate the drug before reaching the target. For other organisms, there are diminished expression of an outer-membrane porin required for beta-lactam uptake. In *Klebsiella pneumoniae,* decreased expression of outer-membrane porins often accompanies ESBL production and may allow a TEM or SHV-type ESBL to express resistance to cefepime (Bradford *et al*., 1997; Martinez-Martinez *et al*., 1999).

The production of β-lactamase enzymes is the main resistance mechanism against β-lactam drugs that inhibits the drugs from binding to the penicillin-binding proteins (James *et al*., 2009). Introduction of these antibiotics in any population can result in the evolution of bacterial strains capable of producing new penicillin-binding proteins, to which no β-lactam antibiotic can bind. The main mechanism is the production of enzymes called penicillinase, which has the ability to attack other β-lactam drugs such as the cephalosporins, carbarpenems and monobactams, and so they are most appropriately called β-lactamases (Rastogi, 2010). The most important activity of these β-lactamase enzymes is the alteration of the β-lactam ring of the drug (Samaha-Kfoury and Araj, 2003). The introduction of newer classes of β-lactam antibiotics such as the third generation cephalosporins and aztreonam and their widespread use, have co-evolved with the new enzymes that

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have led to the Extended Spectrum Beta-lactamases among the *Enterobacteriaceae* (Thomson and Smith, 2000; Samaha-Kfoury and Araj, 2003). These enzymes have an extended substrate among the cephalosporin and monobactam antibiotics together with the ability to confer resistance to other non-β-lactam antibiotics and so these enzymes were labeled Extended Spectrum β-lactamases, thus ESBLs (Chaudhary **and** Aggarwal, 2004). The spread of bacterial resistance is most often by plasmids. These are transferred between Gram positive and Gram negative bacteria by transducing phages and conjugation respectively (Thompson and Smith, 2000). This mechanism of transferability is primarily responsible for outbreaks of resistance (Dbaibo, 1999; Sarma and Ahmed, 2010).

2.4 Laboratory Detection of ESBL Phenotypes

Currently, the recommended procedure for ESBL-producing *E. coli* and *K. pneumoniae* detection by the Clinical and Laboratory Standards Institute (CLSI, 2012) involves an initial disk-diffusion or broth dilution screening test with one or more oxyimino-β-lactams. Confirmatory test follows, to measure the susceptibilities to cefotaxime (CTX), ceftazidime (CAZ) or cefpodoxime (CPD) alone and in combination with an ESBL inhibitor, usually clavulanic acid (CA) (Jacoby and Munoz-Price, 2005). Other ESBL inhibitors include sulbactam and tazobactam. Aside these recommended tests for detecting ESBLs in enteric bacteria (especially *E. coli* and *K. pneumoniae*), there are no recommended test for detecting ESBLs in *Pseudomonas aeruginosa* (Jacoby and Munoz-Price, 2005).

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2.4.1 Screening Methods for Detecting ESBL Phenotypes

According to the Health Protection Agency (HPA) (2004), cefpodoxime or ceftazidime are the best third generation cephalosporin substrates for TEM and SHV-derived ESBLs, qualifying them to be included in the first line routine susceptibility testing of isolates. Cefotaxime is considered appropriate for CTX-M genes and it is also used as a first line routine susceptibility testing agent. Based on these recommendations, the British Society for Antimicrobial Chemotherapy (BSAC) developed and approved the zone sizes for the routine disc diffusion tests and Minimum Inhibition Concentration (MIC) breakpoints (mm) for the broth dilution method (Table 2.1) (Jonathan, 2005).

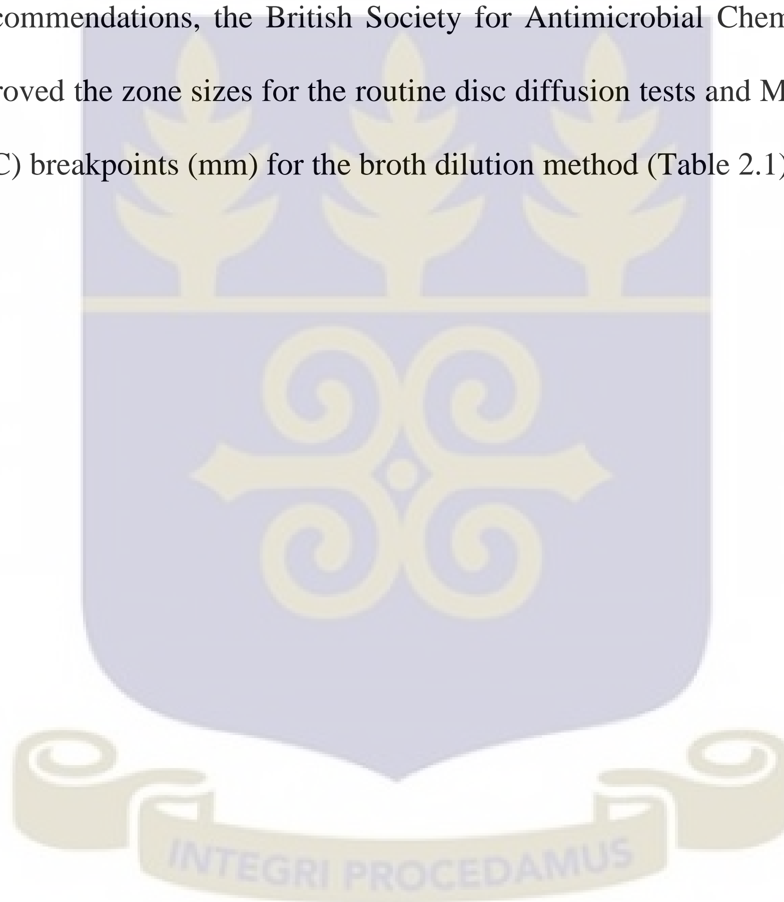

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Table 2.1 Recommended breakpoints for detecting ESBLs courtesy BSAC

Antibiotic/ dics contents	**Zone breakpoints (mm) for the disc diffusion test**		**MIC (mg/L) for broth dilution or E-test**	
	Resistant, ≤	**Susceptible, ≥**	**Resistant, ≥**	**Susceptible, ≤**
Cefotaxime, 30µg	29	30	1	1
Ceftazidime, 30µg (*E. coli* and *Klebsiella*)	21	22	2	2
Ceftazidime, 30µg (other species)	27	28	2	2
Cefpodoxime, 10µg	25	26	1	1

(Courtesy; HPA, 2004)

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The Clinical and Laboratory Standards Institute (CLSI) reviewed the BSAC methods for testing and recommended both disc diffusion and MIC methods for screening ESBL-producing *E. coli* and *Klebiella* as well as other coliforms (CLSI, 2012). Alternatively, CLSI suggested that laboratories that make use of the disc diffusion method for antimicrobial susceptibility testing during ESBL screening, must adopt the BSAC breakpoints for more than one testing antibiotic (cefotaxime, ceftazidime, cefpodoxime and ceftriaxone) (CLSI, 2012). The following general traits of cephalosporins for screening ESBL-producing isolates, were proposed by HPA in 2004 as guidelines, owing to the numerous test methods available and the confusion involved:

- TEM and SHV ESBL-producing enterobacteria are obviously resistant to ceftazidime (CAZ), with variable results when tested using cefotaxime (CTX).
- CTX-M ESBLs producers are obviously resistant to cefotaxime; with variable results to ceftazidime.
- All ESBL-producers, however, have obvious resistance to cefpodoxime (CPD), and so this antibiotic may be used as the drug of choice for the screening of ESBLs.
- Cefuroxime, cephalexin and cephradine are unreliable indicators. In any case the suspected organisms must be confirmed for ESBL production (HPA, 2004).

2.4.2 Confirmatory Test Methods for ESBL Phenotypes

Enterobacteriaceae suspected to be ESBL-producers, may be submitted to the **under listed** confirmatory tests. These tests **make** it possible to evaluate the inhibition of ESBL activity by the use of clavulanic acid. Examples of the confirmatory test methods include:

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2.4.2.1 Combined-Disc Diffusion Method

For the combined-disc diffusion method, cefotaxime or ceftazidime may be used alone as an indicator cephalosporins **or** in combination with clavulanic acid (CLSI, 2012). This method was proposed by both Oxoid and the BSAC for the detection of ESBL-producing *Enterobacteriaceae*. Using the same procedure, cefpodoxime (CPD) only as well as CPD in combination with clavulanic acid are placed at appreciable distance (about 30mm) apart on Mueller-Hinton agar (MHA) inoculated with the test organism and incubated aerobically at 37°C for 18 to 24 hours. The zones of inhibition for both discs (used alone and in combination with CA) are measured using a vernier caliper or measuring rule and compared. Between the two discs (single and combined disc), a difference of greater than 5mm increase in zone diameter is considered positive for ESBL production (Carter *et al*., 2000).

With regard to controls when performing confirmatory testing, *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 must be tested routinely (daily or weekly) or for every batch. For *K. pneumoniae* ATCC 700603, a difference of greater than 5mm increase in CAZ plus CA zone diameter and greater than 3mm increase in CTX plus CA zone diameter is considered positive. Likewise, for *E. coli* ATCC 25922, a difference of greater than 2mm increase in zone diameter of the cephalosporin agent tested alone verses its zone when tested in combination with clavulanic acid is considered positive.

Aside the combined-disc diffusion method described, there are several other test methods for confirming ESBL-producers. Few of these test methods are mentioned below but not exhaustive.

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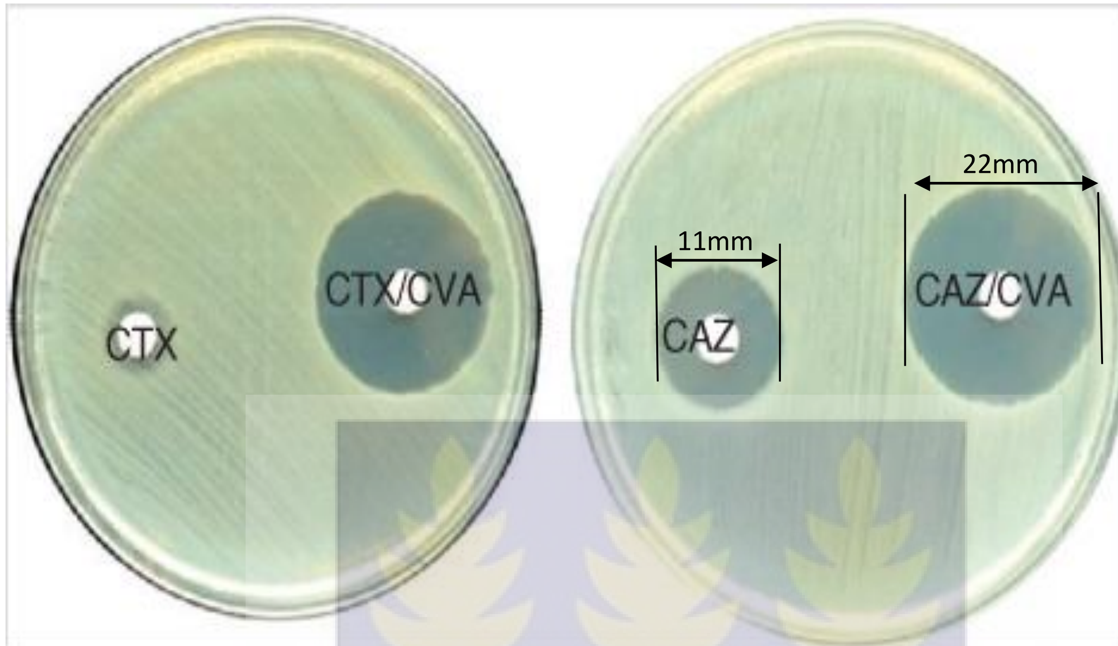

Figure 2.1 The Combined-Disc Diffusion Method

Zone size of combined disc (CAZ/CA) = 22mm, whereas that of ceftazidime alone (CAZ) = 11mm. Thus, 22mm – 11mm = > 5mm. Therefore, phenotypically the isolate is an ESBL-producer (Carter *et al*., 2000)

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2.4.2.2 Double-Disc Synergy Method

In this method, discs containing cephalosporins (cefotaxime, ceftriaxone, ceftazidime, cefepime) are applied next to a disc with clavulanic acid, amoxicillin plus clavulanic acid or ticarcillin plus clavulanic acid. Positive result is indicated when the inhibition zones around any of the cephalosporin discs are augmented in the direction of the disc containing clavulanic acid. The distance between the discs is crucial, such that 30mm center-to-center has been found to be optimal for 30µg cephalosporin discs; however it may be reduced to 25mm or expanded to 35mm for strains with very high or low resistance levels, respectively.

2.4.2.3 Vitek ESBL Test

This method is an automated test method for ESBL detection, produced by BioMeriex Vitek (bioMerieux Vitek, Inc. Hazelwood, Missouri). This method employs cephalosporin and cephalosporin inhibitors in wells on a card, for the detection of ESBLs, which can be determined within 4-15 hours (Livermore *et al.,* 2002).

2.4.2.4 The E-test ESBL Strip Method

For this method, cefotaxime or ceftazidime are the frequently used E-test ESBL strips, seen on one side of the strip with the other end having the corresponding antibiotics plus clavulanate as confirmatory agents. For a phenotically positive ESBL-production test, the Minimum Inhibition Concentration (MIC) ratio of cephalosporin alone to cephalosporin plus clavulanic acid is MIC ≥ 8, or a decrease in the MIC of cephalosporin of more than three dilutions in the presence of clavulanic acid (Sridhar *et al.,* 2008).

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2.5 Molecular Characterization of ESBL Genes

With the availability of PCR followed by sequencing, it is now possible to differentiate between different variants of ESBL genes and non-ESBL parent enzymes, making this system the method of choice (Fluit and Schmitz, 2001). Owing to the challenging and laborious nature of the molecular procedures, they are not performed routinely for clinical diagnostic purposes, rather they are restricted to reference laboratories and molecular surveillance studies in many countries (Woodford *et al*., 2006). Frantic attempts have, however, been made to develop very simple, affordable and easily accessible molecular techniques (rapid-cycle sequencing and microarrays for genotyping ESBLs) for diagnostic laboratories (Pitout and Laupland, 2008) but this hope is yet to be realized.

2.6 The Problem of ESBL Detection

The ability of clinical laboratories to identify and characterize organisms producing ESBL is a major challenge owing to the fact that, ESBL enzymes have variable affinity for different substrates (HPA, 2004). This substrate variability and inoculums effect make some ESBL-producing organisms difficult to detect (HPA, 2004). The World Health organization (WHO) has expressed concerns about the current abilities of some laboratories to fully detect ESBL-producing organisms (Tenover *et al*., 2001). Additionally, many clinical laboratories may not be fully aware of the importance of organisms producing ESBLs. The current detection of ESBL-producing pathogens in microbiology laboratories therefore remains a controversial issue with some people questioning its clinical relevance in view of the financial demands involved (Pitout and Laupland, 2008).

In response, the Clinical and Laboratory Standards Institute (CLSI) has a publication on the guidelines for ESBL detection (Paterson and Bonomo, 2005). With reference to the guidelines,

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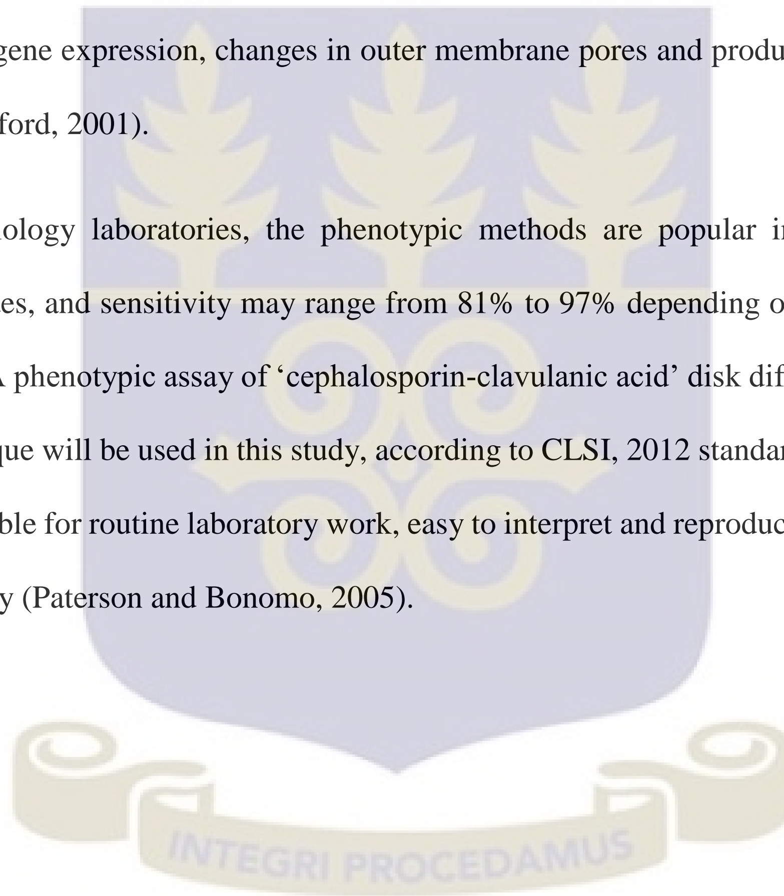
molecular methods are used to identify the presence of specific ESBL genotypes in clinical isolates but the procedures are complicated with variability in data interpretations (Bradford, 2001). Many researchers use the genotypic methods to ascertain the geographical relatedness of ESBL-producing organisms in epidemiological studies (Paterson and Bonomo, 2005). It is, however, worth noting that, clinically no precise association can be established between ESBL genotypes and the susceptibility of ESBL-producing *Enterobacteriaceae* to different β-lactams, since susceptibility depends on ESBL gene expression, changes in outer membrane pores and production of additional β-lactamases (Bradford, 2001).

In clinical microbiology laboratories, the phenotypic methods are popular in detecting ESBL expression in isolates, and sensitivity may range from 81% to 97% depending on the method used (Bradford, 2001). A phenotypic assay of 'cephalosporin-clavulanic acid' disk diffusion on Mueller-Hinton agar technique will be used in this study, according to CLSI, 2012 standards. This method is cost effective, suitable for routine laboratory work, easy to interpret and reproducible in results with over 93% sensitivity (Paterson and Bonomo, 2005).



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CHAPTER THREE

MATERIALS AND METHODS

3.0 Study Design

This study was a cross-sectional study that involved convenient sampling of *E. coli* and *K. pneumoniae* isolates collected randomly from patient's clinical specimen at the Tamale Teaching Hospital (TTH) between April and June 2015.

3.1 Study Setting

The Tamale Teaching Hospital is located in the Eastern part of the Tamale Metropolis, in a catchment area which has a population of approximately 2.1 million. The Hospital was established to serve as a Medical Referral Centre for the Northern, Upper East and Upper West Regions, the Northern parts of Brong Ahafo Region and neighboring countries, including La Cote D'Ivoire, Burkina Faso and Togo. The hospital has a bed capacity of four hundred and fifty-two (452). The hospital has many wards/units and blocks comprising of maternity block, obstetrics and gynecological block, intensive care unit (ICU), males and females wards, trauma and surgical wards and out-patient department (OPD). The microbiological laboratory provides routine bacteriological services to the hospital and the population in general with over 8,000 clinical cultures annually (http://www.tamaleteachinghospital.org).

3.2 Collection of Clinical Isolates

3.2.1 Inclusion Criteria

Clinical isolates clearly identified as *E. coli* and *K. pneumoniae* were investigated in the study.

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3.2.2 Exclusion Criteria

Multiple isolates per species from the same patient and having the same antibiogram pattern were excluded from the study to avoid duplication.

3.2.3 Minimum Sample Size

Clinical isolates of *E. coli* and *K. pneumoniae* were used for this study. The minimum sample size of isolates screened for ESBL production was determined using the formula:

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

Where: Z = 1.96, is the standard score for the confidence interval at 95%.

P = 0.493, is the sample proportion of the prevalence of ESBL-producing isolates in Ghana, (Obeng-Nkrumah *et al.*, 2013).

A 9% allowable error was used.

Our sample size, $\mathrm{N} = \frac{(1.96)^2(0.493)(1-0.493)}{(0.09)^2} = 118.54$

= 119 *E. coli* and *K. pneumoniae* isolates collected randomly.

Overall, 140 isolates were screened and confirmed for ESBL producers and non-producers.

3.3 Data Collection

During the study period, clinical isolates of *E. coli* and *K. pneumoniae* recovered as etiologic agents of infections from clinical specimen submitted by patients to the Microbiology laboratory (TTH) for bacteriological investigations were randomly collected. For each clinical isolate, the respective

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patient data including personal data (age, gender), diagnosis and type of specimen submitted for investigation, were obtained using structured questionnaire. Additional information covering history on antibiotic usage, patients' assessing a hospital facility and comorbid conditions (diabetes, dialysis and chemotherapy treatment) were also collected to determine if these variables were more or less often associated with ESBL-producing *E. coli* and *K. pneumoniae*.

3.4 Laboratory Investigations

The laboratory investigations were in four phases:

PHASE I – Bacteriological confirmation of *E. coli* and *K. pneumoniae* isolates received from Tamale Teaching Hospital (TTH) using MINIBACT-E micro-test kits (SSI, Denmark).

PHASE II – General antibiotic susceptibility testing and stocking of characterized isolates.

PHASE III – Phenotypic determination of ESBL-producing isolates.

PHASE IV – Molecular identification of ESBL genes responsible for phenotypes in phase III.

3.4.1 Phase I: Bacteriological Confirmation of E. coli and K. pneumoniae Isolates received from TTH using MINIBACT-E micro-test kits

The work was done in the Medical Microbiology Department (Korle-Bu) and Noguchi Memorial Institute for Medical Research (NMIMR, Legon). Phases I, II and III were carried out in the Korle-Bu and Phase IV in Legon. Isolates received from TTH were sub-cultured onto MacConkey agar plate (Mac) and incubated in ambient air at 35±2°C for 16 to 18 hours. After overnight incubation, the identities of all the clinical isolates were confirmed using the MINIBACT-E micro-test kits

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according to the manufacturer's instructions (Appendix III) and results read after four hours' incubation.

The MINIBACT-E is a ready-to-use four hour chromogenic micro-test kit embedded with substrates in the various wells, which works on the principle of colour change for bacteria identification. This was used to confirm clinical isolates of *E. coli* and *K. pneumoniae* from TTH. The micro-test kit takes up to four cultures per plate with 16 wells each. The use of the kit **saved** time, labour and cost.

3.4.2 Phase II: General Antibiotic Susceptibility Testing and Stocking of Characterized Isolates

Antibiotic susceptibility testing for each isolate studied was determined using the Clinical and Laboratory Standard Institute (CLSI, 2012). Purity plates were prepared as working stock for study isolates sub-cultured onto MHA. Four to six morphologically similar colonies from the pure cultures were touched using sterile inoculating loops and transferred into 3ml peptone broth, to make a suspension of 0.5 McFarland standard (10^{7-8}cfu/ml). After 15 minutes incubation to enhance appreciable growth, sterile cotton tipped swabs were dipped into the standardized inoculums and swabbed carefully in three dimensions on the Mueller-Hinton agar (MHA) plates (Oxiod, UK). The moisture was allowed to be absorbed for at least 15 minutes. The antibiotic discs were placed firmly on the surface of the agar plate and incubated at 35±2°C for 16 to 18 hours in ambient air, after allowing the agar plate to be on the bench at room temperature for about 15 minutes to enhance adequate antibiotics absorption. Susceptibilities of all isolates studied to the following antibiotics (MAST, UK) listed below were recorded after overnight incubation. They included: ampicillin (10µg), amikacin (30µg), meropenem (10µg), gentamicin (10µg), ciprofloxacin (5µg), cefuroxime (30µg), cefotaxime (30µg), chloramphenicol (30µg), and tetracycline (30µg). Nalidixic acid (30µg) was used for urine isolates. Susceptible reference strains of *E. coli* ATCC 25922 and *K. pneumoniae*

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ATCC 700603 as controls were performed for every batch carried out and the zones of inhibition recorded after 16 to 18 hours of incubation. *Escherichia coli* and *K. pneumoniae* isolates characterized for ESBL production were stored in trypticase soy broth containing 10% glycerol (v/v) in 1.5ml cryovial tubes (Sigma, UK) and **stored** at -20°C for further workup. Working cultures of pure isolates were kept on solid agar slants at -5°C.

3.4.3 Phase III: Phenotypic Determination of ESBL-producing Isolates

Phenotypic determination of ESBL-producing isolates was carried out using the Kirby-Bauer's method of susceptibility testing on Mueller Hinton agar (MAST, UK) (CLSI, 2012). The inhibition zone diameters were interpreted according to the reference breakpoints of the Clinical and Laboratory Standard Institute (CLSI, 2012). *Escherichia coli* ATCC 25922 (ESBL-negative) and *K. pneumoniae* ATCC 700603 (ESBL-positive) were used as controls. This phase comprised of screening and confirmation of ESBL-producing isolates using the combined disc synergy method.

With reference to the CLSI screening guidelines, isolates with zones inhibition diameters of less than or equal to 27mm for Cefotaxime (CTX) (30µg), less than or equal to 22mm for Ceftazidime (CAZ) (30µg) and less than or equal to 17mm for Cefpodoxime (CPD) (10µg) were considered as potential ESBL-producers. Isolates that were resistant at these breakpoints to at least one of the three screening antibiotics were described as positive for ESBL screening. These potential ESBL-producers were further investigated in order to confirm ESBL production.

The test was carried out using the Kirby-Bauer disc diffusion method according to the CLSI recommendation. Cefotaxime (30µg), Ceftazidime (30µg) and Cefpodozime (10µg) antibiotic discs with or without Clavulanic acid (10µg) were placed at a distance of 25mm on MHA plate inoculated

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with bacteria suspension of 0.5 McFarland turbidity standards and incubated overnight at 37°C. As determined by the CLSI reference, the study isolates that demonstrated clavulanic acid effect defined by the increase in zone diameter greater than 5mm for at least one test antibiotic, were confirmed ESBL-producers.

3.4.4 Phase IV: Molecular Characterization of ESBL-producing Isolates

Phenotypically documented ESBL-producing *E. coli* and *K. pneumoniae* were characterized by PCR to confirm the presence of gene families (TEM, SHV and CTX-M) that encode ESBLs. In this phase, two extraction procedures (Boiling and Qiagen extraction) were used with the aim of achieving optimum DNA concentrations and purity for amplification. The DNA concentrations and purity were quantified using the Nanodrop device (Thermo Scientific, USA).

The boiling suspension method was used to extract DNA from bacteria samples (Holmes and Quingely, 1981). About 4 - 6 morphologically similar colonies of test isolates were touched and suspended in 200µl nuclease free water in a 1.5ml eppendorf screw-capped tubes. The suspension was heated at 98°C for about 20 minutes using a waterbath incubator to obtain cell lysate. The mixtures were centrifuged at 2,000 rpm for 15 minutes and the supernatant transferred into sterile 1.5ml eppendorf screw-capped tubes as the DNA template leaving behind the pellet. Qiagen extraction kits were also used to extract DNA from the test isolates following the manufacturers protocol as provided in Appendix III.

Isolates were screened for TEM, SHV and CTX-M-1, 2 and 9 cluster groups of ESBLs. PCR was carried out using thermal cycler (BioRad, USA) with a total volume of 25.0µl containing 2.0µl DNA template and 23.0µl Master Mix (Qiagen, UK) (Apendix III). The primer sequences and cycling conditions used for the different PCRs are shown in Table 2. The PCR products for each reaction

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was electrophoresized on 2% agarose gel with a 100 base pair molecular DNA marker (Biolabs, New England). Gels were visualized using the UV-transilluminator after staining in ethidium bromide.

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Table 3.1 Primers and cycling conditions used for amplification of TEM, SHV and CTX-M genes

Resistant gene	Sequence (5' to 3')	Size (bp)	Cycling conditions
TEM	FP:GTATCCGCTCATGAGACAATAACCCTG RP: CCAATGCTTAATCAGTGAGGCACC	918	Initial heat activation temperature at 94°C for 15minutes; denaturation for 30 cycles at 94°C for 30seconds; annealing at 63°C for 90seconds; extension at 72°C for 60seconds; final extension at 72°C for 10 minutes
SHV	FP: CGC CTG TGT ATT ATC TCC CTG TTA GCC RP: TTG CCA GTG CTC GAT CAG CG	842	Initial heat activation temperature at 94°C for 15minutes; denaturation for 30 cycles at 94°C for 30seconds; annealing at 63°C for 90seconds; extension at 72°C for 60seconds; final extension at 72°C for 10 minutes
CTX-M 1	FP: GACAGACTATTCATGTTGTTGTTAWTTCG RP: CCGTTTCCSCTATTACAAA	940	Initial heat activation temperature at 94°C for 15 minutes; denaturation for 30 cycles at 94°C for 30seconds; annealing at 50°C for 90seconds; extension at 72°C for 60seconds; final extension at 72°C for 10 minutes
CTX-M 2	FP: ACAGTTGGTGACGTGGCTTAAGG RP : TCAGAAACCGTGGGTTACGA	253	Initial heat activation temperature at 94°C for 15 minutes; denaturation for 30 cycles at 94°C for 30seconds, annealing at 50°C for 90seconds, extension at 72°C for 60seconds; final extension at 72°C for 10 minutes
CTX-M 9	FP: ATGGTGACAAAGAGAGTGCAACG RP: ATGATTCTCGCCGCTGAAGC	860	Initial heat activation temperature at 94°C for 15 minutes; denaturation for 27 cycles at 94°C for 30seconds, annealing at 50°C for 90seconds, extension at 72°C for 60seconds; final extension at 72°C for 10 minutes

(Primers designed with modifications as published by Hackman *et al*., 2014)

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3.5 Ethics

The work received ethical clearance from the Ethical and Protocol Review Committee of the University of Ghana Medical School (MS-Et/M.7-P3.2/2014-2015) (Appendix IV). Clinical isolates recovered from patients' specimens were assigned arbitrary numbers to ensure anonymity. Informed consent were sought from appropriate authorities of Tamale Teaching Hospital and the Department of Microbiology, TTH, Tamale.

3.6 Statistical Analysis of Data

Data obtained were entered in Microsoft Office Excel, and results analyzed using Statistical Package for Social Sciences (SPSS) (Version 20) in order to address the objectives of the study. Point estimates of statistical significance were indicated with 2 tailed *p*-values <0.05. Descriptive statistics (frequencies and cross-tabulations) were used to determine the prevalence of ESBLs. For continuous variables, standard weighted-mean statistics using Kruskal Wallis or Mann Whitney test (respectively for normalized and non-normalised distributions) were used to estimate differences in population means. Categorical data were compared across study parameters using Chi-square or the Fisher's exact test where appropriate. Univariate comparisons between study outcomes and covariates were computed with Chi-square tests and unadjusted Odds ratios (OR) at 95% confidence interval (CI).

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CHAPTER FOUR

RESULTS

4.0 General Characteristics of Study Participants

A total of 140 isolates of *E. coli* (83.6%; n=117) and *K. pneumoniae* (16.4%; n=23) were randomly obtained from patients' clinical specimen at the Tamale Teaching Hospital (TTH). The isolates were mainly from cultures of urine (55.7%; n=78), HVS/ endocervical swab (17.1%; n=24), aspirates (8.6%; n=12), wound (7.1%; n=10), sputum (6.4%; n=9) and blood (5.0%; n=7). The mean age of patients was 32.7±19.2 and comprised 44 males (40.8±22.3) and 96 females (29.0±16.4). Out of the 117 patients infected with *E. coli*, 31.6% (n=37) were males whereas 68.4% (n=80) were females. Also, out of the 23 patients infected with *K. pneumoniae*, 30.4% (n=7) were males whereas 69.6% (n=16) were females.

Figure 4.1 illustrates the general antibiotic susceptibility pattern of the study isolates as per Kirby-Bauer method of susceptibility testing. A total of 10 antimicrobials (ampicillin, gentamicin, cefotaxime, cefuroxime, chloramphenicol, nalidixic acid, tetracycline, ciprofloxacin, amikacin and meropenem) were tested against the study isolates. Generally, there was high percentage resistance to ampicillin 96% and tetracycline 89%. Resistance to nalidixic acid and ciprofloxacin were 77% and 74% respectively, while chloramphenicol, cefuroxime, gentamicin and cefotaxime were 60%, 56%, 54% and 46% respectively. Relatively lower resistance was noted for amikacin 36%, however, none of the isolates was resistant to meropenem, using CLSI inhibition zone size interpretation criteria (CLSI, 2012). Isolates resistant to at least one agent in three or more antimicrobial categories is referred to as Multidrug Resistant (MDR) (Magiorakos *et al*., 2012). In this study, 19.7% of *E. coli* and 26.1% of *K. pneumoniae* were determined to be MDR.

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Table 4.1 General Characteristics of Study Participants

Variables	Total (n=140)	*E. coli* (n=117)	*K. pneumoniae* (n=23)
Socio-demographic data			
Age (yrs)	32.7±19.2	32.9±19.2	31.5±18.4
Male	44 (31.4%)	37 (31.6%)	7 (30.4%)
Female	96 (68.6%)	80 (68.4%)	16 (69.6%)
Locality/ Region			
Northern	138 (98.6%)	115 (83.3%)	23 (16.7%)
Upper East	2 (1.4%)	2 (100%)	0 (0.0%)
Diagnosis queried by clinicians			
Wound abscess	10 (7.1%)	10 (8.5%)	0 (0.0%)
Sepsis	7 (5.0%)	4 (3.4%)	3 (13.0%)
Chest pain	2 (1.4%)	0 (0.0%)	2 (8.7%)
PID	4 (2.9%)	1 (0.9%)	3 (13.0%)
BPH	5 (3.6%)	5 (4.3%)	0 (0.0%)
Chronic cough	7 (5.0%)	2 (1.7%)	5 (21.7%)
Peritonitis	10 (7.1%)	10 (8.5%)	0 (0.0%)
Ascites	2 (1.4%)	2 (1.7%)	0 (0.0%)
Lower abdominal pain	2 (1.4%)	2 (1.7%)	0 (0.0%)
UTI/ painful urination	73 (52.1%)	69 (59.0%)	4 (17.4%)
Vaginal discharge	18 (12.9%)	12 (10.3%)	6 (26.1%)
Sample types			
Urine	78 (55.7%)	74 (63.2%)	4 (17.4%)
HVS	24 (17.1%)	15 (12.8%)	9 (39.1%)
Aspirates	12 (8.6%)	12 (10.3%)	0 (0%)
Wound	10 (7.1%)	10 (8.5%)	0 (0%)
Sputum	9 (6.4%)	2 (1.7%)	7 (30.4%)
Blood	7 (5.0%)	4 (3.4%)	3 (13.0%)

PID-Pelvic inflammatory disease, BPH-Benign prostatic hyperplasia, UTI-Urinary tract infection, HVS-High vaginal swab, n-Number of isolates.

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100
80
60
40
20
0
Overall % resistance among study isolates
Ampicillin
Tetracycline
Nalidixic acid
Ciprofloxacin
Chloramphenicol
Cefuroxime
Gentamicin
Cefotaxime
Amikacin
Meropenem
Antimicrobial agents
Overall resistance

Figure 4.1 Antibiogram of *E. coli* and *K. pneumoniae* in TTH

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4.1 Phenotypic Expression of ESBLs

Sixty two (44.3%) of the 140 clinical isolates were ESBL-producers. Of these, 83.9% (n=52) were *E. coli* and 16.1% (n=10) were *K. pneumoniae* isolates as shown in Table 4.2. The proportion of ESBL-producing isolates were found to be relatively higher (p=0.14) in adults (15-65 years) than in neonates (< 28 days) as illustrated in Figure 4.2. However, age had no significant association with respect to ESBL-producing enterobacteria (p=0.14). Univariate analysis using patient's characteristics and their association with ESBLs were performed as shown in Table 4.3. Diagnosis and gender showed no significant association with ESBL-producing enterobacteria. Patients who reported their previous medication as having been prescribed by a Physician as well as those who completed their previous medication showed significant associations with ESBL-producing enterobacteria (p=0.0008 and 0.0399 respectively).

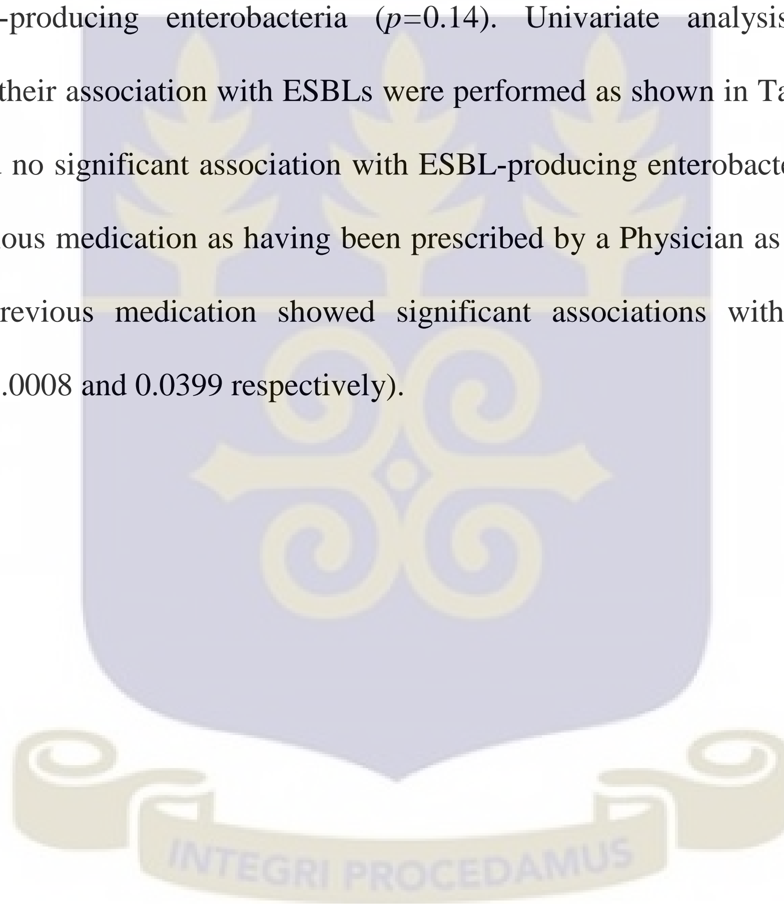

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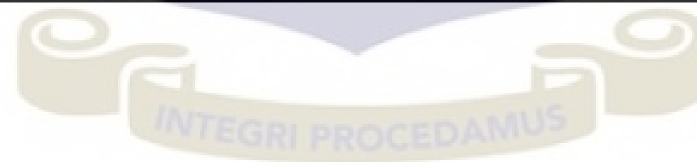
Table 4.2 Phenotypic Expression of ESBLs

Isolated species		ESBL-producers as determined by phenotypic tests						
		Urine	HVS	Aspirate	Wound	Sputum	Blood	Total ESBL (%)
Escherichia coli	n (*ESBL*%)	33 (44.6%)	4 (26.7%)	8 (66.7%)	5 (50%)	1 (50%)	1 (25%)	**52 (83.9%)**
Klebsiella pneumoniae	n (*ESBL*%)	2 (20%)	5 (55.6%)	0 (0.0%)	0 (0.0%)	1 (14.3%)	2 (66.7%)	**10 (16.1%)**
Total ESBL-producers		**35 (44.9%)**	**9 (37.5%)**	**8 (66.7%)**	**5 (50.0%)**	**2 (22.2%)**	**3 (42.9%)**	**62 (44.3%)**

*n is the number of isolates.



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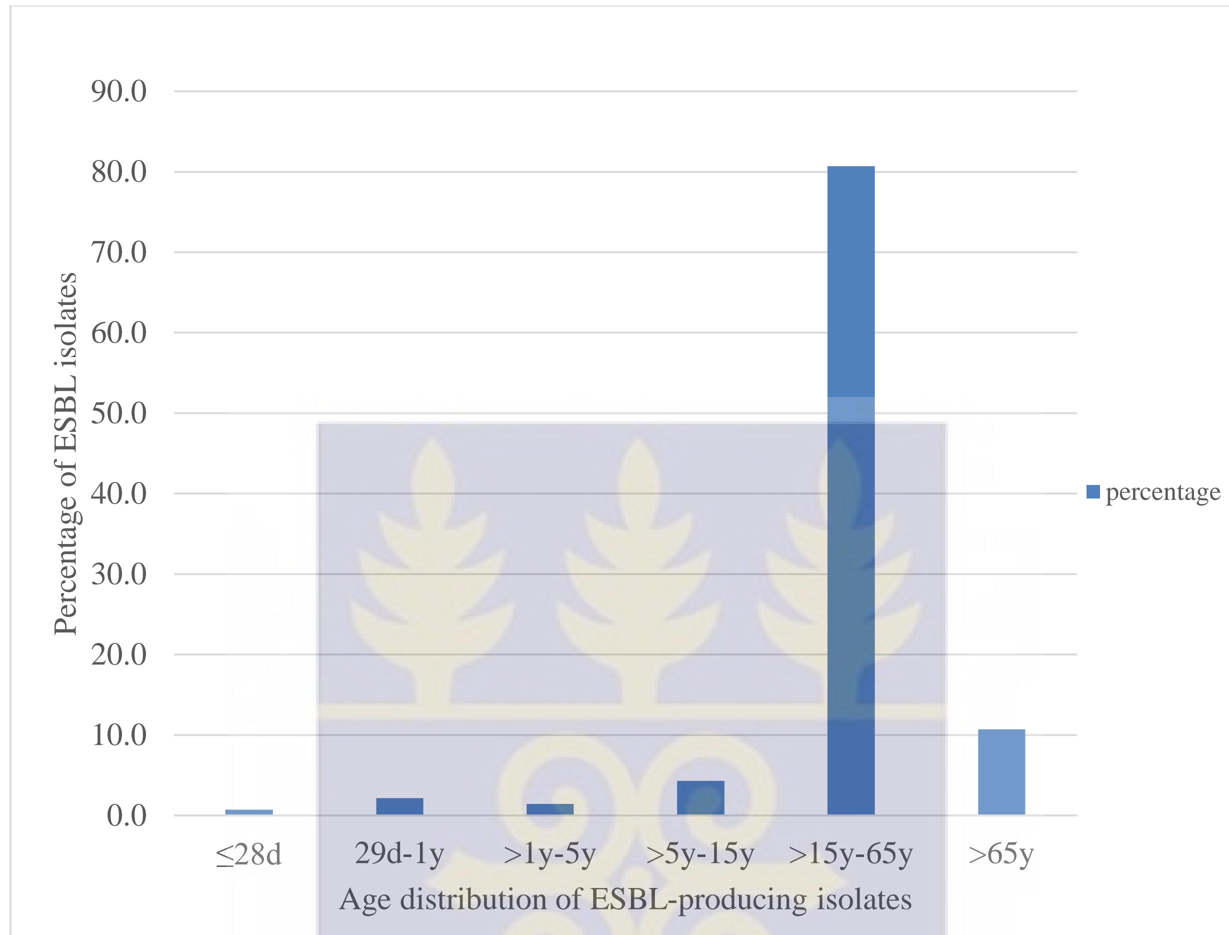
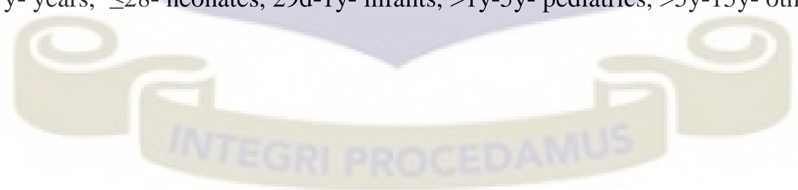


Figure 4.2 ESBL-producers isolated from various age groups



* Where d- days and y- years, ≤28- neonates, 29d-1y- infants, >1y-5y- pediatrics, >5y-15y- other children, > 15y-65y- adults, >65y- elderly

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Table 4.3 Univariate Analysis of Patient's Characteristics in Relation to ESBL

Variables	Total n=140	ESBL Producers n= 62	Non-producers n=78	Odds ratio (95% CI)	*P-value*
Socio-demographic data					
Age group					
≤28d		0 (0.00%)	1 (1.28%)	0 (0-0)	1
29d-1y		0 (0.00%)	3 (3.85%)	0 (0-0)	0.26
>1y-5y		2 (3.23%)	0 (0.00%)	0 (0-0)	0.2
>5y-15y		4 (6.45%)	1 (1.28%)	5.03 (0.55-46.18)	0.18
>15y-65y		49 (79.03%)	65 (83.33%)	0.95 (0.58-1.56)	0.9
>65y		7 (11.29%)	8 (10.26%)	1.10 (0.38-3.20)	1
Male		18 (29.0%)	26 (33.3%)	0.82 (0.40-1.69)	0.71
Female		44 (71.0%)	52 (66.7%)		
Diagnosis queried by Clinician					
Wound abscess		5 (8.1%)	5 (6.4%)	1.30 (0.36-4.70)	0.75
Sepsis		3 (4.8%)	4 (5.1%)	0.94 (0.20-2.23)	1
Chest pain		0 (0.0%)	2 (2.6%)	0.00 (0.00-0.00)	0.5
PID		3 (4.8%)	1 (1.3%)	3.92 (0.40-38.60)	0.32
BPH		2 (2.3%)	3 (3.8%)	0.83 (0.13-5.15)	1
Chronic cough		3 (4.8%)	4 (5.1%)	0.94 (0.20-4.37)	1
Peritonitis		7 (11.3%)	3 (3.8%)	3.18 (1.05-12.86)	0.11
Ascites		1 (1.6%)	1 (1.3%)	1.26 (0.08-20.60)	1
Lower abdominal pain		1 (1.6%)	1 (1.3%)	1.26 (0.08-20.60)	1
UTI/ painful urination		32 (51.6%)	41 (52.6%)	0.96 (0.49-0.88)	1
Vaginal discharge		5 (8.1%)	13 (16.7%)	0.44 (0.15-1.31)	0.2
Sample type					
Urine		34 (54.8%)	44 (56.4%)	0.97 (0.56-1.70)	1
HVS		9 (14.5%)	15 (19.2%	0.75 (0.31-1.84)	0.66
Aspirates		8 (12.9%)	4 (5.1%)	2.52 (0.72-8.74)	0.23
Wound		5 (8.1%)	5 (6.4%)	1.26 (0.35-4.54)	0.75
Sputum		3 (4.8%)	6 (7.7%)	0.63 (0.15-2.62)	0.73
Blood		3 (4.8%)	4 (5.1%)	0.94 (0.20-4.37)	1
Previous antibiotics medication					
-Prescribed by doctor		53 (85.5%)	47 (60.3%)	3.88 (1.68-8.99)	*0.0008
-Self-prescribed		9 (14.5%)	31 (39.7%)		
Completion of previous medication		49 (79.0%)	50 (64.1%)	2.11 (0.98-4.54)	*0.0399

*Shows significant association in relation to ESBLs.
CI-Confidence interval, PID-Pelvic inflammatory disease, BPH-Benign prostatic hyperplasia, UTI-Urinary tract infection, HVS- High vaginal swab.

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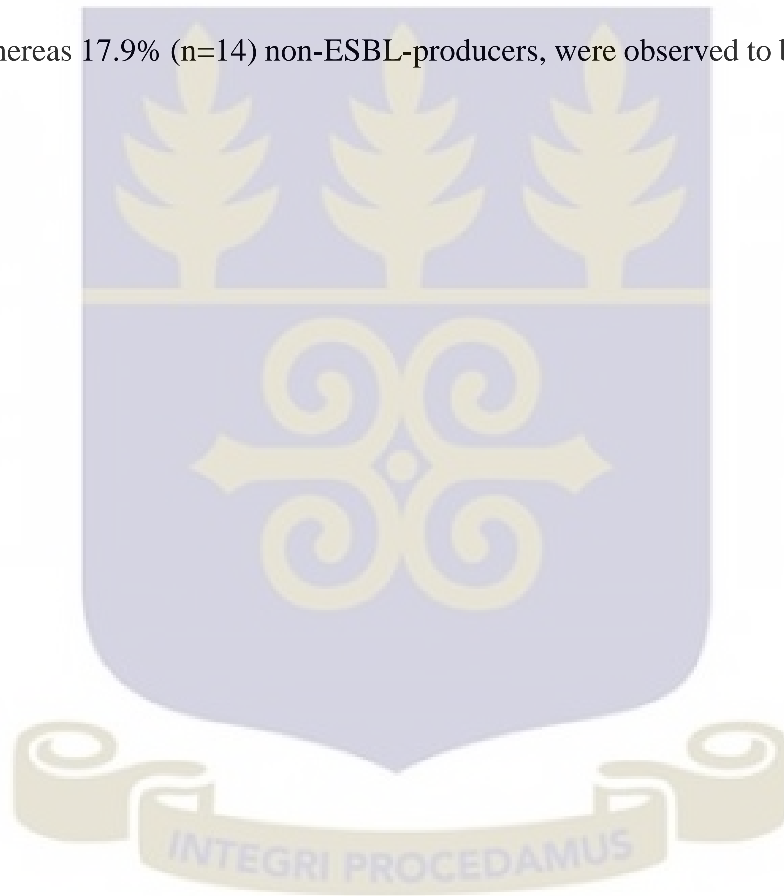
4.2 Antimicrobial Resistance among ESBL-Producers and Non-ESBL Producers

Comparison of antibiograms of ESBL-producers and non-ESBL-producers as per Kirby-Bauer susceptibility testing method is illustrated in Figure 4.3. Significant differences in antibiotic resistance between ESBL-producers and non-ESBL-producers were observed for cefuroxime (*p*=0.00001), gentamicin (*p*=0.004) and amikacin (*p*=0.02). However, all isolates used in this study were susceptible to meropenem. Of the ESBL-producers, 22.6% (n=14) were multidrug resistant (MDR) strains whereas 17.9% (n=14) non-ESBL-producers, were observed to be MDR (Table 4.8).



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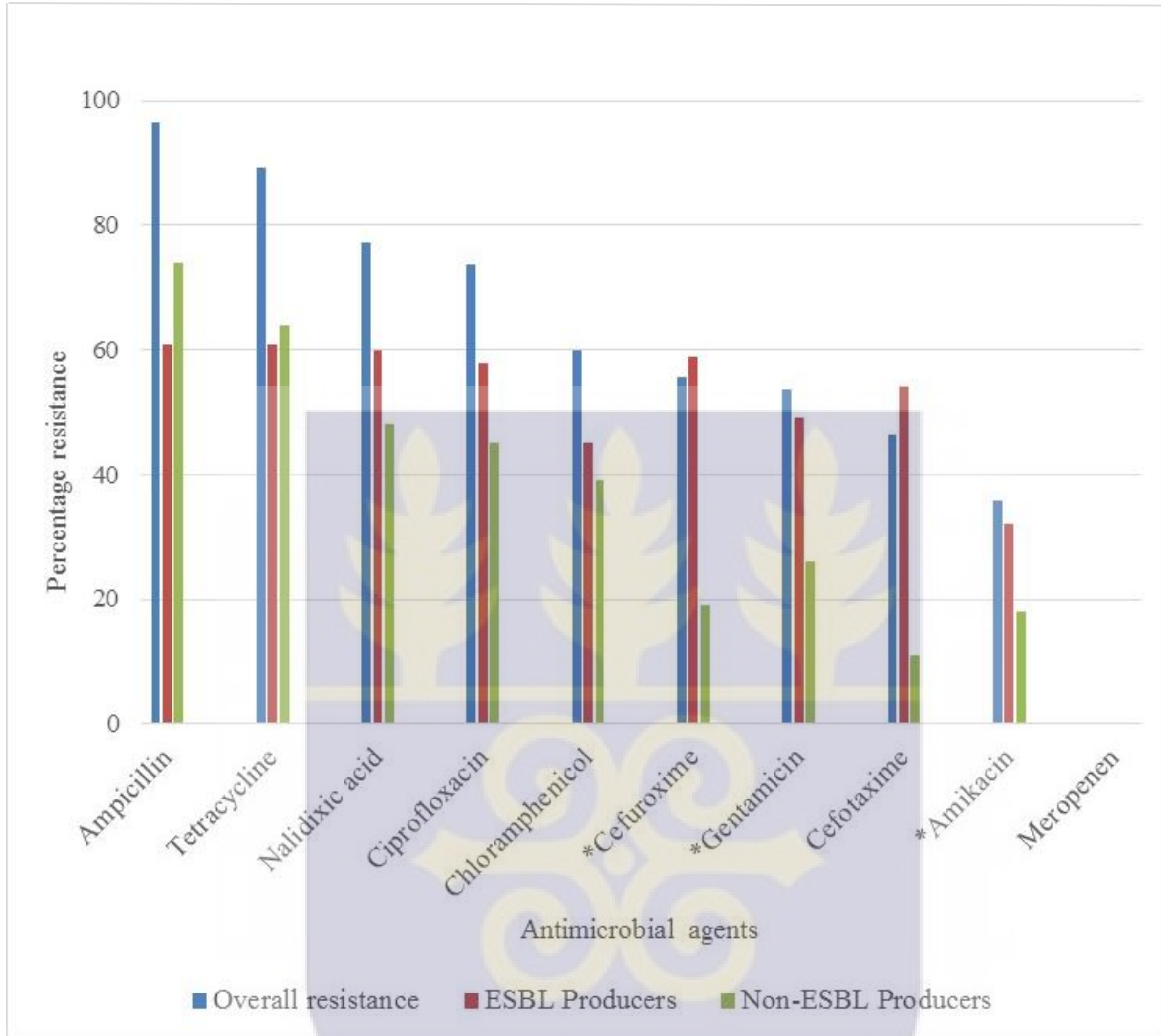

Figure 4.3 Antibiogram of ESBL-producers and non-ESBL producers (April to June 2015) in TTH

*Antimicrobial resistance showing significant difference between ESBL-producers and non-ESBL-producers

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4.3 Detection of ESBL Genotypes

In this study, two DNA extractions methods (Qiagen extraction and boiling lysate) were assessed to determine their performance in providing DNA templates for a positive ESBL amplification test. Altogether, ESBL PCR positivity based on either of the extraction procedures was crudely considered as the gold standard. Both Qiagen and boiling extraction methods used, showed good concordance as extraction methods for DNA templates for TEM, SHV and CTX-M-1 gene amplification (K=97%, K=96% and K=93% respectively). Both extraction methods had good (>83%) sensitivity and specificity with high negative predictive values (>82) for each ESBL gene. The total number of ESBL gene positivity determined from DNA templates prepared with either of the extraction methods did not significantly differ (p=0.61).

Extended Spectrum β-Lactamase-producing *E. coli* and *K. pneumoniae* isolates were characterized for Bla_{TEM}, Bla_{SHV} and $Bla_{CTX\text{-}M}$ genes. About seventy four percent (74.2%) of the ESBL genotypes expressed $Bla_{CTX\text{-}M\text{-}1}$ genes followed by 62.9% Bla_{TEM} and 16.1% Bla_{SHV}. None of the isolates expressed genes for CTX-M 2 and CTX-M 9. About 6.5% (n=4) of the isolates harbored all three genes (Bla_{TEM}, Bla_{SHV} and $Bla_{CTX\text{-}M\ 1}$). Overall, 12.9% (n=8) of the isolates that phenotypically expressed ESBLs, did not harbour any identifiable Beta-lactamase genes as shown in Table 4.5.

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Table 4.4 Performance of DNA Extraction Procedures for ESBL Genotypic Detection

Resistant genes	Methods	Number of true positives	Prevalence (%)	Sensitivity (%)	Specificity (%)	Positive predictive value	Negative predictive value	Kappa value
TEM (n=62)	QIAGEN	36	58	100	88	92	100	96.5
	BOILED	36	63	92	100	100	88	
SHV (n=62)	QIAGEN	10	19	83	100	100	96	96.3
	BOILED	10	16	100	96	83	100	
CTX-M 1 (n=62)	QIAGEN	42	71	95	98	91	88	92.9
	BOILED	42	74	91	88	95	78	

*n is the number of isolates.

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Table 4.5 ESBL Genotypes in *E. coli* and *K. pneumoniae* Isolates that Phenotypically Expressed ESBLs

Resistance genes	Number of positive genotypes
TEM only	4 (6.5%)
SHV only	2 (3.2%)
CTX-M 1 only	11 (17.7%)
CTX-M 2 only	0 (0.0%)
CTX-M 9 only	0 (0.0%)
TEM + SHV	2 (3.2%)
TEM + CTX-M-1	29 (46.8%)
SHV + CTX-M-1	2 (3.2%)
TEM + SHV + CTX-M-1	4 (6.5%)
ESBL expressing isolates without identifiable ESBL genes	8 (12.9%)
Total	**62 (44.3%)**
Total TEM	39 (62.9%)
Total SHV	10 (16.1%)
Total CTX-M-1	46 (74.2%)

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CHAPTER FIVE

DISCUSSION

A relatively high proportion (44.3%) of ESBL-producing *E. coli* and *K. pneumoniae* in TTH, Ghana was recorded in the present study. The level of ESBL reported is higher compared to that reported by Aibinu *et al.*, (2003) in Nigeria (20%, 8 of 40). The prevalence recorded in this study is comparable to that documented by Obeng-Nkrumah *et al.*, (2013) in Korle-Bu Teaching Hospital (49.3%; n=148/300, *p*=0.32) but lower than the prevalence reported by Feglo *et al.*, (2013) from Komfo-Anokye Teaching Hospital (57.8%; n=234/405, *p*=0.006).

Escherichia coli and *Klebsiella pneumoniae* are responsible for common community and hospital acquired infections. These organisms have become multidrug resistant over time, making infections they cause difficult to treat (Blomberg *et al.*, 2005). The major resistance mechanism expressed by *E. coli* and *K. pneumoniae* are ESBLs; and they remain the commonest ESBL producing organisms worldwide (Jacoby and Munoz-Price, 2005). The ESBL prevalence amongst clinical isolates within institutions varies greatly from country to country. Across Europe there is varying prevalence, with low occurrence of 1% and 3% in the Netherlands and Sweden respectively (Rodriguez-Villalobos *et al.*, 2011), to as high as 42% in an intensive care unit in France (Branger *et al.*, 1998).

The general antimicrobial susceptibility pattern in the present study showed an overall high resistance prevalence among the antibiotics used. Significant difference in antimicrobial resistance between ESBL-producers and non-ESBL-producers were observed for cefuroxime, gentamicin and amikacin (*p*=0.00001, 0.004 and 0.02 respectively). The higher antibiotic resistance levels recorded in the present study is similar to the study conducted by Obeng-Nkrumah *et al.*, (2013), where they

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documented high antimicrobial resistance among *Enterobacteriaceae* in KBTH and reported that, prevalence is on the rise. Other works elsewhere in Ghana have reported similar conclusions (Adu-Sarkodie, 2010; Feglo *et al*., 2013). In the present study, all the isolates were susceptible to meropenem. This may be attributed to the fact that, meropenem is a very expensive board-spectrum antimicrobial agent usually prescribed for serious infections. More so, meropenem administration is parenteral and is less likely to be abused.

Multidrug Resistant (MDR) is an acquired non-susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos *et al*., 2012). In the present study, isolates that expressed resistance to three or more antimicrobials categories (penicillins, aminoglycosides, cephalosporins, carbapenems, tetracyclines, fluoroquinolones, quinolones and phenicol) were referred to as multidrug-resistant. Approximately 23% ESBL-producers were observed to be MDR, compared to non-ESBL-producers (18%). These figures are similar to those reported by Obeng-Nkrumah *et al*., (2013) in KBTH, with statistically no significant difference between ESBL-producers and non-ESBL-producers in association with MDR. The levels of MDRs reported in this study could be attributed to the indiscriminate sale and misuse of antibiotics in Ghana (Newman *et al*., 2011), the failure to complete antibiotic treatment and the use of antimicrobials in animal husbandry (Tajick, 2006).

In the present study, majority of the isolates expressed *Bla*$_{CTX\text{-}M\text{-}1}$ genes (74.2%). Similar findings were reported in a study by Hackman *et al*., (2014) in Accra, where 78% of their isolates were positive for CTX-M-1 group ESBL genes. These results are, however, lower compared to the prevalence reported by Feglo *et al.,* (2013) in Kumasi with 93.5% (n=72) of their isolates expressing *Bla*$_{CTX\text{-}M\text{-}1}$ genes in *E. coli*. Additionally, Feglo *et al.,* (2013) reported that about 90% of the isolates

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co-harboured CTX-M-1 and TEM. The percentage reported in their study was higher compared to the figure (approximately 50%) observed in the present study. The CTX-M ESBLs commonly found in *E. coli* isolates are increasingly reported (Coque *et al*., 2008) and the level of resistance conferred by these enzymes to cefotaxime and ceftazidime is high (Bonnet, 2004). The predominance of these enzymes among the study isolates may explain the widespread antimicrobial resistance among the isolates. The spread of these resistant organisms could have occurred through hospital cross infections, improper hand hygiene practices especially after visiting the lavatory, improper use of disinfectants and overcrowding in communities (Raymond *et al*., 2007).

Among the ESBL expressing isolates, none expressed genes for CTX-M 2 and CTX-M 9. Also, ESBL expressing isolates without identifiable ESBL genes were found to be 12.9%. This observation suggests that the isolates could possess other ESBL enzyme types besides TEM, SHV and CTX-M-1 genes which were not sought for in the present study.

Often, seriously ill patients stand the risk of developing infections caused by ESBL-producing organisms. This is due to prolonged hospital stays and use of invasive medical devices (urinary catheters, endotracheal tubes, central venous lines) (Paterson and Bonomo, 2005). In the present study, univariate analysis on some patient's characteristics in relation to ESBLs was conducted. Patients who reported their previous treatment with antibiotics prescribed by a Physician were more likely to be infected with ESBL-producing organisms. Patients who also completed their previous medication were also found to be associated with ESBL-producing enterobacteria. Heavy antibiotic use has been reported as a factor for acquisition of an ESBL-producing enterobacteria (Pena *et al*., 1997; Ariffin *et al*., 2000; Lautenbach *et al*., 2001). However in the present study, this risk factor was not determined owing to the limited information from the patient's questionnaire.

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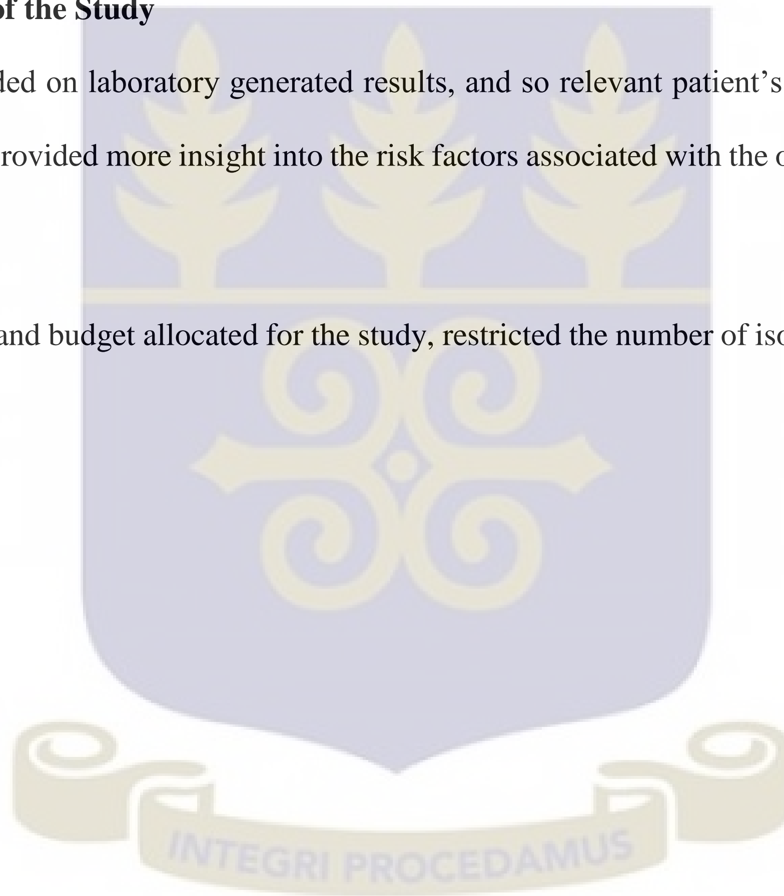
In order to ensure empirical therapy, reduction in antimicrobial abuse and not compromising patients' health, there is an urgent need for routine laboratory detection of ESBL-producing isolates. Proper antimicrobial administration to buttress the usefulness of active hospital surveillance programs for drug resistant bacteria is also warranted.

5.1 Limitations of the Study

The study depended on laboratory generated results, and so relevant patient's clinical information that would have provided more insight into the risk factors associated with the occurrence of ESBLs were missing.

The limited time and budget allocated for the study, restricted the number of isolates (140) involved in the study.



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CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The aim of the study was to determine the prevalence of ESBLs in clinical isolates of *E. coli* and *K. pneumoniae* from patients attending the Tamale Teaching Hospital (TTH). There is relatively high proportion (44.3%) of ESBL-producers among *E. coli* and *K. pneumoniae* in TTH. The results generated during the study period, revealed that, there is high antimicrobial resistance amongst *E. coli* and *K. pneumoniae* to the commonly prescribed antimicrobial drugs in TTH, due to ESBL-production. However, resistance levels of isolates to amikacin is low. Overall, meropenem proved useful against the isolates tested and is therefore recommended in the treatment for infections caused by these isolates.

Extended Spectrum Beta-Lactamase producing isolates that harbored all three genes (Bla_{TEM}, Bla_{SHV} and $Bla_{CTX\text{-}M\text{-}1}$) were 4 (6.5%). Those without identifiable genes were 8 (12.9%). Meanwhile, among the ESBL expressing isolates, none expressed genes for CTX-M 2 and CTX-M 9.

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6.2 Recommendations

1. It is recommended that more data covering a wider sample type (including non-fermenters like, *Pseudomonas* and *Proteus mirabilis*) be collected to determine more accurately the situation of ESBL-producers and provide a better understanding of the epidemiology associated with ESBL-producing strains in TTH in further studies.
2. It is also recommended that further studies with the sequencing of ESBL-producing isolates obtained from TTH be performed to determine their relatedness.

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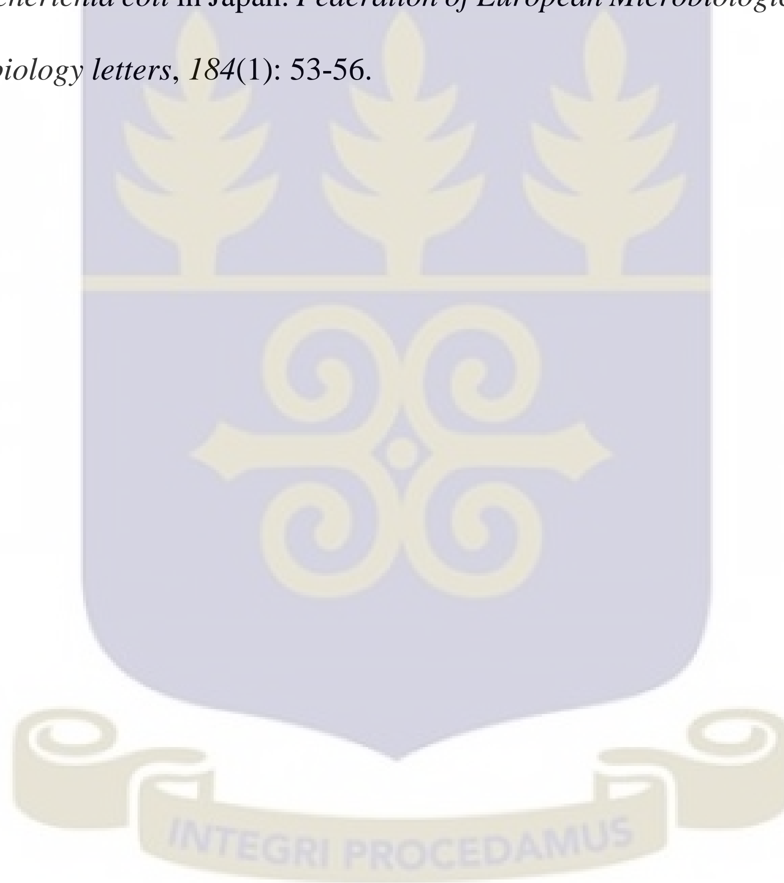

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APPENDICES

Appendix I

Informed Consent Form

Subject Path. Number: ………………… **Date: ……..………**

CONSENT TO PARTICIPATE IN A RESEARCH PROJECT

TITLE OF PROJECT: Extended Spectrum Beta-Lactamase Genes in Clinical Isolates of *Escherichia coli* and *Klebsiella pneumoniae* from the Tamale Teaching Hospital.

The contents will be explained to the participants in the language that he/she is most comfortable with. Patients/ Guardians will be requested to assist in the provision of information for the study. This form describes the nature and purpose of the study, explanation of procedures, benefits, refusal/withdrawal, rights and complaints, confidentiality, research authorization for use, and disclosure of your health care information.

Nature and Purpose of the Study

The study is been conducted by the team of investigators from Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon.

You are being invited to participate in a research project because you are within the area of interest under study and this is the first of its kind to be conduct in the Northern part of Ghana. The purpose of the study is to determine the occurrence of ESBLs in clinical isolates of *E. coli* and *K. pneumoniae* in Tamale Teaching Hospital (TTH).

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Benefits

There are no direct benefits by participating in this project. However, you can also request for your ESBL test results. In case you want your ESBL test results, please prompt the investigator explaining the study to you. We will also request that you make your telephone number available which we will use to contact you to come for your ESBL test results which would be in a sealed envelope for your collection from the Doctor in Charge of the ward/unit. The result will also be accompanied with the antibiogram outcome to know the appropriate antibiotics for effective treatment.

Explanation of Procedures:

The procedure will be well explained to you in the language you are most comfortable with before commencement. You will be asked to answer a questionnaire about your age, education level, occupation, ethnicity, marital status, income and risk factors for ESBL infection such as indiscriminate abuse of antibiotics, under/ over-use of antibiotics and the quality of antibiotics on the Ghanaian market. Based on your clinical diagnosis, the appropriate clinical specimen will be collected and investigated for ESBL-producers in the laboratory.

Confidentiality and Research Authorization for Use and Disclosure of Your Health Care Information. All information gathered from the study will remain confidential to the fullest extent of the law. Your identity as a participant will not be disclosed to any unauthorized persons; only the researchers will have access to the research materials, which will be kept under lock. The study participants will be coded with the code known only to the principal investigator and research staff. The principal investigator will only break the code if you require further treatment.

Refusal/Withdrawal

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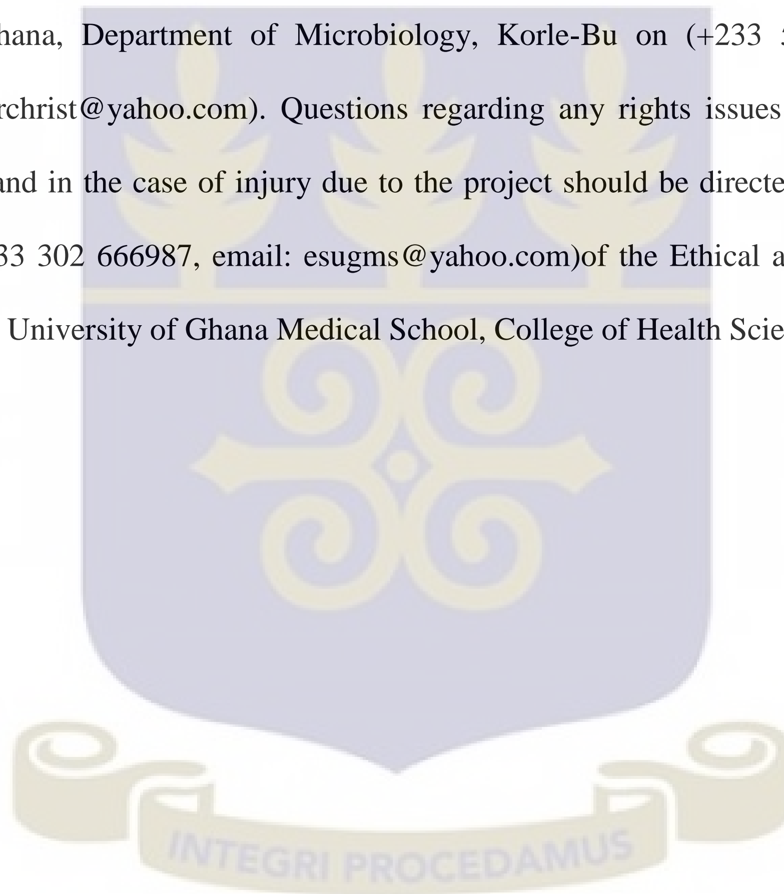
Participation in this study is voluntary; refusal to participate will involve no penalty or loss of medical care. You are free to withdraw consent and discontinue participation in this project at any time. Withdrawal or refusal to participate will not affect the care you receive from the ward/ unit.

Rights and Complaints

Any questions concerning the research project, participants can call Noah Obeng-Nkrumah, University of Ghana, Department of Microbiology, Korle-Bu on (+233 548 394763, email: successfulnoahforchrist@yahoo.com). Questions regarding any rights issues as a person in this research project and in the case of injury due to the project should be directed to the chairperson (Contact Tel; +233 302 666987, email: esugms@yahoo.com)of the Ethical and Protocol Review Committee of the University of Ghana Medical School, College of Health Sciences.



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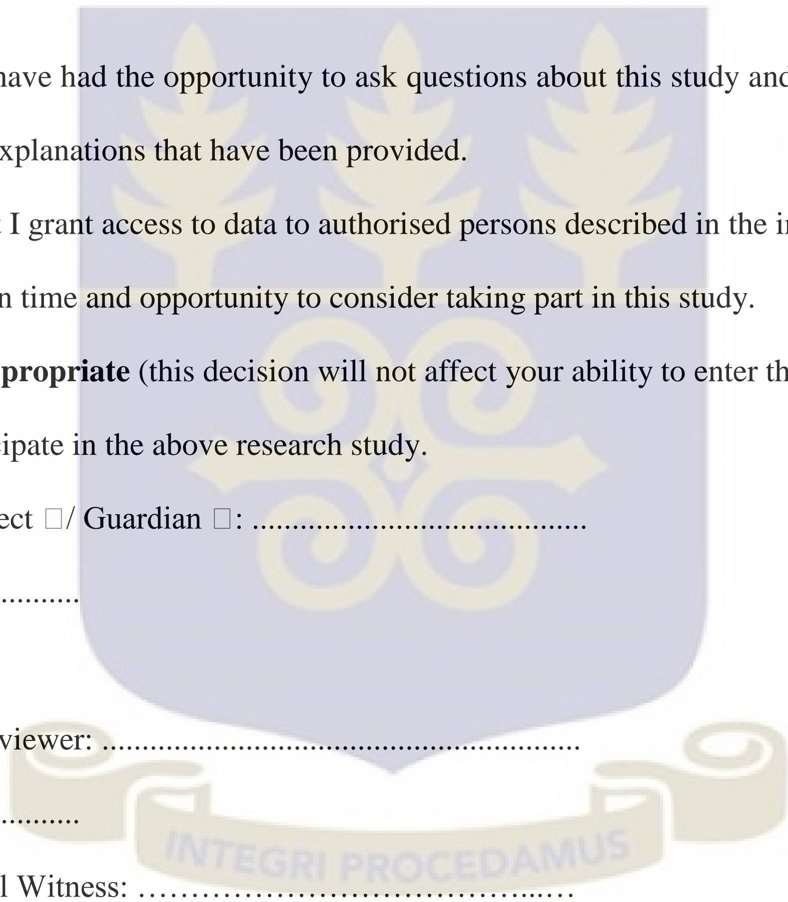
Consent to participate in Research

I, …………………………………………………………………………………………..…………

□ confirm that I have read the written information (or have had the information read to me) for the study on **Extended Spectrum Beta-Lactamase Genes in Clinical Isolates of *Escherichia coli* and *Klebsiella pneumoniae* from the Tamale Teaching Hospital** and that the study procedures have been explained to me by study staff during the consent process for this study.



□ confirm that I have had the opportunity to ask questions about this study and I am satisfied with the answers and explanations that have been provided.

□ understand that I grant access to data to authorised persons described in the information sheet.

□ have been given time and opportunity to consider taking part in this study.

Tick above as appropriate (this decision will not affect your ability to enter the study):

I consent to participate in the above research study.

Signature of Subject □/ Guardian □: ....

Date..............................

Signature of Interviewer: ....

Date..............................

Name of Impartial Witness: …………………………………..…

Signature of Impartial Witness: ………………………………

Date..............................

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Appendix II

Questionnaire

Project Title: Extended Spectrum Beta-Lactamase Genes in Clinical Isolates of *Escherichia coli* and *Klebsiella pneumoniae* from the Tamale Teaching Hospital.

Subject Path. Number ………..… Date ……………

In-patient □ Ward/ Folder number ……..…………………...… Date/ Time admitted ………………

Period stayed ………… Brief diagnosis (from folder)………………………………..……………

Out-patient □ Ghanaian □ Non Ghanaian □

Patient's information for the study provided by Patient □/ Guardian □

1) **Personal Profile**

Age ……………… Gender ………..………

Educational Level: None □ Primary □ Secondary □ Tertiary □

Occupation………………………………………………………………………………………

Region……………………………………………………………………………………………

Marital status: Single □ Married □ Separated □ Divorced □ Widowed □

Level of income per month: <GH¢ 100 □ GH¢ 100-250 □ GH¢ 250-500 □ > GH¢ 500 □

2) **Medical History**

Number of hospital admission(s) in a year? …………………………………………......

Number of days spent for hospital admission in a year? ………………………………..

Number of hospital contacts within three months? ……………………………………..

Admission to ICU? Yes □ No □

If yes, length of admission in ICU………………………………………………………

Admission time /stage of infection? ……………………………………………………

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3) **History on healthcare exposure**

Out-patient □/ In-patient □ at the time of culture?

Specimen type: blood □, urinary tract □, wound/ abscess □, respiratory tract □, vaginal discharge □, cerebrospinal fluid □, others □ (please state) ………………………………..……

Etiological agent: *E. coli* □/ *K. pneumoniae* □ (**To be filled by interviewer**)*

Please note the following conditions:

Community onset infections (cultured samples from patients <48 hours after admission, test positive)**, Healthcare-associated infections** (positive cultures <48 hours on admission from another health facility)**, Hospital acquired or Nosocomial infections** (positive cultures from patients after ≥48 hours on admission)

Patient infection: Community acquired □/ Hospital acquired □ (**To be filled by interviewer**)*

Number of patients on the ward? ……………………………………….……………….

Number of household contacts with the outpatient? ……………………………….

4) **History on antibiotics usage**

(**To be filled by interviewer**)*

Who prescribed your previous antibiotics? Physician □, Self-diagnosed □, Unknown □

Did you complete your previous medication? Yes □ No □

If no, why not? (Please state)……………………………………………………………………..

Are you currently on medications? Yes □ No □

If yes, which drugs? (Please state)………………………………………………………………..

How long have you been on the drug? < 1 month □ 3 to 6 months □ > 6 months □

Number of antibiotics used in previous three months……………………………………………….

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Previous antibiotics therapy in three months: Cephalosporins □, days of use………..; Flouroquionolones □, days of use…………; Aminoglycosides □, days of use………..; Cotrimoxazole □, days of use...........................…; others □, days of use…………………………..

Intake of antibiotics before sample was collected for culture? Yes □ No □

5) Life style characteristics

Do you abuse drugs? Yes □ No □

If yes, which type of drugs? (Please state)...

Do you know the side effect of the drugs you abuse? Yes □ No □

If yes, (Please state)…………………………..

Chronic alcoholic? Yes □ No □

Chronic smoker? Yes □ No □

6) Comorbid conditions within 30 days prior to culture date:

Neutropenia (absolute neutrophil count < 500/mm^3)? ………; White blood cell count (…..x10^9/L)

Chemotherapy treatment? Yes □ No □; Dialyses? Yes □ No □;

Supplementary oxygen? Yes □ No□; Diabetes? Yes □ No □;

Respiratory disease? Yes □ No □; Surgical procedures? Yes □ No □;

Underlining infections? Yes □ No □ and Renal disease? Yes □ No □

The presence of indwelling devices such as: Central or arterial venous catheter? Yes □ No □ and Urinary catheter? Yes □ No □ prior to culture date.

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Any other information

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INTEGRI PROCEDAMUS

Please note

*** To be filled by interviewer.**

Thank you for the sample and your time!!!

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Appendix III

Laboratory Protocols

A. *Enterobacteriaceae* Identification Protocol using MINIBACT-E micro-test kits

1. About 4 to 6 morphologically similar colonies of test isolates were used to prepare dense suspension in a 2ml dH_2O and mixed gently to obtain a homogenous suspension (in comparison with 1.0 McFarland as the standard).
2. 200µl of the solution was pipetted into each well of the micro-test kit embedded with their respective substrates (16 wells in all), covered and incubated aerobically at 35 - 37°C for 4 hours.
3. After the 4 hour incubation, about 1 to 2 drops of Nitrite (N), Phenyl-alanine (PD) and Indole (I) reagents were added to wells G, F and E respectively. Again, 2 drops of Voges Proskauer (VP 1) reagent was added to the VP-test well followed by 2 drops of (VP 2) reagent and mixed by puffing vigorously 5 times using the 'puff flask'. This important part of the reaction was done by holding the flask 5-10 minutes, after which the panels were read.
4. The result form the VP test well was recorded first, followed by the other wells and outcome documented in terms of octal codes (4, 2 or 1).
5. The recorded octal codes were used to locate the corresponding strains of the enterobacteriaceae under investigation from the manufacturer's identification manual to know their identity.

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B. Raw Antimicrobial Susceptibility Data (zone sizes in mm)

DATE	SERIAL/ ISOLATE NUMBER	SPECIMEN TYPE FROM WHICH ISOLATE WAS OBTAINED	GRAM	ISOLATE ID USING MINIBACT®	ANTIMICROBIAL SENSITIVITY TEST										PHENOTYPIC ID						ESBL	
															SCRENNING			CONFIRMATION				
					AMPICILLIN	GENTAMICIN	CEFOTAXIME	CEFUROXIME	CHLORAMPHENICOL	NITROFURANTOIN	TETRACYCLINE	CIPROFLOXACIN	AMIKACIN	MERPENEM	CTX	CAZ	CPD	CTX/CA	CAZ/CA	CPD/CA	POSITIVE (> 5mm)	NEGATIVE (< 5mm)
	001	STOOL	GNR (LF)	EC	0	16	0	0	22	0	14	10	18	30	10	12	0	28	26	22	√	
	002	MSU	GNR (LF)	EC	0	10	0	0	0	0	0	0	18	30	10	12	0	30	28	26	√	
	003	MSU	GNR (LF)	EC	0	18	28	20	24	0	0	24	18	30	30	26	26	30	26	26		√
	004	MSU	GNR (LF)	EC	18	0	28	0	0	0	0	0	20	32	30	26	26	30	26	26		√
	005	MSU	GNR (LF)	EC	0	16	26	20	0	0	0	0	22	28	30	30	24	32	30	26		√
	006	STOOL	GNR (LF)	EC	0	18	26	20	0	0	0	0	16	28	28	28	24	30	28	24		√
	007	STOOL	GNR (LF)	EC	0	18	0	20	24	0	0	0	16	30	10	12	0	26	26	20	√	
	008	STOOL	GNR (LF)	EC	0	0	28	24	22	20	0	24	18	28	30	24	24	30	26	26		√
	009	WOUND	GNR (LF)	EC	0	18	22	16	0	0	0	0	20	30	28	24	24	28	26	24		√
	010	MSU	GNR (LF)	EC	0	18	0	0	24	0	0	0	16	30	30	22	28	32	26	28		√
	011	MSU	GNR (LF)	EC	0	14	28	22	0	0	0	22	16	30	32	26	28	32	30	28		√
	012	HVS	GNR (LF)	EC	0	0	8	24	26	0	0	0	18	34	8	10	0	26	24	20	√	
	013	MSU	GNR (LF)	EC	0	18	30	20	0	20	0	20	18	30	30	24	26	30	26	28		√
	014	HVS	GNR (LF)	EC	0	18	0	0	0	0	20	0	16	28	28	24	22	28	26	26		√
	015	MSU	GNR (LF)	EC	0	18	26	20	0	0	0	12	18	30	30	24	28	30	26	28		√

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	016	HVS	GNR (LF)	KP	0	20	28	20	20	24	22	26	20	24	28	24	28	30	26	28		√
	017	HVS	GNR (LF)	EC	12	20	26	22	22	22	22	26	20	26	30	14	26	32	16	30		√
	018	HVS	GNR (LF)	EC	0	0	26	16	18	0	0	0	18	26	28	24	24	28	24	24		√
	019	HVS	GNR (LF)	EC	0	0	0	0	22	0	0	0	16	26	0	12	0	26	26	26	√	
	020	MSU	GNR (LF)	EC	0	18	0	0	26	18	0	20	16	28	0	10	0	24	24	20	√	
	021	MSU	GNR (LF)	EC	0	16	0	0	0	0	0	22	16	26	10	14	0	26	24	22	√	
	022	MSU	GNR (LF)	EC	0	12	0	0	0	0	0	0	14	28	10	10	0	28	26	24	√	
	023	MSU	GNR (LF)	EC	0	0	28	22	22	0	0	0	16	30	30	26	26	30	26	26		√
	024	MSU	GNR (LF)	EC	0	10	24	18	0	0	0	0	14	30	24	24	20	26	26	22		√
	025	MSU	GNR (LF)	EC	0	18	28	0	24	0	0	0	18	28	30	20	28	30	22	28		√
	026	MSU	GNR (LF)	EC	0	0	0	0	0	0	0	0	0	26	0	0	0	26	22	22	√	
	027	SPUTUM	GNR (LF)	EC	0	0	0	0	10	0	14	0	14	26	0	0	0	20	20	18	√	
	028	MSU	GNR (LF)	KP	0	16	0	0	0	0	0	0	16	30	10	18	0	28	28	24	√	
	029	MSU	GNR (LF)	KP	0	20	28	20	0	0	0	0	16	26	30	24	28	32	24	30		√
	030	HVS	GNR (LF)	EC	0	0	30	24	26	0	0	0	24	30	30	26	26	30	26	26		√
	031	MSU	GNR (LF)	EC	0	0	22	22	0	0	0	26	16	26	30	22	22	30	30	28		√
	032	MSU	GNR (LF)	EC	0	16	20	0	20	20	0	24	18	28	28	24	24	28	24	24		√
	033	BLOOD	GNR (LF)	KP	0	0	0	0	0	0	22	0	14	26	10	12	0	28	26	24	√	
	034	SPUTUM	GNR (LF)	KP	0	16	20	20	20	20	14	25	22	26	30	24	26	30	26	28		√

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	035	HVS	GNR (LF)	EC	0	0	28	18	22	0	0	0	18	26	28	26	26	28	26	26		√
	036	MSU	GNR (LF)	EC	0	0	17	20	24	0	0	0	16	28	18	20	14	18	20	14		√
	037	MSU	GNR (LF)	EC	0	10	30	22	18	20	8	14	22	30	32	28	28	34	28	28		√
	038	MSU	GNR (LF)	KP	8	8	30	24	10	20	10	18	16	26	30	26	26	30	26	26		√
	039	MSU	GNR (LF)	EC	0	0	0	8	10	0	0	24	18	26	8	10	0	24	24	20	√	
	040	MSU	GNR (LF)	EC	0	0	22	20	20	18	8	18	14	24	28	24	26	28	26	26		√
	041	MSU	GNR (LF)	EC	0	0	10	0	0	0	0	0	16	26	10	12	0	28	26	24	√	
	042	MSU	GNR (LF)	EC	10	14	28	22	14	10	8	10	28	30	30	26	28	30	26	28		√
	043	MSU	GNR (LF)	EC	0	0	0	8	0	0	0	0	14	24	0	14	0	26	22	0	√	
	044	MSU	GNR (LF)	EC	0	0	0	0	0	0	0	8	14	26	0	14	0	26	26	0	√	
	045	MSU	GNR (LF)	EC	0	0	0	10	0	0	0	8	14	24	0	10	0	24	20	0	√	
	046	HVS	GNR (LF)	KP	0	10	24	22	14	16	10	12	12	22	22	20	20	26	26	26	√	
	047	HVS	GNR (LF)	KP	0	0	28	28	18	18	12	18	16	28	28	20	28	28	26	26		√
	048	MSU	GNR (LF)	KP	0	8	26	22	18	20	0	10	22	30	28	20	20	30	26	26	√	
	049	SPUTUM	GNR (LF)	KP	0	0	24	18	10	16	0	8	18	24	24	16	20	24	22	26	√	
	050	MSU	GNR (LF)	EC	0	0	0	14	0	8	12	18	20	22	0	14	0	28	28	0	√	
	051	SPUTUM	GNR (LF)	KP	0	0	26	20	0	12	0	10	14	26	21	16	24	26	24	22		√
	052	MSU	GNR (LF)	EC	0	0	0	8	0	0	0	10	18	28	0	18	0	28	26	12	√	
	053	MSU	GNR (LF)	EC	0	0	0	10	0	0	0	8	22	24	0	16	0	26	24	10	√	

	054	MSU	GNR (LF)	EC	0	0	0	0	0	8	0	0	18	24	0	14	0	22	24	0	√	
	055	MSU	GNR (LF)	EC	0	0	0	18	0	0	0	10	10	22	8	10	0	30	26	26	√	
	056	HVS	GNR (LF)	KP	0	0	0	14	0	18	10	0	14	26	0	10	0	24	24	20	√	
	057	MSU	GNR (LF)	EC	0	0	0	8	0	0	0	0	12	20	0	0	0	26	22	18	√	
	058	STOOL	GNR (LF)	EC	0	0	8	10	8	0	0	18	20	28	10	16	0	28	26	24	√	
	059	STOOL	GNR (LF)	EC	0	0	0	10	0	0	0	10	20	28	0	14	0	28	26	8	√	
	060	MSU	GNR (LF)	EC	0	8	28	20	18	16	20	18	22	30	28	26	26	28	26	26		√
	061	MSU	GNR (LF)	EC	8	20	30	20	18	24	26	20	26	30	34	30	30	34	30	30		√
	062	MSU	GNR (LF)	EC	0	0	0	8	0	10	12	10	14	24	0	12	0	24	24	10	√	
	063	MSU	GNR (LF)	EC	0	0	0	18	0	0	0	10	16	22	0	16	0	24	24	10	√	
	064	MSU	GNR (LF)	EC	8	18	26	22	12	20	18	26	24	28	30	26	28	30	28	28		√
	065	MSU	GNR (LF)	EC	0	0	0	0	0	0	0	0	20	20	0	0	0	14	14	0	√	
	066	BLOOD	GNR (LF)	EC	0	20	30	30	0	26	0	36	22	26	36	32	32	36	32	30		√
	067	MSU	GNR (LF)	EC	0	20	26	28	0	24	0	26	14	30	34	28	30	32	28	30		√
	068	SPUTUM	GNR (LF)	KP	0	8	10	0	8	22	0	16	20	30	12	16	0	30	26	26	√	
	069	HVS	GNR (LF)	KP	0	10	10	0	0	0	0	0	14	24	12	14	0	30	28	26	√	
	070	HVS	GNR (LF)	KP	12	18	26	26	26	26	22	30	22	26	32	28	30	32	30	30		√
	071	STOOL	GNR (LF)	EC	0	0	24	26	26	0	0	0	22	30	34	26	28	34	26	26		√
	072	STOOL	GNR (LF)	EC	0	22	28	24	0	0	28	0	24	32	30	26	26	30	28	26		√

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	073	MSU	GNR (LF)	EC	0	0	26	20	0	0	0	0	18	26	30	28	26	30	28	26		√
	074	BLOOD	GNR (LF)	KP	6	20	24	22	22	24	22	30	22	28	30	26	28	30	26	28		√
	075	WOUND	GNR (LF)	EC	0	6	8	0	22	0	0	0	26	28	10	12	0	26	26	22	√	
	076	MSU	GNR (LF)	EC	0	0	0	0	30	0	0	0	22	30	8	14	0	28	28	26	√	
	077	BLOOD	GNR (LF)	KP	0	22	26	26	0	26	0	28	22	30	30	26	30	28	30	30		√
	078	MSU	GNR (LF)	EC	24	20	24	26	26	8	10	26	20	30	32	30	28	32	30	30		√
	079	STOOL	GNR (LF)	EC	0	22	0	0	24	0	0	0	24	30	0	0	0	26	26	24	√	
	080	MSU	GNR (LF)	EC	0	0	14	0	26	0	0	0	22	32	14	18	0	32	30	28	√	
	081	HVS	GNR (LF)	EC	0	22	30	28	0	28	0	34	26	34	38	34	34	38	34	34		√
	082	HVS	GNR (LF)	EC	0	22	30	28	26	0	0	0	26	34	36	34	34	38	34	30		√
	083	WOUND	GNR (LF)	EC	0	20	28	26	0	0	0	22	24	34	34	30	30	34	30	30		√
	084	HVS	GNR (LF)	EC	0	22	30	28	0	26	0	32	26	34	36	30	34	36	32	34		√
	085	MSU	GNR (LF)	EC	0	22	26	26	0	26	0	34	26	34	32	30	30	32	28	30		√
	086	MSU	GNR (LF)	EC	0	26	28	10	0	34	0	36	26	36	34	34	26	34	30	22		√
	087	MSU	GNR (LF)	EC	0	24	8	0	0	0	0	0	26	36	10	12	0	30	30	26	√	
	088	HVS	GNR (LF)	EC	0	0	0	0	0	0	0	0	26	32	0	10	0	30	30	26	√	
	089	MSU	GNR (LF)	EC	0	20	26	22	22	0	0	0	26	34	32	26	26	32	26	26		√
	090	MSU	GNR (LF)	EC	0	0	26	20	0	0	0	0	24	34	30	28	26	30	28	26		√
	091	MSU	GNR (LF)	EC	20	20	8	8	0	0	0	0	26	36	14	16	0	36	36	32	√	

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	092	MSU	GNR (LF)	EC	0	0	0	0	0	0	0	0	0	24	0	0	0	14	10	10	√	
	093	HVS	GNR (LF)	EC	0	22	30	24	24	0	0	20	24	34	34	28	28	34	28	30		√
	094	MSU	GNR (LF)	EC	0	22	30	28	26	30	28	38	22	36	38	34	34	38	34	32		√
	095	WOUND	GNR (LF)	EC	0	10	0	0	0	0	0	0	22	28	0	12	0	26	24	22	√	
	096	MSU	GNR (LF)	EC	0	0	8	0	0	0	0	0	20	30	12	12	0	30	28	28	√	
	097	HVS	GNR (LF)	EC	0	20	30	24	0	28	0	30	22	30	32	28	30	32	28	20		√
	098	MSU	GNR (LF)	EC	0	0	8	0	20	0	0	0	20	28	10	12	0	26	26	24	√	
	099	MSU	GNR (LF)	EC	0	20	24	22	0	24	0	28	20	28	28	24	26	28	26	24		√
	100	MSU	GNR (LF)	EC	0	0	30	24	26	26	0	30	20	28	32	28	26	32	28	26		√
	101	BLOOD	GNR (LF)	EC	0	18	0	0	0	0	0	0	20	26	0	10	0	24	24	24	√	
	102	MSU	GNR (LF)	EC	20	20	30	20	24	24	20	30	20	30	32	28	30	32	28	26		√
	103	MSU	GNR (LF)	EC	0	16	20	18	20	20	0	24	22	28	26	26	26	26	26	26		√
	104	SPUTUM	GNR (LF)	KP	0	20	30	24	0	18	0	20	22	30	30	28	30	30	28	30		√
	105	MSU	GNR (LF)	EC	0	20	26	20	0	0	0	0	18	30	30	26	28	30	26	28		√
	106	MSU	GNR (LF)	EC	0	20	24	20	18	0	0	0	16	28	28	24	24	28	24	24		√
	107	WOUND	GNR (LF)	EC	0	10	0	0	0	0	0	18	18	18	0	14	0	26	26	14	√	
	108	SPUTUM	GNR (LF)	KP	0	0	6	0	0	0	0	0	18	30	12	10	0	26	26	18	√	
	109	MSU	GNR (LF)	EC	0	0	20	18	0	0	0	0	14	28	26	24	22	26	24	22		√
	110	MSU	GNR (LF)	EC	0	10	0	0	26	0	0	0	20	26	0	15	0	26	22	22	√	

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	111	WOUND	GNR (LF)	EC	0	22	24	20	0	0	0	0	20	28	28	30	24	30	30	30		√
	112	HVS	GNR (LF)	KP	10	20	24	20	24	16	12	16	16	22	26	24	26	26	24	26		√
	113	STOOL	GNR (LF)	EC	0	12	0	0	0	0	0	0	14	26	0	14	0	24	22	24	√	
	114	HVS	GNR (LF)	KP	0	20	20	20	22	20	10	24	20	22	26	24	26	26	22	24		√
	115	WOUND	GNR (LF)	EC	0	14	0	0	20	0	0	0	14	24	0	14	0	24	22	22	√	
	116	MSU	GNR (LF)	EC	0	10	0	0	20	0	0	0	14	26	0	12	0	22	22	18	√	
	117	MSU	GNR (LF)	EC	0	22	26	22	0	0	0	30	22	28	30	26	28	28	26	26		√
	118	MSU	GNR (LF)	EC	0	14	0	0	0	0	0	0	16	26	8	14	0	24	22	22	√	
	119	HVS	GNR (LF)	KP	0	20	8	0	24	10	0	14	18	24	10	14	0	24	22	22	√	
	120	WOUND	GNR (LF)	EC	0	20	0	0	0	0	0	18	20	26	0	14	0	26	22	22	√	
	121	MSU	GNR (LF)	EC	0	10	0	0	16	0	0	0	16	26	0	10	0	22	18	20	√	
	122	MSU	GNR (LF)	EC	20	16	26	22	24	20	10	24	22	30	28	22	28	28	26	26		√
	123	MSU	GNR (LF)	EC	20	16	26	22	24	20	10	24	22	30	28	22	28	28	26	26		√
	124	MSU	GNR (LF)	EC	14	18	26	25	20	20	18	28	24	26	30	26	28	30	26	28		√
	125	WOUND	GNR (LF)	EC	0	14	20	18	0	0	0	0	20	30	26	30	24	30	30	24		√
	126	BLOOD	GNR (LF)	EC	0	16	20	20	0	0	0	0	18	30	30	30	26	32	30	26		√
	127	HVS	GNR (LF)	EC	0	20	26	22	0	24	0	28	22	30	32	26	28	32	28	28		√
	128	MSU	GNR (LF)	EC	0	18	0	0	20	0	0	0	20	26	0	10	0	28	26	18	√	
	129	SPUTUM	GNR (LF)	KP	0	20	20	20	0	14	0	16	18	26	24	24	22	24	24	22		√

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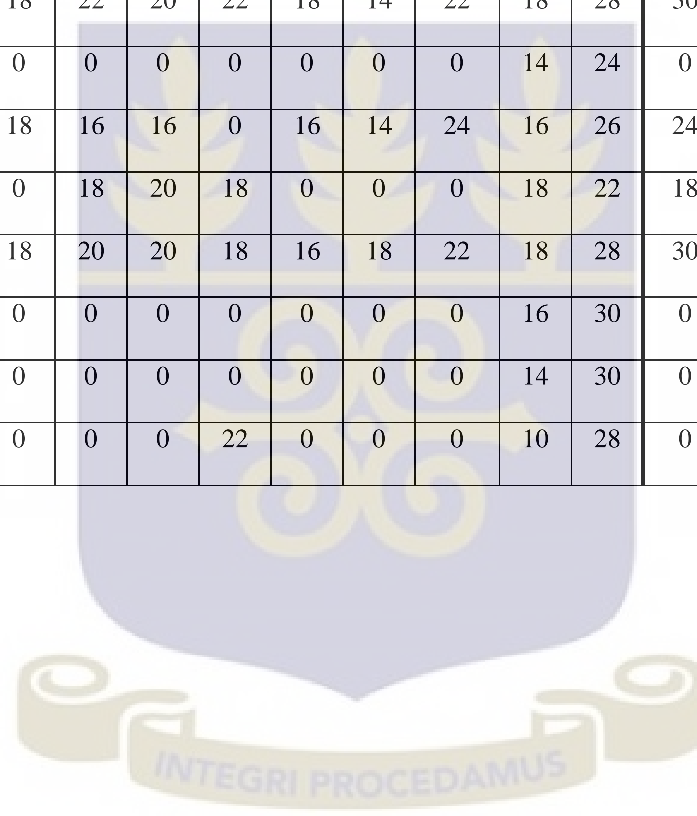
	130	MSU	GNR (LF)	EC	0	18	24	22	22	24	18	30	20	28	30	28	28	30	28	28		√
	131	HVS	GNR (LF)	EC	0	0	0	0	0	0	00	0	16	28	0	10	0	26	24	20	√	
	132	WOUND	GNR (LF)	EC	12	18	14	14	22	0	0	0	16	24	20	18	18	20	18	18		√
	133	MSU	GNR (LF)	EC	0	18	22	20	22	18	14	22	18	28	30	26	26	30	26	26		√
	134	STOOL	GNR (LF)	EC	0	0	0	0	0	0	0	0	14	24	0	0	0	28	24	20	√	
	135	MSU	GNR (LF)	EC	0	18	16	16	0	16	14	24	16	26	24	26	22	28	26	26		√
	136	SPUTUM	GNR (LF)	EC	0	0	18	20	18	0	0	0	18	22	18	16	16	18	16	16		√
	137	BLOOD	GNR (LF)	EC	0	18	20	20	18	16	18	22	18	28	30	24	26	30	26	26		√
	138	STOOL	GNR (LF)	EC	0	0	0	0	0	0	0	0	16	30	0	10	0	30	26	20	√	
	139	MSU	GNR (LF)	EC	0	0	0	0	0	0	0	0	14	30	0	10	0	26	26	22	√	
	140	MSU	GNR (LF)	EC	0	0	0	0	22	0	0	0	10	28	0	14	0	28	24	24	√	



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B. Preparation of DNA Templates using Qiagen Extraction kits

1. About 4 to 6 morphologically similar colonies of test isolates were suspended in 200µl nuclease free water in a 1.5ml microcentrifuge tube and mixed gently to obtain a homogenous suspension.
2. Bacterial cells were lysed by adding 20µl of Qiagen protease (or proteinase K), followed by 200µl Buffer AL and pulse-vortexing, for 15 seconds ensuring a homogeneous solution and efficient cell lysis.
3. The solution was incubated at 56°C for 10 minutes to ensure maximum DNA yield and centrifuged at 6,000xg for 1 minute.
4. Absolute ethanol (200µl) was added to the lysed sample and mixed again by pulse-vortexing for 15 seconds, followed by brief centrifugation at 6,000xg for 1 minute.
5. Lysed sample were carefully applied to the QIAamp Mini spin column (in a 2ml collection tube) without wetting the rim and the cap closed tightly. This was centrifuged at 6,000 x g (8,000 rpm) for 1 minute. The QIAamp Mini spin column was then removed and placed in a clean 2ml collection tube, discarding the previous tube containing the filtrate.
6. Buffer AW1 (500µl) was added to the QIAamp Mini spin column without wetting the rim and centrifuged at 6,000 x g (8,000 rpm) for 1 minute. The QIAamp Mini spin column was then removed and placed in a clean 2ml collection tube, discarding the previous tube containing the filtrate.
7. Buffer AW2 (500µl) was also added to the QIAamp Mini spin column without wetting the rim and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 minutes. Again, the QIAamp Mini spin column was removed and placed in a clean 2ml collection tube, discarding the

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previous tube containing the filtrate. This was further centrifuged at full speed for 1 minute to eliminate the chances of possible Buffer AW2 carryover.

8. The QIAamp Mini spin column was placed in a clean 1.5ml microcentrifuge tube, discarding the previous tube containing the filtrate. DNA was eluted by adding 100µl buffer AE and incubated at room temperature for 1 minute followed by centrifugation at 6,000 x g (8,000 rpm) for 1 minute. The eluted DNA was stored at -20°C.

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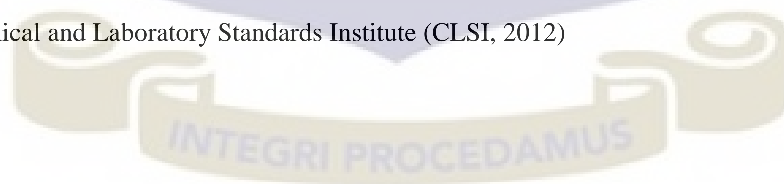
Appendix IV

Performance Breakpoints

Table 2.2 Performance Breakpoints of Reference Strain *Escherichia coli* ATCC 25922 to ESBL Detection Agents

Acceptable zone range (mm)		Total zone size (mm) of *Escherichia coli* ATCC 25922
Screening		
Cefotaxime	(31-39)	34
Ceftazidime	(34-44)	38
Cefpodoxime	(28-32)	28
Confirmation		clavulanate effect ≤ 2mm
Cefotaxime/clavulanate		1.3
Ceftazidime/clavulanate		1.0
Cefpodoxime/clavulanate		1.5

*according to Clinical and Laboratory Standards Institute (CLSI, 2012)



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Appendix V

Preparation of Master Mix

Table 3.2 Preparation of Master Mix from Qiagen

PCR operation	Volume (µl)/ 1 reaction	Master mix for 61 reactions (µl)
2x Qiagen multiplex PCR master mix	12.5	762.5
10x primer mix	2.5	152.5
5x Q solution	2.5	152.5
DNAs/RNAs free PCR water from Qiagen	5.5	335.5
Template DNA	2.0	X
Total mix	25.0	

Volumes of master mix were prepared for a number of PCR reaction tubes plus 1 extra unit to compensate for pipetting errors.

Calculation for Primer mix

Given that:

$C_1=100\mu M$

$C_2 = 4\mu M$

$V_1 = ?$

$V_2=200\mu l$

using $C_1V_1 = C_2V_2$

$$V_1 = \frac{C_2V_2}{C_1}$$

$$V_1 = 8\mu l$$

thus, $V_1 = 8\mu l + 192\mu l\ dH_2O$

Thus, 8µl of the primer stock was pipetted from the whole primer stock after thawing and added to 192µl of dH_2O to make up the 4µl primer working concentration. The primer working solution was then vortexed and kept at -20°C to be used for the general PCR and ESBL genotyping.

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Appendix VI

Supplementary Results

Table 4.6 List of Clinical Isolates Studied Between April and June, 2015

Clinical isolates	Overall	
	Total number	Percentage (%)
Escherichia coli	117	83.6
Klebsiella pneumoniae	23	16.4
Total	140	100

Table 4.7 DNA Concentration and Purity Comparison of Extraction Methods

Method	Mean±SD	P value	95% CI	
			Lower limit	Upper limit
Qiagen DNA Conc	26.9±11.4	<0.001	-211.8	-145.8
Boiled DNA Conc	205.7±130.9	<0.001	-212.2	-145.3
Qiagen DNA purity	1.8±0.2	0.35	0.0065	0.16931
Boiling DNA purity	1.7±0.2	0.35	0.0065	0.16931

SD - Standard deviation, CI - Confidence interval.

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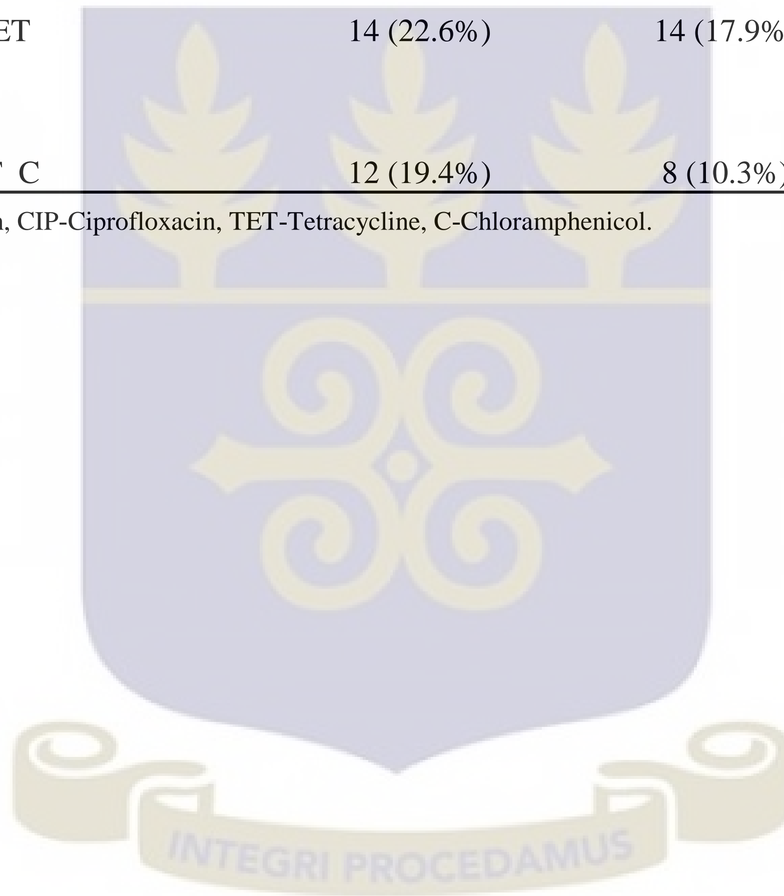
Table 4.8 Enterobacteria Expressing Multidrug Resistant Phenotypes to Amikacin, Ciprofloxacin, Tetracycline and Cotrimoxazole

Antimicrobial drugs combinations	Multidrug resistant (MDR) phenotypes	
	ESBL-producers n=62	Non-ESBL-producers n=78
AMK CIP TET	14 (22.6%)	14 (17.9%)
AMK CIP TET C	12 (19.4%)	8 (10.3%)

* AMK-Amikacin, CIP-Ciprofloxacin, TET-Tetracycline, C-Chloramphenicol.



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Appendix VII

Gel photograph of TEM genes amplified

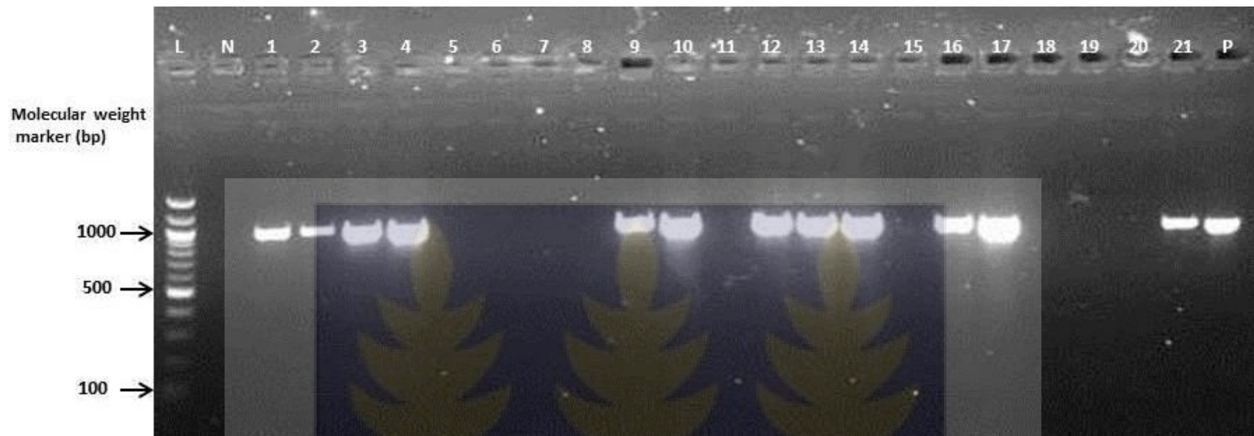

Figure 4.4 PCR Profile for *Bla*$_{TEM}$ of Enterobacteria Isolates

Lane L – Ladder. Lanes N and P - Negative and Positive controls respectively.

Lanes numbered 1 – 4, 9, 10, 12 – 14, 16, 17 and 21 are positive for *Bla*$_{TEM}$ genes. Lanes numbered 5 – 8, 11, 15 and 18 – 20 are negative for *Bla*$_{TEM}$ genes

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Appendix VIII

Gel photograph of CTX-M-1 genes amplified

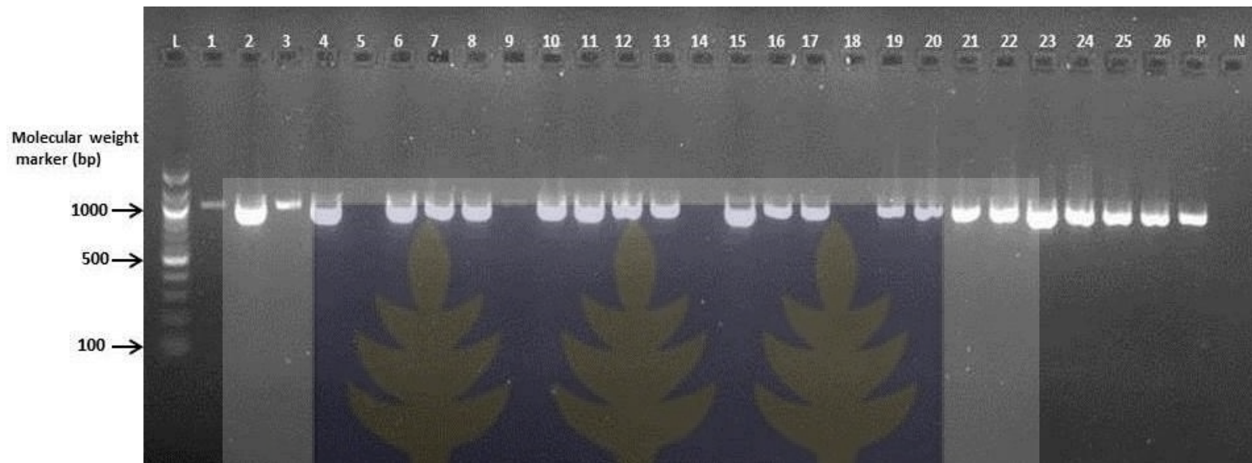

Figure 4.5 PCR Profile for *Bla*$_{\text{CTX-M-1}}$ of Enterobacteria Isolates

Lane L – Ladder. Lanes P and N - Positive and Negative controls respectively.

Lanes numbered 1 – 4 and 6 – 26 are positive for *Bla*$_{\text{CTX-M-1}}$ genes. Lane numbered 5 is negative for *Bla*$_{\text{CTX-M-1}}$ genes

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Appendix IX

Gel photograph of SHV genes amplified

Figure 4.6 PCR Profile for *Bla*$_{SHV}$ of Enterobacteria Isolates

Lanes L, P and N – Ladder, Positive and Negative controls respectively.

Lanes numbered 26, 27, 33, 46 and 47 are positive for *Bla*$_{SHV}$ genes. Lanes numbered 2, 7, 12, 19, 20, 21, 22, 28, 31, 39, 41, 43, 44, 45 and 51 are negative for *Bla*$_{SHV}$ genes

INTEGRI PROCEDAMUS

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Appendix X

Ethical Clearance

UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES
ETHICAL AND PROTOCOL REVIEW COMMITTEE

My Ref. No. **MS-AA/C.2/Vol.18^A** 10th April, 2015

Mr. Francis Morgan Tetteh
Department of Microbiology
SBAHS
Korle-Bu

ETHICAL CLEARANCE

Protocol Identification Number: **MS-Et/M.7 – P 3.2/2014-2015**

The Ethical and Protocol Review Committee of the College of Health Sciences on 26th March, 2015 unanimously approved your research proposal.

TITLE OF PROTOCOL: **"Extended Spectrum Beta-Lactamase in Clinical Isolates of *Escherichia Coli and Klebsiella Pneumoniae* in Tamale Teaching Hospital"**

PRINCIPAL INVESTIGATOR: **Mr. Francis Morgan Tetteh**

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Ethical and Protocol Review Committee at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study during and after implementation.

Please note that any significant modification of this project must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the Ethical and Protocol Review Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

This ethical clearance is valid till October, 2015.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed:
PROFESSOR ANDREW A. ADJEI
CHAIRMAN, ETHICAL AND PROTOCOL REVIEW COMMITTEE

cc: Provost, CHS
Head of Department
Research Office