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INTESTINAL CARRIAGE OF EXTENDED-SPECTRUM BETA-LACTAMASES PRODUCING ENTEROBACTERIA IN IMMUNOCOMPETENT PATIENTS

TAWIAH GLORIA DELLA
(10551508)

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MSC MEDICAL LAB. SCIENCE DEGREE

JULY, 2017

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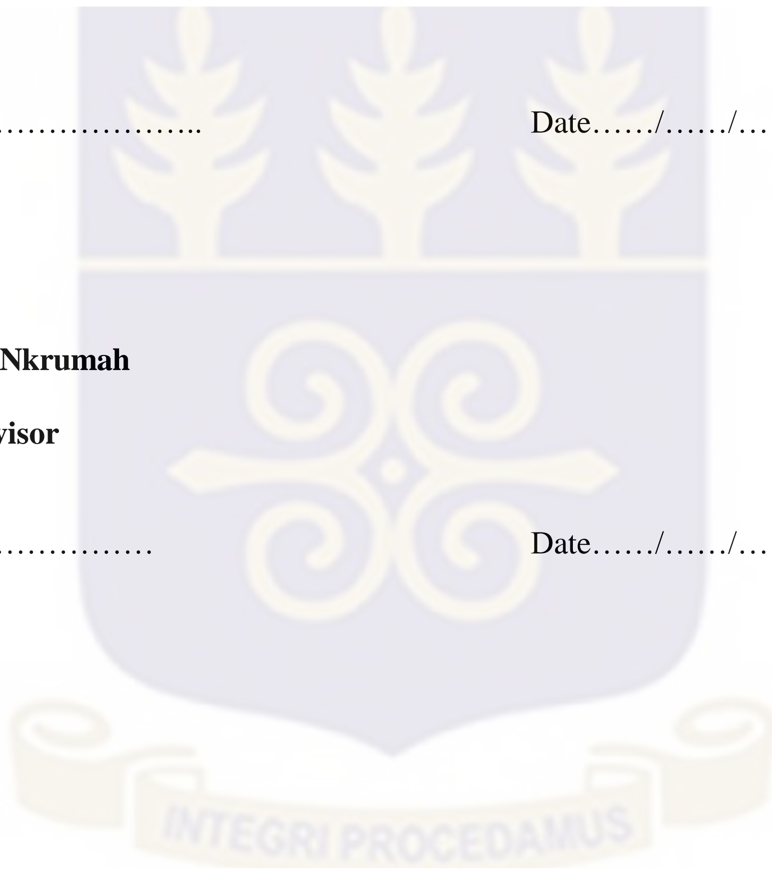
DECLARATION

I hereby declare that this is the product of my own research undertaken under the supervision and has neither been presented in whole nor in part for another degree elsewhere. I am solely responsible for any residual flaws in the work.

Tawiah Gloria Della (10551508)
(Student)

Signature……………………….. Date……/……/………….



Dr Noah Obeng-Nkrumah

Principal Supervisor

Signature…………………… Date……/……/………………

Dr Enid Owusu

(Co-supervisor)

Signature…………………… Date……/……/………………

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ABSTRACT

Background: The intestinal tract serves as a reservoir for ESBL–producing *Enterobacteriaceae* and colonized patients act as a source of dissemination and infections. What is not clear in literature is whether the immune status of patients influences ESBL intestinal colonization patterns. Available studies on ESBL faecal colonization have not clearly distinguished between immunocompetent and suppressed patients.

Aim: The aim of the study was to examine immunocompetent patients for faecal carriage with extended-spectrum beta-lactamase-producing enterobacteria at a district care hospital setting in Ghana.

Method: Between March and May 2017, a cross-sectional sampling was performed to enrol patients and conduct questionnaire-structured interviews for factors that predispose patients to ESBL faecal carriage. Faecal samples from study patients were quantified to determine the predominant ESBL-producing enterobacteria. PCR and sequencing were used to characterize ESBL genes.

Results: The overall faecal carriage prevalence of ESBL was 35.5% (n = 38/107). The $bla_{CTX\text{-}M\text{-}}$ gene, mostly CTX-M-15, was detected in 79% (n = 30/38) of the ESBL-producing isolates. Other ESBL types detected include $bla_{SHV(n=3)}$ and $bla_{OXA(n=1)}$. The CTX-M-15-positive isolates, when present in a faecal sample, constituted the predominant faecal enterobacteria-with significantly higher colony counts than all other enterobacteria in a faecal sample. In multivariate regression, the following were identified as independent risk factors for faecal carriage with ESBL-producing enterobacteria: hospitalization in the past 1 year, infections since admission, use of antibiotics in past 6 weeks, and admission from another hospital.

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Conclusion: Nearly one in every 3 patients included in the study was colonized by ESBL-producing enterobacteria. The high colonization level is worrying, therefore prudent antimicrobial use should be adopted in the hospital.

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DEDICATION

This work is dedicated to Almighty God,

my parents DSP/Mr. Christopher Tawiah and Mrs Jemima Tawiah,

my mentor, Dr Noah Obeng Nkrumah

Your inspirations have been most helpful and dearly appreciated.

To God be the glory, great things He has done

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ACKNOWLEDGEMENTS

My sincere gratitude goes to the Almighty God for giving me the grace and strength to go through this programme successfully. I am also grateful to my family especially to my Parents, Mr and Mrs Tawiah for the support and encouragement they gave me during the period of study. The financial, emotional, psychological and moral support from my parents, sisters and brother cannot be overlooked. May the good Lord richly bless you. I also thank the Department of Medical Laboratory Science, School of Allied Health Sciences, University of Ghana, for the training, exposure and experience they offered me during my postgraduate studies. I am exceptionally grateful to my supervisor, Dr. Noah Obeng- Nkrumah and my Co-supervisor, Dr. Enid Owusu for their excellent supervision, patience and dedication towards my training in scientific research. To Loretta Antwi of Public Health Reference Laboratory, Mr Michael Olu Taiwu of School of Allied Health Science Microbiology Laboratory, I say a very big thank you for guiding me through the culturing and identification aspects of my laboratory work. I also want to thank all the teaching and non-teaching staff at the Department of Medical Laboratory Science and other departments. Finally, I appreciate the assistance of both Madam Ruth and Mr Asamoah of Achimota Government Hospital-Achimota, for their great help during my sample collection and being there whenever I needed them. Thank you all very much. May the good Lord continue to bless you.

INTEGRI PROCEDAMUS

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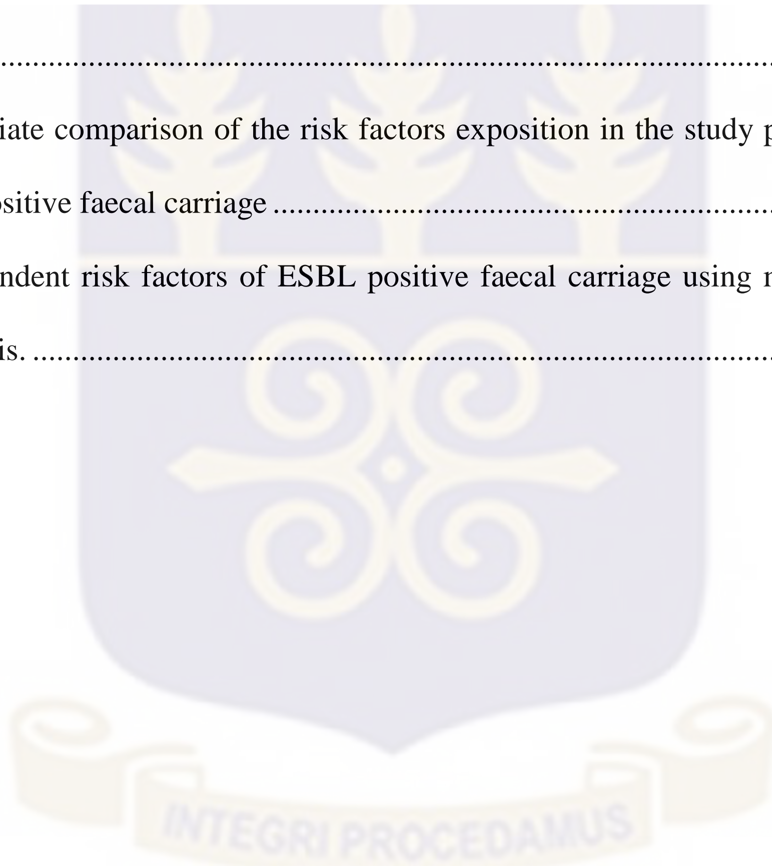

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LIST OF ABBREVIATIONS

ADH	Achimota District Hospital
AMpC	Ampicillin hydrolyzing Cephalosporinase
BES	Brazillian extended -spectrum Beta-lactamases
CAZ	Ceftazidime
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
CTX	Cefotaxime
CTX-M	Cefotaximase
CV	Clavulanic acid
ESBLs	Extended spectrum Beta-lactamase
OXA	Oxacillinase
PCR	Polymerase Chain reaction
PER	Pseudomonas Extended Resistance
SHV	Sulfhydryl variable active sites
SSI	Staten serum Institut
TEM	Temoniera
ul	Micro litre
VEB	veitnamese extended-spectrum Beta-lactamase
GES	Guiana Extended-Spectrum
ug	Microgram
g	Gram
MIC	microgram
AOR	Adjusted Odd Ratio

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CHAPTER ONE

INTRODUCTION

1.1 Background

The most preferred antibiotic in the treatment of infectious diseases globally is the Beta-lactam antibiotics. Although, there are several resistance mechanisms against Beta-lactam antibiotics, Beta-lactamases. are the most common (Lakshmi *et al.*, 2014; Nigam, Gupta & Sharma, 2014). Generally, three broad groups of Beta-lactamases are differentiated: class C cephalosporinases (AmpC), extended-spectrum beta-lactamases (ESBLs) and Beta-lactamases with carbapenemase activity (Ghafourian *et al.*, 2015). Among these enzymes, the ESBLs are the most prominent Beta-lactamase resistant mechanism against Beta-lactam antibiotics. ESBLs enzymes are equipped for hydrolyzing an extensive variety of Beta-lactams, including the most newly developed cephalosporins, yet are not mediated against cephamycins and carbapenems (Dhillon & Clark, 2012). Though, ESBLs are characterized as Beta-lactamases that present resistance to the penicillins, cephalosporins, and monobactams by hydrolysis, however, are sensitive to inhibitors such as clavulanic acid, sulbactam and tazobactam (Brolund, 2014). ESBLs are encoded by point mutations in genes such as TEM, SHV and CTX. A newly-emerging Beta-lactamase group CTX-M ESBLs, are gradually becoming popular around the world (Cantón, González-Alba & Galán, 2012).

The *Enterobacteriaceae* frequently referred to as enterobacteria, are the most prominent Gram-negative bacteria found to be associated with ESBLs (Östholm, 2014). Enterobacteriaceae are represented by over 40 genera with 110 recognized species. Of these, *Escherichia coli* and

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Klebseilla pneumoniae are the widely spread ESBL-producing isolates (Östholm, 2014). Intestinal carriage with ESBL-producing isolates poses serious threats to infection control and prevention (Ebrahimi *et al.*, 2016 ; Titelman *et al.*, 2014). This is because ESBL-producing isolates in carriers is a threat to other individuals (non-carriers) via human to human transmission or through the environment. This reportedly leads to increase in the resistance pool and also aid in the acquisition of resistance mechanisms by susceptible bacteria (Kumar & Babu, 2012). ESBL-producing *Enterobacteriaceae* intestinal carriage is widespread especially in Europe and America (Östholm, 2014), ESBL-producing *Escherichia coli* and *Klebseilla pneumoniae* investigations have been done across hospitalized patients and healthy community persons (Brolund, 2014). In contrast, little research has been conducted in Africa including sub-Saharan countries, where there appears to be the significant spread of infections and colonization with ESBL-producing isolates (Tansarli *et al.*, 2014). In Ghana, there is a limited report. The first published data of ESBL was an acknowledgement of high levels in one of the largest referral hospitals (Obeng-Nkrumah, 2013).

There is the need to conduct more epidemiological surveillance studies for local data to aid in the implementation of infection control and prevention measures which is crucial for monitoring resistance pattern at the local level to help clinical administration (Andriatahina *et al.*, 2010). The present study focuses on the faecal colonization with ESBL-producing *E. coli* and *K. pneumoniae* among patients in a hospital care setting.

1.2 The ESBL problem

ESBLs in *Enterobacteriaceae* renders Beta-lactam antibiotics ineffective. This compels the use of non-Beta-lactam antimicrobials such as ciprofloxacin and amikacin (Andriatahina *et al.*, 2010) in the treatment of infections due to these pathogens. Nonetheless, there is increasing evidence

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that the occurrence of ESBL is associated with the antimicrobial co-resistance to both Beta-lactam and non-Beta-lactam antibiotics, considerably reducing treatment options available for these pathogens (Ebrahimi *et al.*, 2016; Titelman *et al.* 2014). Consequently, the duration of hospital stays, charges and mortality rates are also increased. Plasmids remain the significant wellspring of ESBL transmission adding to the selection of antimicrobial co-resistance among *Escherichia coli* and *Klebsiella pneumoniae* (Baudry *et al.*, 2009). ESBL-producing enterobacteria are increasingly becoming associated with transferable genetic elements, including integrons and transposons, which travel alongside the ESBL-containing plasmids.

In *Escherichia coli* and *Klebsiella pneumoniae*, the genes encoding ESBLs are sometimes carried by transposons borne on transmissible plasmids, which facilitate the rapid transfer of genetic material between unrelated *Enterobacteriaceae* strains (Baudry *et al.*, 2009). The ESBL genes could likewise be discovered incorporated inside integrons as a feature of multi-drug resistance cassettes that present mechanisms for resistance to extended-spectrum cephalosporins as well as to other antibiotic classes such as aminoglycosides, macrolides, sulphonamides and chloramphenicol (Pfeifer, Cullik & Witte, 2010). In addition, the integrons live on conjugative plasmids and are consequently effortlessly transmitted from one strain to the other (Wang *et al.*, 2015).

Extended Spectrum Beta-Lactamase producing organisms have been accounted for as a worldwide rising public health issue. ESBL infections are mostly associated with seriously ill patients who have had a prior hospitalization or prolonged stays in health care facilities, especially intensive care units (Esteve-Palau *et al.*, 2015). Likewise, the presence of indwelling medical devices such as catheters, invasive tubes or arterial lines increases the risk of ESBL infections (Garland, 2014; Chen *et al.*, 2013). Other documented risks, many of which are

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related, include increased severity of illness, a severe underlying disease like malignancy and heart failure, administration of total parenteral nutrition, poor nutritional status, mechanical ventilatory assistance, hemodialysis and recent surgery (Quan *et al.*, 2017; Nakai *et al.*, 2016). The consequences have been several treatment failures and outbreaks of multidrug resistance which required expensive control efforts. Recent works have additionally highlighted faecal carriage as a noteworthy pool of ESBL-positive bacteria in the hospital (Davido *et al.*, 2017; Stedt *et al.*, 2015). Colonization of the intestinal tract with ESBL-positive isolates leads to infection. Therefore, a faecal carriage with ESBL-positive enterobacteria is of clinical importance (Davido *et al.*, 2017; Stedt *et al.*, 2015).

1.4 Problem statement

Beta-lactams (e.g., cefotaxime) remains the most broadly utilized antibiotics in sub-Saharan Africa including Ghana. However, resistance to Beta-lactam antibiotics is progressively turning into a worldwide problem in the management of infections caused by members of the family *Enterobacteriaceae* particularly *E. coli* and *K. pnuemoniae*. The presence of ESBLs in *E. coli* and *K. pneumoniae* remain the chief resistance mechanism against the Beta-lactam antibiotics. ESBL-producing organisms may be endemic in Ghana. Indeed, it is the experience in Ghana that about 40% of the infecting *E.coli* and *K. pneumoniae* isolates in Korle-Bu Teaching Hospital (the nation's largest tertiary care facility) are ESBL-producing (Obeng-Nkrumah *et al.*, 2013). Similar prevalence rates have been reported elsewhere in Ghana. Infections with ESBL-producing *Enterobacteriaceae* increases the risk of antibiotic treatment failure, morbidity and mortality, length of hospital stay, and cost of hospitalization (Quan *et al.*, 2017; Nakai *et al.*, 2016).

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Prior intestinal colonization with ESBL-producing isolates has been identified as significant risk factor for ESBL infections (Davido *et al.*, 2017; Stedt *et al.*, 2015). Intestinal colonization with ESBL—producing *Enterobacteriaceae* are reportedly a frequent occurrence among patients admitted in the hospitals. Such colonized patients remain reservoirs − serving as conduits for the dissemination ESBL-producing *Enterobacteriaceae* among patients and the hospital environments − and as revolving doors for the spread of resistant pathogens between the community and hospital (Davido *et al.*, 2017; Stedt *et al.*, 2015). This phenomenon increases the hospital potential as a repository of multidrug resistant pathogens, with a consequential increase in hospital acquired infections and its associated economic burden to the patient, hospital and country (Quan *et al.*, 2017; Nakai *et al.*, 2016). Despite these menace, limited studies have reported on the intestinal carriage of ESBLs in Africa. In Ghana, there are no published article on infections and intestinal colonization with ESBL-producing isolates.

Many efforts for reducing the ESBL menace are proposed in literature. One of such approaches involves reducing the effect of patient characteristics that may predispose them to intestinal colonization with ESBLs (Bar-Yoseph *et al.*, 2016; Rieg *et al.*, 2015; Kumar *et al.*, 2014). An important factor that predisposes patients to ESBL intestinal colonization is the misuse and abuse of antibiotics. This is more pronounced in immunosuppressed patients where antibiotics are systematically used as prophylaxis to prevent infections. There is, however, a paucity of data to infer correlations between immunosuppression, immunocompetence, and ESBL intestinal colonization. The present study is part of a larger explorative study on immunocompetence and ESBL intestinal colonization. The larger project is a comparative study to examine ESBL intestinal colonization in immunosuppressed and immunocompetent patients in age and time matched patient cohorts. This present study was designed to report the occurrence of intestinal

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colonization with ESBL-producing *Enterobacteriaceae* among immunocompetent patients — with a particular focus on quantification of ESBL-producing isolates and on genotypes of ESBLs. Furthermore, risk factors for intestinal colonization with these resistant bacteria were analyzed.

1.5 Justification

The primary objective of this study is to examine the occurrence of intestinal colonization with ESBL-producing enterobacteria in immunocompetent patients in a hospital setting and determine factors predictive of this phenomenon. This is important in understanding the possible mechanisms for dissemination of ESBL resistance and risk factors for colonization (Nakai *et al.*, 2016; Reuland *et al.*, 2016). Foremost such awareness can lead to successful infection control and proper patient management efforts which is especially crucial in the settings of developing countries where there is limited financial tolerance for longer hospital stays due to persistent illness (Bar-Yoseph *et al.*, 2016; Kumar *et al.*, 2014). Also, the study will add up to knowledge on the poorly defined diversity of ESBL genes in enterobacteria from Africa. In hospital care settings, ESBL faecal carriage status is also essential for instituting interventions and provision of guidance for research. In addition, such information provides baseline data to help in the implementation of active surveillance for multidrug-resistant bacteria.

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1.6 Aim

To determine the faecal carriage of extended-spectrum beta-lactamase producing enterobacteria in immunocompetent inpatients at a district care hospital setting in Ghana.

1.5 Specific Objectives

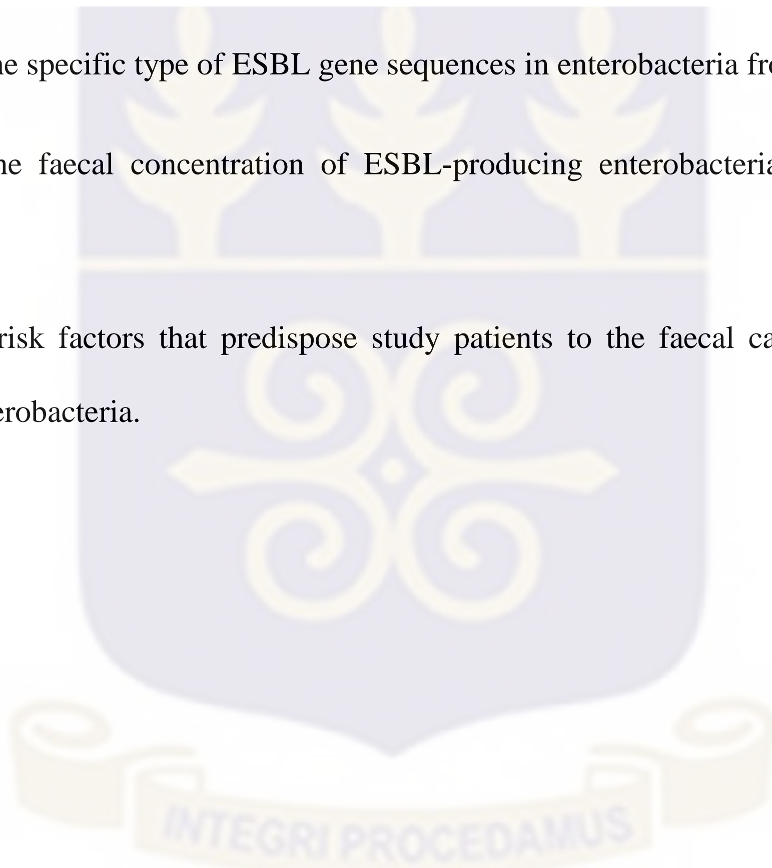
The specific objectives of the study were:

a) to determine the occurrence of ESBL-producing enterobacteria among study patients.

b) to determine the specific type of ESBL gene sequences in enterobacteria from (a) above.

c) to examine the faecal concentration of ESBL-producing enterobacteria among the study patients.

d) to determine risk factors that predispose study patients to the faecal carriage with ESBL-producing enterobacteria.



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CHAPTER TWO

LITERATURE REVIEW

2.1 EXTENDED SPECTRUM β-LACTAMASES (ESBLS)

2.1.1 Background

The introduction of the first Beta-lactam antibiotic, Penicillin, helped greatly incurring infections caused by bacterial species. Prolong use of this antibiotic led to the production of a resistance mechanism, Penicillinase (Beta-lactamase) that hydrolyzed the beta-lactam antibiotic. The resistance mechanism was later found to spread to same or other bacterial species. through a mobile genetic material called Plasmid. The first plasmid-mediated Beta-lactamase (inhibited by clavulanic acid) was described and named TEM-1 in the early 1960s from a Greek patient named Temoniera within an *Escherichia coli* that showed resistance mainly to amino penicillin (Datta & Kontomichalou, 1965). Other types of plasmids encoded Beta-lactamases such as SHV-1 was found in *Klebseilla pneumonia* and later in other *Enterobacteriaceae* (Chaves *et al.*, 2001).

TEM-1 Beta-lactamase also spread to *Klebsiella pneumonia* and other Gram-negative bacterial (Kiratisin, Apisarnthanarak, Laesripa & Saifon, 2008). The rapid spread of TEM-1 and SHV-1 was as a result of their carriage in conjugative transposons and plasmids. TEM-1 producing *K. pneumonia* has progressively become endemic in many hospitals (Khosravi, Hoveizavi & Mehdinejad, 2013; Chang, Siu, Fung, Huang & Ho, 2001).

Over the past years, overcoming the menace of beta-lactamases has led to the development of many new generations of Beta-lactam antibiotics with increased spectrum and more resistance to Beta-lactamases enzymes. Nevertheless, new mutants of Beta-lactamase emerge from a narrow spectrum to an extended-spectrum, hydrolyzing many types of Beta-lactam antibiotics including the third generation cephalosporins used to treat patients (Newire, Ahmed, House, Valiente &

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Pimentel, 2013). These enzymes spectrums of activity against the oxyimino-cephalosporins (extended-spectrum cephalosporins) termed Extended Spectrum Beta-lactamases (ESBLs). Since then, the prevalence of ESBL-producers has gradually increased in acute care hospitals (Bradford, 2001). ESBL, therefore, mediate resistance to extended-spectrum cephalosporins with an oxyimino side chain: cefotaxime, ceftriaxone, and ceftazidime and oxyimino-monobactam aztreonam. The rates of hydrolysis of these Beta-lactam antibiotics are greater than ten percent , evidently more than that of benzylpenicillin, Beta-lactam inhibitors including the clavulanic acid, tazobactam or sulbactam which essentially mediate activity against these enzymes but not cephamycin and carbapenems (Bush, 2013).

2.1.2 Characterization of Extended-Spectrum β-Lactamases (ESBLs)

The Bush, Jacoby and Medeiros classification of 1995 which correlates molecular structures of Beta-lactamases based on nucleotides and amino acid sequences to their phenotypic functioning now remains the most up-to-date classification scheme for Beta-lactamases (Bush & Jacoby, 2010; Bush, Jacoby & Medeiros, 1995). There are four main groups (1-4) and multiple subgroups in this system. For the most part, Beta-lactamases of a specific group correlate with a particular molecular class in the Ambler's classification system. Technically, ESBLs are Bush-Jacoby-Medeiros group 2be and 2d enzymes belonging to Ambler's class A or class D Beta-lactamases with a serine active site which can hydrolyze oxyimino-Beta-lactam compounds at a rate that is equivalent to or higher than 10% that of benzylpenicillin yet are restrained by active site beta-lactamase inhibitors, for example, clavulanate, sulbactam and tazobactam (Bush, Jacoby & Medeiros, 1995). ESBLs have been grouped into major evolutionary families in view of judgments of their deduced amino acid sequences (Bush & Jacoby, 2010; Bush, Jacoby & Medeiros, 1995).

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Most ESBLs are derivatives of TEM and SHV Beta-lactamase families (Ghafourian *et al.*, 2015). There are over 80 TEM ESBLs and are found mostly in *Escherichia coli* and *Klebsiella pneumoniae* (Brolund & Sandegren, 2016; Storberg, 2014). ESBL-producers were mostly nosocomial isolates of klebsiella, and sometimes *Escherichia coli*, which almost entirely harboured TEM or SHV ESBLs (Thenmozhi *et al.*, 2014). But CTX-M enzymes are replacing TEM and SHV ESBLs as the transcendent ESBLs over Europe (D'Andrea *et al.*, 2013). The CTX-M ESBLs have additionally turned into the most common type of ESBLs described amid the previous 5 years from Canada and South American nations (Brolund & Sandegren, 2016; Storberg, 2014; D'Andrea *et al.*, 2013; Livermore *et al.*, 2007) . Among the CTX-M ESBLs, 11.7% were from South Africa and this denoted the primary report of CTX-M enzymes in the country (Govinden *et al.*, 2006). In another study from Tanzania by Blomberg and others (Tansarli *et al.*, 2014), CTX-M ESBLs were the most predominant ESBL type in *Escherichia coli* strains. Elsewhere in Southwest Nigeria, ESBLs were categorized from 30 selected multidrug-resistant *Klebsiella pneumonia* strains isolated from patients. All the isolates produced at least one type of ESBL, with 57% producing CTX-M enzymes (Storberg, 2014; Tansarli *et al.*, 2014).

2.1.2.1 Functional and molecular grouping

Characterization of the ESBLs will require the need for a short review of Beta-lactamase classification. The classification of Beta-lactamase was based on their hydrolytic spectrum, susceptibility to inhibitors, and whether they were encoded for by the chromosome or plasmids. This first scheme classification was informed by irregularities established during the subsequent fifteen (15) year period from which evolved two distinct approaches were evolved. This included the Ambler molecular classification scheme and the Bush-Jacoby-Medeiros functional

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classification system. A major restructuring as recommended by Bush in 1989 with more updates in 1995. has been the basis for Ambler's scheme ,this scheme, however, lacks differentiation between ESBLs and their progenitors, including details in enzymatic activities (Bush, Jacoby & Medeiros, 1995). This classification system corresponds to the Ambler scheme which was the first classification system used. Ambler and colleagues in 1991 identified four molecular classes A to D beta-lactamases according to amino acid sequences. Class A, C, D are serine Beta-lactamases revealed to have structural similarity whereas Class B are all metalloenzymes with zinc active sites (Bush & Jacoby, 2010).

The Bush, Jacoby and Medeiros classification of 1995 which associates molecular structures of Beta-lactamases based on nucleotides and amino acid sequences to their phenotypic functioning now remains the most contemporary classification scheme for Beta-lactamases. There are four main groups (1-4) and multiple subgroups in this scheme. Essentially, Beta-lactamases of a specific group correlate with a particular molecular class in the Ambler's classification system. Technically, ESBLs are Bush-Jacoby-Medeiros group 2be and 2d enzymes belonging to Ambler's class A or class D Beta-lactamases with a serine active site which is able to hydrolyze oxyimino-Beta-lactam compounds at a frequency explicitly equivalent to or greater than 10% that of benzylpenicillin but remain inhibited via active site Beta-lactamase inhibitors such as clavulanate, sulbactam and tazobactam (Pitout & Laupland, 2008; Bush *et al.*, 1995).

2.1.2.2 Diversity of ESBLs types

The pace of growth of ESBL from parent Beta-lactamases in addition to pre-existing ESBL presently known are more than 200 natural ESBL variants. Generally, they are put together into major and minor ESBL types. Major ESBLs exist frequently and are expressed and detected in many clinical isolates in any parts of the world while the minor types are sometimes challenged

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(Naas, Poirel & Nordmann, 2008). Diversity results after alteration of their narrow spectrum complement. Many of these novel ESBLs which remain typically plasmid-encoded rise naturally from occurring enzymes that are intrinsically broad spectrum (Poole, 2004). The major types which are the typical ESBLs developed from class A TEM (TEM-1or TEM-2) and SHV (SHV-1) enzymes, together with class D ESBLs (OXA family) which have been acknowledged severally. Yet, other non-TEM, non-SHV ,non-OXA ESBLs such as BES, GES, PER, TLA, VEB and CTX-M have been reported worldwide with the most prevalent being CTX-M-type ESBLs (Stürenburg & Mack, 2003; Rupp & Fey, 2003).

2.1.2.2.1 TEM –type ESBLS (Class A)

TEM-type products of ESBL were first reported in 1965 from Athens, Greece of a plasmid-encoded Beta-lactamase in Gram-negative bacilli (Datta & Kontomichalou, 1965). TEM-1 was a non-ESBL resistance to Penicillin and first-generation cephalosporin but not oxyimino-cephalosporin or monobactams (Stürenburg & Mack, 2003). Single amino acid substitution at position 39 of TEM-1 led to the development of TEM-2. TEM-2 hydrolytic properties remained related to that of the TEM-1 enzyme (Barthélémy, Peduzzi & Labia, 1985). TEM -3 was discovered in a strain of *K. pneumoniae* with transferable resistance to higher cephalosporin. Its hydrolytic activity was against cefotaxime and hence the first TEM-type beta-lactamase that showed ESBL phenotypic characteristics. It had the ability to hydrolyze extended-spectrum cephalosporin due to two amino acid substitutions: Lys104Glu and Ser238Gly differing from an amino acid sequence of TEM-2 (Paterson & Bonomo, 2005).

Generally, the bla_{TEM} gene is a 286 amino acid peptide of 23 principal amino acid after the signal sequence on the N-terminal cleaved to form the complete enzyme. Amino acid substitution positions 104 (glutamate to lysine), 238 (glycerin to serine) or 240: (glutamate to lysine) are the

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results of ESBL phenotype among TEM enzymes (figure 2.1.2.2.1). TEM types of ESBL are most often found in *Escherichia coli* and *Klebsiella pneumonia* but are also described in other Gram-negative bacteria. TEM-1 are reportedly the most prevalence in this group (Perez, Endimiani, Hujer & Bonomo, 2007; Stapleton, Shannon & French, 1999).

2.1.2.2.2 SHV Type ESBL

SHV Beta-lactamases are a plasmid-mediated group that were known to comprise at least twenty-three variants besides SHV-1. Most SHV retains extended-spectrum activity against the newer broad-spectrum cephalosporins (Tzouvelekis & Bonomo, 1999). The most prominent description of plasmid-mediated Beta-lactamases in Klebsiella species recounted was named SHV-1 (sulfhydryl variable). The comprehensive sequence of SHV-1 amino acid has been discovered to have 68% amino acid homology with TEM-1enzyme (Bois, Marriott & Amyes, 1995). It is reported that SHV type Beta-lactamase has their likely antecedent in chromosomal penicillinase in *K. pneumonia* (Tzouvelekis & Bonomo, 1999). The SHV-2 enzyme was found to vary from SHV-1 by substitution of glycine with serine at position 238. This is as a consequence in development of affinity of SHV-1 Beta-lactamase to oxyimino-cephalosporin (Barthélémy, Péduzzi, Ben Yaghlane & Labia, 1988). At position 234, Serine residue is efficiently hydrolyzed by ceftazidime and lysine residue at position 240 by cefotaxime (Bonnet, 2004). Currently, SHV-type of more than 40 has been described where charges in amino acid sequence confer the ability to hydrolyze new cephalosporin. Globally SVH-2, SHV-2a SHV-5 SHV-12 are the common SHV type ESBLs (Liakopoulos, Mevius & Ceccarelli, 2016).

2.1.2.2.3 Others

Over the years, the upsurge of non-TEM and non-SHV descent has developed in ESBLs (Paterson & Bonomo, 2005). Noticeable among these are the CTX-M (cefotaximase), OXA

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(oxacillinases), PER (pseudomonas extended resistance), VEB (Vietnamese extended Beta-lactamases) and BES (Brazilian extended Beta-lactamases).

Researchers have identified 7 types of PER ESBL with the first reported in 1991 from a patient in turkey. It was shown to be resistant to ceftazidime and aztreonam but susceptible to carbapenems (Vahaboglu *et al.*, 2001; Danel, Hall, Gur, Akalin & Livermore, 1995) and associated with infections caused by Salmonella species, Acinetobacter species and *Proteus vulgaris* transmissible in a large plasmid (154 kb in size) or in chromosome depending on the different bacterial strains. VEB-1 first originated in *E. coli* isolate from Vietnam (Poirel *et al.*, 1999) while GES from *K. pneumoniae* isolates obtained in France in 1998 (Poirel *et al.*, 2000). GES has in a way been detected in other enterobacteria such as *Pseudomonas aeruginosa* (Poirel *et al.*, 2000).

2.2 GROWING CHALLENGE OF CTX-M TYPE ESBL

In Germany, the first CTX-M was observed within an *E. coli* clinical isolate (Bauernfeind, Schweighart & Grimm, 1990). Subsequently in 2000, dramatic changes arose in the prevalence and types of ESBLs worldwide. CTX-M enzymes have been known to encode genes that often harbor insertion sequence IS*EcpI* which enable them to easily transfer and move to other genetic locations. It is also transmissible by conjugation with high transfer frequencies of 10^7 to 10^2 per donor cell (D'Andrea *et al.*, 2013). These properties make CTX-M not only limited to nosocomial infections but also have the potential to spread beyond the hospital environment heightening public health concerns.

Previously, ESBL -producers were ordinarily nosocomial isolates of Klebseillae, and occasionally *E. coli*, which nearly completely sheltered TEM and SHV ESBLs (Pitout & Laupland, 2008). Nonetheless, CTX-M enzymes are substituting TEM and SHV ESBLs as the

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major ESBLs across Europe (Blanco *et al.*, 2009; Livermore *et al.*, 2007; Hernandez *et al.*, 2005). CTX-M ESBLs had been the most prevalent type of ESBLs described from Canada and South American countries (Livermore *et al.*, 2007; Cantón & Coque, 2006) . However, presently, CTX-M enzymes are recognized to be the predominant ESBL worldwide and also including Africa.Over 150 more CTX-M genes have been identified (www.lahey.org/studies), and classified into five families according to amino acid sequence: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25; recently two groups:CTX-M-74 and CTX-M-75 have been added (Bonnet *et al.*, 2003; Karim, Poirel, Nagarajan & Nordmann, 2001).

In addition, *Escherichia coli* have promptly joined *Klebseillae* as major hosts for CTX-M and this development is worrying. Research by Pagani *et al.*, (2003) indicated that diverse types of CTX-M enzymes could be found in a single hospital. The impact of CTX-M producing enterobacteria causing infections is indicated through colonization of gastrointestinal tract (Ben-Ami *et al.*, 2006). Patients colonized (carriers) become the route of CTX-M producers in health-care settings besides other merit vehicles aimed at dissemination to the community (Cantón & Coque, 2006) and worldwide dissemination through international travellers (Lübbert *et al.*, 2015).

2.3 PREVALENCE AND RISK FACTORS FOR CARRIAGE OF ESBL- PRODUCING *ENTEROBACTERIACEAE.*

ESBL over the past years has increased rapidly with *Enterobacteriaceae* due to the fact that their ESBL genes are carried on plasmids enabling dissemination across species barriers (Ozgumus, Tosun, Aydin & Kilic, 2008). Faecal carriage of ESBL-producing *Enterobacteriaceae* amongst asymptomatic individuals is increasing in the hospital as well as in outside the hospital (Ben-Ami *et al.*, 2006). The gastrointestinal tract is the primary ecological niche for *Enterobacteriaceae*.

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Herein, inter-and intra-species exchange of resistance can happen and upon suitable selective pressure, resistant species can emerge and dominate (Carlet, 2012). Although faecal carriage of antibiotic-resistant bacteria is not an instantaneous menace to the immunocompetent individual, it poses two threats: treatment becomes considerably difficult to treat when auto-infection of a sterile body site occurs and resistant organism may be transmitted to other individuals (Donskey, 2004).

Colonization with ESBL producing isolates remains a possible origin of cross-transmission and privilege for infection. Thus colonizing bacteria may serve as a source for later infection, and consequently will affect the choices of empirical antimicrobial treatment. This was clear in a research conducted by Ben-Ami *et al*., (2016) which proved that 4 of the patients with ESBL-producing *Enterobacteriaceae* colonization (15.4%) developed subsequent ESBL infection. Another study reported, 35 (8.5%) of the patients colonized developed ESBL infection (Reddy *et al.*, 2007). Friedmann *et al*., (2009) had reported the frequency of 8% faecal carriage of ESBL-producing *Enterobacteriaceae* at admission. Notable studies have been documented on the increase in intestinal rates with ESBL-producing *E. coli* and *K. pnemoniae* in various countries (Girlich, Bouihat, Poirel, Benouda & Nordmann, 2014; Schaumburg *et al.*, 2013).

A fecal carriage surveillance studies in Europe indicated that the rate of inpatients colonized by ESBL producers has been addressed by few national studies, yet the few including a Spanish analysis demonstrated that frequency of fecal carriers had increased from 1% to 12% among hospitalized patients between 1991 and 2003 with continuous increase since 2000 in invasive *E coli* and *K. pneumoniae* isolates resistance to cephalosporins (Coque, Baquero & Canton, 2008). In Korea, the prevalence was 12% in *E. coli* and 20% to 30% for *K. pneumonia* (Ko *et al.*, 2008) and 45% faecal carriage in hospitalized high-risk patients (Ko, Moon, Hur & Cho, 2012). In the

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time period between 2011 to 2013, a study conducted showed hospitalized patients faecal carriage with *E. coli* and *K. pneumoniae* rate was 62.7% (Babu *et al.*, 2016).

Certain parts of the world are classified as high-risk areas, and travel to these areas becomes one major risk factor for acquisition of asymptomatic faecal carriage of ESBL-producing *Enterobacteriaceae*. These areas include Indian subcontinent, Middle East, Asia and North Africa (Östholm-Balkhed *et al.*, 2013; Tärnberg *et al.*, 2011; Peirano & Pitout, 2010; Tängdén, Cars, Melhus & Löwdin, 2010). High acquisition with travellers is largely linked to the international spread of *E. coli* clone ST131, a carriage which mostly occurs through ingesting of contaminated food and water (Peirano and Pitout, 2010). Östholm-Balkhed*et al.*, (2013) confirmed one common risk factor for acquisition of ESBL among faecal flora was travel to an international country. The geographical area visited had the greatest impact although there were other factors that significantly affect the risk of ESBL-producing *Enterobacteriaceae* colonization. Healthcare contact and antimicrobial exposure including the cephalosporins and fluoroquinolones have the identification of risk factors for endemic regions for asymptomatic carriage (Wener *et al.*, 2010).

Within hospitals, the spread has been facilitated by carriers with diarrhea, gastroenteritis, urinary catheter and other type of catheters (Spadafino, Cohen, Liu & Larson, 2014; Tängdén *et al.*, 2010). ESBL–producing *Enterobacteriaceae* frequency of nosocomial acquisition is increased with extended duration of hospitalization, doubling to 17% by day 4 or 5 after admission, gradually increasing up to 33 after 10 day or more days of hospitalization (Friedmann *et al.*, 2009). New patients admission to a gastric unit risked factors determined for ESBL-*Enterobacteriaceae* colonization were multiple contacts with hospital within the previous year, chronic catheter use, and high-level dependency. Risk factors such as recent antibiotic treatment,

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multiple hospitalization, old age, crowding of hospital patients in the hospital and broad-spectrum antibiotic were mentioned by other studies for fecal carriage (Isendahl *et al.*, 2012; Friedmann *et al.*, 2009). Additionally, the use of vancomycin and piperacillin-tazobactam has been described as being related to ESBL colonization, by the initial decolonization of the normal flora and subsequent colonization with ESBL strains (Rieg *et al.*, 2015).

2.4 DETECTION OF ESBL CARRIAGE

The emergence of ESBLs has enabled the development of numerous detection strategies found all over the world. The detection of ESBLs could be a major challenge for microbiologists owing to the upsurge of phenotypic differences among strains. Clinical laboratory standard Institute (CLSI) has accordingly, developed screening methods for ESBL-producers including disk diffusion and dilution antimicrobial susceptibility tests (Wayne & Pa, 2007). Comparable results with reported sensitivities of over 94% and specificities over 98% are produced by both methods. Other methods, some of which are commercially available methods of ESBL detection include Vitek ESBL test, E tests, Minimum Inhibitory Concentration (MIC), genetic methods, and Isoelectric focusing (Pitout *et al.*, 1998).

2.4.1 Disk diffusion methods

The CLSI guidelines published in January 2010 (M100-S20) by The Clinical and Laboratory Standards Institute, (2016) involve semi-confluent to confluent growth of Enterobacteria isolates on Mueller-Hinton agar. It mentions a primary screening of *Escherichia coli*, Klebseilla species and *Proteus* species for ESBL production with zone inhibition diameters of < 22 mm for ceftazidime (30 µg), < 27 mm for cefotaxime (30µg). CLSI also created allowance for use of 10µg cefpodoxime disk in ESBL screening using zone diameters of ≤ 22 mm for *Proteus*

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mirabilis and ≤ 17 mm for *Escherichia coli, Klebsiella oxytoca* and *Proteus mirabilis*. Concurrent use of all reagents increases the chances of ESBL detection.

2.4.2 Broth –dilution method

The CLSI procedures (M100-S20, 2016) include dilution methods for screening of ESBL-production in Klebsiella, *Escherichia coli* and Proteus species. A ceftazidime, aztreonam, cefotaxime, or ceftriaxone minimum inhibitory concentration (MIC) of ≥ 2 ug/ml is indicative of ESBL expression. Cefpodoxime MIC remains at ≥ 8ug/ml. Standardization have also been made for *Proteus mirabilis*, which is selected for confirmation at MIC of ≥ 2 ug/ml.

2.4.3 Confirmatory tests for ESBL-producing Enterobacteriaceae

Clinical Laboratory Standard Institute (2016) recommend a combined disk method and an MIC method for ESBL confirmation. This confirmatory test now rest on demonstrating synergy between cefotaxime, ceftazidime or cefpodoxime with or without clavulanic acid.

2.4.3.1 Combined Disk synergy test

Clinical Laboratory Standard Institute protocols employ susceptibility to cefotaxime (30 µg) and cefotaxime (30 µg)/clavulanic acid (10 µg), ceftazidime (30 µg) and ceftazidime (30 µg)/clavulanic acid (10 µg). A zone increase of 5mm or more, in the presence of clavulanic acid for any antibiotic disks indicates ESBL production

2.4.3.2 Broth dilution test

Extended Spectrum Beta-Lactamase confirmation is equally applicable on standard broth dilution methods. ESBL production outcomes are an equal or greater than threefold serial dilution decrease in MICs in the presence of clavulanic acid. This standardization is maintained by use of ceftazidime (0.25 to 128 µg/ml), ceftazidime plus clavulanic acid (0.25/4 to 128/4

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μg/ml), cefotaxime (0.25 to 64 μg/ml), and cefotaxime plus clavulanic acid (0.25/4 to 64/4μg/ml). This method, conversely, is arduous particularly for routine examinations.

2.4.4 Other detection methods

Moreover, other detections methods are broadly categorized into two groups: phenotypic methods and genotypic methods have been considered and recommended by several workers aside CLSI. The phenotypic methods detect the expression of ESBL enzymes in vitro, and the genotypic methods use molecular techniques to detect the presence of ESBL genes.

2.4.4.1 Phenotypic methods

Fundamentally the Kirby-Bauer disk diffusion test methodology has been the backbone behind the phenotypic methods of which the principle is employ that ESBLs hydrolyze third-generation cephalosporins, but are inhibited by Beta-lactamase inhibitors mainly clavulanic acid.

2.4.4.2 Double disk approximation test

First routine ESBL detection method was described in 1987 by Brun-Buisson and colleague. Synergy between 30 μg antibiotic disks of ceftazidime, ceftriaxone, cefotaxime and cefpodoxime placed 30 mm (center to center) from an amoxicillin/clavulanic acid (20 μg/10 μg) was suggestion of ESBL production by this method. For the reason that this method is affected by low ESBL activities which widen inhibition zones, some studies have altered the standard approximation distance to 35 mm for optimum results (Ho, Tsang, Que, Ho & Yuen, 2000; Thomson & Sanders, 1992). In addition, heavy inocula also incline to mask the ESBL synergy. The interpretation of the test hence has been reasonably subjective. Yet, numerous workers have examined this procedure and indicate that, sensitivities and specificities may range from 79 % to 97 % and 94% to 100 % respectively (Mackenzie, Miller & Gould, 2002; Cormican, Marshall & Jones, 1996). A most important advantage of this test is in the fact that the procedure is

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technically simple and remains a convenient method for screening of ESBLs in the laboratory (Ho *et al.*, 2000)

2.4.4.3 Disk replacement method

In 1998, Schooneveldt and colleagues described a modified disk replacement technique for detecting ESBL-producing isolates. The method includes the replacement of three 6mm sterile paper disks inoculated with 20 µl of clavulanic acid (200 µg/ml) at the same spots, after an hour, with ceftazidime, cefotaxime and aztreonam disks on media inoculated with a test organism. Control disks of the antibiotics are instantaneously placed at least 30 mm from these locations. Definition of a positive testis by a zone increase of over 5 mm for the replacement disks compared to the controls. It has been determined that while the sensitivity of this test is similar to that of the double-disk approximation method, the need for a second step render it unfitting for routine laboratory work (Schooneveldt, Nimmo & Giffard, 1998).

2.4.4.4 Three –dimensional method

This method describes that a fully susceptible strain, such as Escherichia coli ATCC 25922 is inoculated onto a Mueller-Hinton agar plate, after which a slit is cut into the media and filled with a heavy inoculum (10^9cfu/mi)of the test organism. Extended-spectrum cephalosporin disks are consequently placed on the surface of the plate 3mm from the slit. A discontinuity in the circular inhibition zone or the production of discrete colonies in the vicinity of the inoculated slit is considered positive. Although this test is not specific for ESBLs, it is more sensitive in detecting ESBLs than the double-disk diffusion test (Thomson and Sanders, 1992). This method is however, technically challenging and labor intensive (Vercauteren, Descheemaeker, Ieven, Sanders & Goossens, 1997).

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2.4.5 ESBL commercial detection methods

Besides the above-mentioned tests some formulations available for ESBL, AmpC and carbapenemase detection have been developed and advertised by commercial institutions. Widely held between the commercially available detection methods are the E-test methods, and other automated detection methods.

2.4.5.1 E-test method

The E-test disk, produced by AB Biodisk in Sweden, is a two-sided plastic strip in which a fixed concentration of clavulanic acid (4 ug/ml) is added to one side of an oxyimino-Beta-lactam MIC gradient. Therefore, ESBL, Amp C and carbapenemase production are indicated by a greater than 8 fold reduction in the MIC of the cephalosporin with clavulanic acid (Cormican *et al.*, 1996). Some workers analyzed this method and have made several important observations (Cormican *et al.*, 1996). They noted that the E-test remains the easiest method for ESBL detection and suitable for routine laboratory work. The reported sensitivities and specificities range from 87 % to 100 % and 95 % to 100 % respectively. A few limitations, nevertheless, were also observed: results are indefinite for weak enzymes, subtle zone deformations are difficult to identify and interpret-test strips are also expensive for routine laboratory detection of ESBL, AmpC and carbapenemase (Perez *et al.*, 2007).

2.4.5.2 Automated ESBL detection method

These are antimicrobial susceptibility test systems automated to perform analyses and interpretations for ESBL phenotypes. They include the Vitek test, produced by Bio Merieux Vitek, (Missouri, United States) which utilizes cephalosporins and cephalosporin-inhibitor combinations in wells on a card to detect ESBLs within 4 to 15 hours (Livermore *et al.*, 2002). MicroScan panels from Dade Behring MicroScan (Sacramento, California, USA) which uses

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dehydrates serial dilutions of cephalosporins and clavulanate combinations in panels and BD Automated Microbiology System from Becton Dickinson Biosciences (Sparks, MO-Maryland, USA) which uses a short incubation system of 6hours to measure growth responses to cephalosporins, with or without clavulanate (Stürenburg, Lang, Horstkotte, Laufs & Mack, 2004). In 2007, Weigand and others matched performance levels of the automated systems and reported that the system with the highest sensitivity was Phoenix (99 %), and then Vitek2 (86 %) and MicroScan (84 %). Specificities were much lower ranging from 52 % (Phoenix) to 78 % (Vitek 2) (Wiegand, Geiss, Mack, Stürenburg & Seifert, 2007).

In an earlier study, Sturenburg *et al*., 2004 compared the abilities of Vitek 2 system and the Phoenix system to correctly detect ESBLs. Whereas the Phoenix system showed 100% detection rate, the Vitek system misidentified 5% of the ESBL producing isolates due to low susceptibilities to either cefotaxime or ceftazidime but not both. These results are comparable to conclusions made by Leverstein-van Hall *et al*., (2002). World Health Organization compared the various automated ESBL tests and documented that the automated systems are capable of detecting the ESBL production with almost the same efficiency as the conventional techniques. The automated systems, however, can be complex and misleading given the underlying software algorithms (Wiegand *et al*., 2007). They also are expensive for routine laboratory work.

2.4.6 Molecular techniques for ESBL detection

Gradually, molecular methods for ESBL detection are gaining recognition. The most common genotypic method has been the Polymerase Chain Reaction (PCR) amplification with oligonucleotides primers to determine genes of specific Beta-lactamases and derivatives of TEM, SHV, OXA and other ESBLs. Thus PCR and subsequent sequencing have become the widely accepted method of choice (Fluit, Visser & Schmitz, 2001; Bradford, 1999).

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The advantage of sequencing is to distinguish between the non-ESBL parent enzymes and different variants of ESBLs. Despite the variable results obtained due to difficulties in reading some sequencing autoradiographs, leading to the introduction of errors in establishing true differences amongst many sequences especially SHV genes, nucleotide sequencing remains the gold standard for the detection of specific ESBL genes present in a strain (Bradford, 1999). For this reason, research and reference laboratories have employed molecular techniques to detect the presence of ESBL genes and their specific subtypes in bacteria isolates.

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CHAPTER THREE

METHODS

3.1 Study Area

This study was conducted among patients recruited from the Achimota District Hospital (ADH) in the Greater-Accra region of Ghana. Achimota is an urban community with a pediatric and adult population of over 200,000 in the Accra Metropolitan Area (Asante *et al.*, 2015). The ADH is approximately a 100-bed capacity primary care government hospital with no intensive care units (Esena & Addo, 2014). The hospital caters for medical and trauma emergencies. There are 7 wards including maternity (15 beds), pre- and post-natal (14 beds), geriatric female (14 beds), children ward (20 beds), male ward (15 beds), and the gastrointestinal ward (15 beds). The hospital has a central laboratory that provides some microbiological services but does not perform procedures in bacteriology.

3.2 Study Design

The study was a cross-sectional survey to document the occurrence of intestinal carriage with ESBL-producing enterobacteria. Questionnaire-based interviews were also conducted at the time of sampling to document factors that may predispose patients to intestinal colonization with ESBL-producing study isolates.

3.3 Participants

The study population comprised immunocompetent in-patients on admission at the ADH. Criteria for immunocompetence was as defined by (Salisbury & Ramsay, 2013) (Appendix 1).

Exclusion criteria. Hospitalized patients living with human immunodeficiency virus (HIV) but immunocompetent were excluded from the study. The HIV patients constitute the study subjects

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in a related ongoing study. Also excluded from the study were patients who provided consent but were unavailable to fill questionnaires or provide stool specimens for laboratory work.

Study isolates. The identification of faecal enterobacteria isolates positive for ESBLs constitutes the primary study outcome.

3.4 Minimum sample size

The sample size was determined using the statistical formula as defined by (Charan & Biswas, 2013);

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

Where Z, 1.96 is the standard score for the confidence interval of 95%; P, is the sample proportion prevalence of faecal carriage with ESBL-producing isolates. Given that there is no published data on the estimate of P in the Ghanaian population, a value of 50% (0.5) will be used.

Using a 9% allowable ERROR,

$$\text{Our minimum sample size, } N = \frac{1.97^2 (0.5)(1-0.5)}{(10/100)^2}$$

$$= \quad 98 \text{ patients}$$

Granting critical factors of time and funding, 107 immunocompetent patients were recruited and examined for intestinal carriage of ESBL-producing *E. coli* and *K. pneumoniae*.

3.5 Procedure for data collection

Data collection were conducted in 3 phases:

a) Phase 1-collection, questionnaire interviews and faecal cultures

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b) Phase 2-phenotypic determination of ESBLs

c) Phase 3-molecular characterizations

3.5.1 Phase-1- Collection, questionnaire interviews and faecal cultures

This phase included patient selection, questionnaire-administered interviews, as well as a collection of faecal samples for laboratory investigations.

Selection of patients: Prior to commencement of the study, appropriate permissions were sought from the hospital authorities. On the day 1 of the survey, all patients hospitalized for ≥ 2 days at the ADH were considered potential study participants. Randomly, the hospitalized patients were introduced to the study. They were provided with copies of the study information. On day 2, the patients previously contacted were requested to join the study. Those who obliged to participate were asked to provide an informed consent (Appendix 2). Their folders were examined with the help of an attending physician to identify those who were immunocompetent (based on the definitions by (Salisbury & Ramsay, 2013) and were without HIV. Only patients who satisfied these criteria were enrolled in the study.

Questionnaire-administered interviews: For all patients entered into the study, data of relevant importance were registered into a standardized questionnaire (Appendix 3). The questionnaire was designed based on ESBL faecal carriage risk factors reported in the literature. These included patients demographic information (age, gender, employment status, educational level), duration of hospital stay until survey day, the source of current hospitalization (home or hospital), and a number of patients in the ward. Some patient lifestyle characteristics were also recorded: use of hand sanitizer at least once in the past 3 months, travel outside Ghana in past 1 year, travel outside a home in past 1 year, pipe water in the household, toilet facility in the household, and animal contact in past three months. Data on patient hospitalization history were

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also collected. These included hospitalization in past 1 year, use of medication that affects intestinal flora, use of antibiotics in past 6 months and bacterial infection since admission. Where necessary, attending physicians were consulted to help clarify patient's hospitalization history.

Faecal culture: Hundred and seven (n = 107) study participants submitted faecal specimens for laboratory investigations. The samples were transported on the ice at 0 ºC to the microbiology laboratory of the Department of Medical Laboratory Sciences, University of Ghana. At the laboratory, 1 g of each faecal specimen was vortexed in 10 ml of 0.9 % sterile saline. Ten-fold serial dilutions of each suspension were prepared at 10^{-1} to 10^{-4}. The serial dilutions were cultured onto Statens Serum Institut (SSI) enteric media (SSI Diagnostica, Denmark) by mixing 1ml of each dilution with 24 ml of molten SSI agar (at 52 ºC) and incubating aerobically at 35-37 ºC for 18-24 hours. The SSI enteric medium combines selective properties with growth differentiation for direct isolation and rapid diagnosis of members of the family *Enterobacteriaceae*.

The faecal concentration of enterobacteria isolates: Quantification was performed by counting growing colonies per enterobacteria morphotype and estimating the number of colony forming units per gram of faecal sample (CFU/g). Estimation of the number of enterobacteria harboured by each faecal specimen (CFU/g) number of colonies per morphotype x 10^1 *(for first faecal dilution)* x $10^{(\text{dilution factor})}$. Each enterobacteria morphotype was sub cultured onto SSI agar for purity.

3.5.2 Phase-2- Phenotypic determination of ESBLs

Each pure culture of an enterobacteria morphotype was subjected to phenotypic ESBL screening and confirmation detection test.

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Screening test for ESBLs: Each enterobacteria morphotype was examined for the presumptive presence of ESBLs using resistance to 3rd generation cephalosporins. The ESBL screening was performed by the agar disc diffusion method of sensitivity testing using cefpodoxime (10ug), cefotaxime (30 ug) and ceftazidime (30 ug) antibiotic discs (RoscoDiagnostica, Tastrup, Denmark). Susceptibility testing was performed according to the Clinical and Laboratory Standard Institute guidelines. Briefly, two to five colonies of each morphotype were vortexed in about 3ml of 0.9% sterile saline. The test inoculum was incubated at room temperature for 15 minutes and the density compared to 0.5 McFarland standard [10^{7-8}Colony forming units (CFU/ml)]. Where necessary, the density of the incubated inoculum was adjusted with sterile 0.9% saline until it equalled that of 0.5 McFarland standard. About 5 µm (loopful) of the inoculum was dispensed to 25 mL of cation balanced Mueller-Hinton agar Mueller Hinton agar (bioMerieux, France) in 90 mm circular plate. The inoculum on the agar was then swabbed in three directions with a cotton-tipped applicator (Oxoid, United Kingdom) to obtain a semi-confluent to confluent growth on the entire agar surface. The moisture was allowed to be absorbed for 15 minutes. Antibiotic disks were then applied firmly to the surface of the agar plate and incubated at 35-37 ^{o}C for 18 to 24 hours aerobically. After incubation, inhibition zone diameters of the various antibiotics were measured and interpreted by CLSI 2016 guidelines. Enterobacteria with zone inhibition diameters of $\leq$ 24 mm for cefpodoxime, $\leq$ 27 mm for ceftazidime and $\leq$ 29 mm for cefotaxime were reported as cephalosporin resistant and positive for ESBL screening.

Confirmation of ESBL production: All cephalosporin enterobacteria were confirmed for the presence of ESBLs using the Kirby-Bauer's method of sensitivity testing according to protocols by the Clinical and Laboratory Standards Institute (CLSI, 2016). ESBL confirmation was done

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by the combined disk synergy assay. This test was performed using ceftazidime antibiotic discs (30 µg) and cefotaxime antibiotic disks (30 µg) with and without clavulanate (10 µg) on cation balanced Mueller-Hinton Agar. The CLSI (2016) interpretative guideline was used to confirm isolates as ESBL-producers. According to the CLSI (2016) reference, the study isolates that demonstrated clavulanic acid effect defined by an increase in zone diameter greater than 5mm for at least one test antibiotic were considered ESBL-producers. *Klebsiella. pneumoniae* ATCC 700603 was used as positive control for ESBL production. *Escherichia coli* ATCC 25922 was used as a negative control.

***Identification of bacteria isolates*:** Enterobacteria isolates determined to ESBL-producing were identified to the species level. Those found to be non-ESBL-producing were identified to the genus level. Representative colonies of each isolate were inoculated into the following biochemical media: peptone broths (Sigma, UK), citrate slants (Sigma, UK), urea agar slants (Sigma, UK), Triple–Sugar-Iron agar slants (Sigma, UK) and motility test semi-solid agar. The inoculated media were incubated aerobically for 18 to 24 hours at 35-37. Following, the media were observed for reactions evident of members of the family *Enterobacteriaceae*. Two to 3 drops of Kovac's reagent (Oxoid, UK) were added to peptone broth cultures to ascertain indole reaction. The biochemical reactions were compared to that of reference strains for presumptive genus identification. Definitive identifications of isolates to the species level were confirmed with the MINIBACT-E ® (SSI Diagnostica, Denmark) according to manufacturer's guidelines, and results read after 5 hours. Only enterobacteria isolates that were ESBL-producing were included in subsequent work.

3.5.3 Phase 3- Molecular characterization

This section involved characterizing cephalosporin resistant *E. coli* and *K. pneumonia* (with

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either ESBL-positive or negative phenotype) by PCR to confirm the presence of gene families encoding these ESBLs. The isolates were subjected to whole DNA extraction. Bacterial DNA template for PCR assays was obtained by the boiling suspension method. This involved suspending two to three colonies of each test isolate in 1 mL for an overnight culture and heating at 90 ºC for 10 minutes using a water bath incubator. The Samples were spun at 12000 rpm for 10 minutes and the resulting supernatant extracted for PCR.

Gene amplification**:** For detection of ESBL genes, SHV, TEM, OXA-2, OXA-10, and the CTX-M group 1, 2, and 9 primers were used (See table 3.1 for primer sequences). The PCR mix for ESBLs included 2 µL of template DNA, 12.5 µL of 2x Multiplex Mastermix (Inqaba, South Africa), 2.5 µL of 10x reverse and forward primer, and 7.5 µL of DNAse/RNase free water (Inqaba, South Africa, South Africa). Previously characterized strains positive for the specific ESBL genes were used as positive controls. *Escherichia coli* ATCC 25922 was used as negative control. All PCR protocols included an initial denaturation of 94 °C for 15 minutes and a final extension at 72 °C for 10 minutes. The TEM PCR was performed at 94 ºC for 15 minutes, 94 ºC for 30 seconds for 30 cycles, 63 ºC for 90 seconds, 72 ºC for 60 seconds, and a final extension at 72 ºC for 10 minutes, with a final hold at 6 ºC. Multiplex PCR assay was performed for CTX-M-1 and CTX-M-2 genes at 94 ºC for 15 minutes and then for 27 cycles of 94 ºC for 30 seconds, 50 ºC for 90 seconds, 72 ºC for 60 seconds, and a final extension at 72 ºC for 10 minutes, with a final hold at 6 ºC in a thermal cycler. Isolates negative for CTX-M-1 and -2 genes examined for CTX-M-9 genes. For CTX-M-9, PCR was conducted at 94 ºC for 15 minutes and then for 27 cycles of 94 ºC for 30 seconds, 50 ºC for 90 seconds, 72 ºC for 60 seconds, and a final extension at 72 ºC for 10 minutes, with a final hold at 6 ºC. Another multiplex PCR assay was also performed for SHV, OXA 2 and OXA 10 genes at 94 ºC for 15 minutes and then for 30 cycles of

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94 ^{o}C for 30 seconds, 63 ^{o}C for 90 seconds, 72 ^{o}C for 60 seconds, and a final extension at 72 ^{o}C for 10 minutes, with a final hold at 6 ^{o}C.

Table 3.1 ESBL PCR Primers

Primer (target)	Primer sequences (5'-3')	T ^{o}C ^{a}	PCR cycles
ESBLs			
TEM (918bp)	FP: GTATCCGCTCATGAGACAATAACCCTG RP: CCAATGCTTAATCAGTGAGGCACC Internal FP CCGGAGCTGAATGAAGCCAT Internal RPCGTTGTTGCCATTGCTGCAG	63°C at 90 seconds	30
SHV (842bp)	FP: CGC CTG TGT ATT ATC TCC CTG TTAGCC RP: TTG CCA GTG CTC GAT CAG CG Internal FP : ACCATGAGCGATAACAGCGC Internal RP: AAGCGCCTCATTCAGTTCCG	63°C at 90 seconds	30
OXA-2 (330bp)	FP: GTTAACAGGGGCTTTGCAGG RP: TGCACGCAGTATCCAGTTGC	63°C at 90 seconds	30
OXA-10 (655bp)	FP: ATGAAAACATTTGCCGCATATGTA RP: ACACCAGGATTTGACTCAGTTCC	63°C at 90 seconds	30
CTX-M-1(940 bp)	FP: GACAGACTATTCATGTTGTTGTTAWTTCG RP: CCGTTTCCSCTATTACAAA Internal FP: GGACGATGTCACTGGCTGAG Internal RP: TTTCGTCTCCCAGCTGTCGGG	50 ^{o}C at 90 seconds	30
CTX-M-2 (253 bp)	FP: ACAGTTGGTGACGTGGCTTAAGG RP: TCAGAAACCGTGGGTTACGA	50 ^{o}C for 90 seconds	30
CTX-M-8/25/26 (690/346bp)	FP1: ACATCGCGTTAAGCGGAT FP2: GCACGATGACATTCGGG RP: AACCCACGATGTGGGTAGC	50 ^{o}C for 90 seconds	30
CTX-M-9 (860bp)	FP: ATGGTGACAAAGAGAGTGCAACG RP: ATGATTCTCGCCGCTGAAGC Internal FP: CAAATTGATTGCCCAGCTCG Internal RP: AAACGTCTCATCGCCGATCG	50 ^{o}C for 90 seconds	30

* T ^{o}C, activation temperature; PCR, polymerase chain reaction

***Gel Electrophoresis*.** All PCR products were analyzed by horizontal gel-electrophoresis in a 2% (weight/volume) self-made agarose gel (SeaKem®GTG®Agarose, Lonza). Gene Ruler 100bp DNA Ladder Plus (Fermentas, Germany) were diluted 1:10 with Mili-Q® water as a size marker.

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Amplification products (5 µl) were diluted 1:4 with water and 0.2 % loading dye. Gels were run at 50 volts for 1 hour plus 15 minutes and stained with Gel Red (Bio-Rad) for 30 minutes. The gels were photographed by use of Ultraviolet trans-illuminator and digital capture system.

***Nucleotide sequencing*:** The PCR products were sent to Macrogen (Seoul, Korea) for nucleotide sequencing. Additional internal primers (Appendix 3) were used for sequencing: CTX-M-1, CTX-M-9, SHV and TEM genes. The DNA sequences from Macrogen were analyzed using the Codon Code analyzer®. Nucleotide- and deduced protein sequences were compared with sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). TEM and SHV beta-lactamase sequences were compared to wild-type *E. coli* TEM-1 and SHV-1 sequences (Gen Bank accession no. AF427133.1 and AF148850 respectively) by using the database at http://www.lahey.org/studies.

3.6 Maintenance of working isolates.

Isolates to be stocked were purified onto 5 % Sheep blood agar and inoculated onto Brain Heart Infusion agar in 1.5 ml cryovial tubes (Sigma, United Kingdom). Inoculated tubes were incubated at 35-37 ^{o}C for 18-24 hours and stored at 5 ^{o}C until further workup.

3.7 Quality control

To establish the quality of media and the potency of antibiotics purchased for this work, quality control assessments were conducted for each batch of study reagents. The Kirby-Bauer method of sensitivity testing was performed with the control strains *Klebsiella pneumoniae* ATCC 700603 and *Escherichia coli* ATCC 25922 for every batch of new antibiotics and media and the results interpreted according to performance standards of CLSI guidelines. Students *t*-test was used to analyze significant differences in inhibition zone sizes of the control strains. Briefly, no

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inhibition zone size of the control strain measured on a representative sample of media or antibiotic should be more than 4 standard deviations away from the midpoints between referenced stated limits of CLSI (2016). Also, not more than 1 in 20 results was outside the stated accuracy limits by CLSI 2016 guidelines. Otherwise, the media or antibiotic is rejected

3.8 Data analysis

Study data was entered into a Microsoft Excel sheet for editing. The results were analyzed using Statistical Package for Social Sciences (SPSS) V16. The study isolates including ESBL-producing isolates were described using relative numbers, proportions or percentages. Similarly, PCR results were discussed using descriptive analysis with proportions and percentages. Overall, point estimates of statistical significance were determined at 2 tailed P-values < 0.05. Categorical data was compared across study parameters using $\chi 2$ or Fisher's exact tests where appropriate. The student's t-test was used to compare continuous data. In this study, faecal colony counts of ESBL-producing *E. coli* and *K. pneumoniae* were compared to that of total non-ESBL-producing enterobacteria. The box and whisker displays were used to illustrate the lower, upper, interquartile range, median and mean faecal colony counts of ESBL-producing *E. coli* and *K. pneumonia* and other ESBL-negative faecal enterobacteria. The odds ratio (OR) was used to quantify the association between independent predictor variables and patients with and without ESBL faecal carriage. From univariate analyses, predictor variables with P-value < 0.05 was examined in multivariate logistic regression models to determine independent risk factors. Predictive accuracy of the models was evaluated by Hosmer and Leme which show goodness-of-fit test with P-value > 0.05 suggesting that the model predicts accurately on average. The area under the ROC (Receiver Operating Characteristic) Curve > 0.7 was used to analyze the discriminatory capability of ESBL faecal carriage versus their respective controls.

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3.9 Ethical consideration

Ethical approval was obtained from the Ethics and Protocol Review Committee of the School of Biomedical and Allied Health Sciences before the study was carried out (Approval number: SBAHS-MD./10551508/AA/5A/2016-2017). Written informed consent was obtained before study patients were enrolled. All study details were explained to participants before requesting for their consent and subsequent enrollment into the study. Information provided to participants included the risk, benefits and the right to refuse participation or withdraw participation from the study at any time. Study participants were assured that enrollment into the study is entirely voluntary and that their clinical care and management would not be jeopardized by refusing to participate in the study. Participants were informed that there will be no financial incentives for participating in this study.

3.10 Financial acknowledgement

This work was supported by the parent project (Intestinal colonization with ESBL in different patient cohorts) led by Dr Noah Obeng-Nkrumah (Department of Medical Laboratory Sciences, University of Ghana) with funds from the Puerperal Infection Work package of the Hospital Acquired Infections (HAI-Ghana) Project (www.haighana.com). The HAI-Ghana project is funded by the Danish International Development Agency financed the whole project.

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CHAPTER FOUR

4.0 RESULT

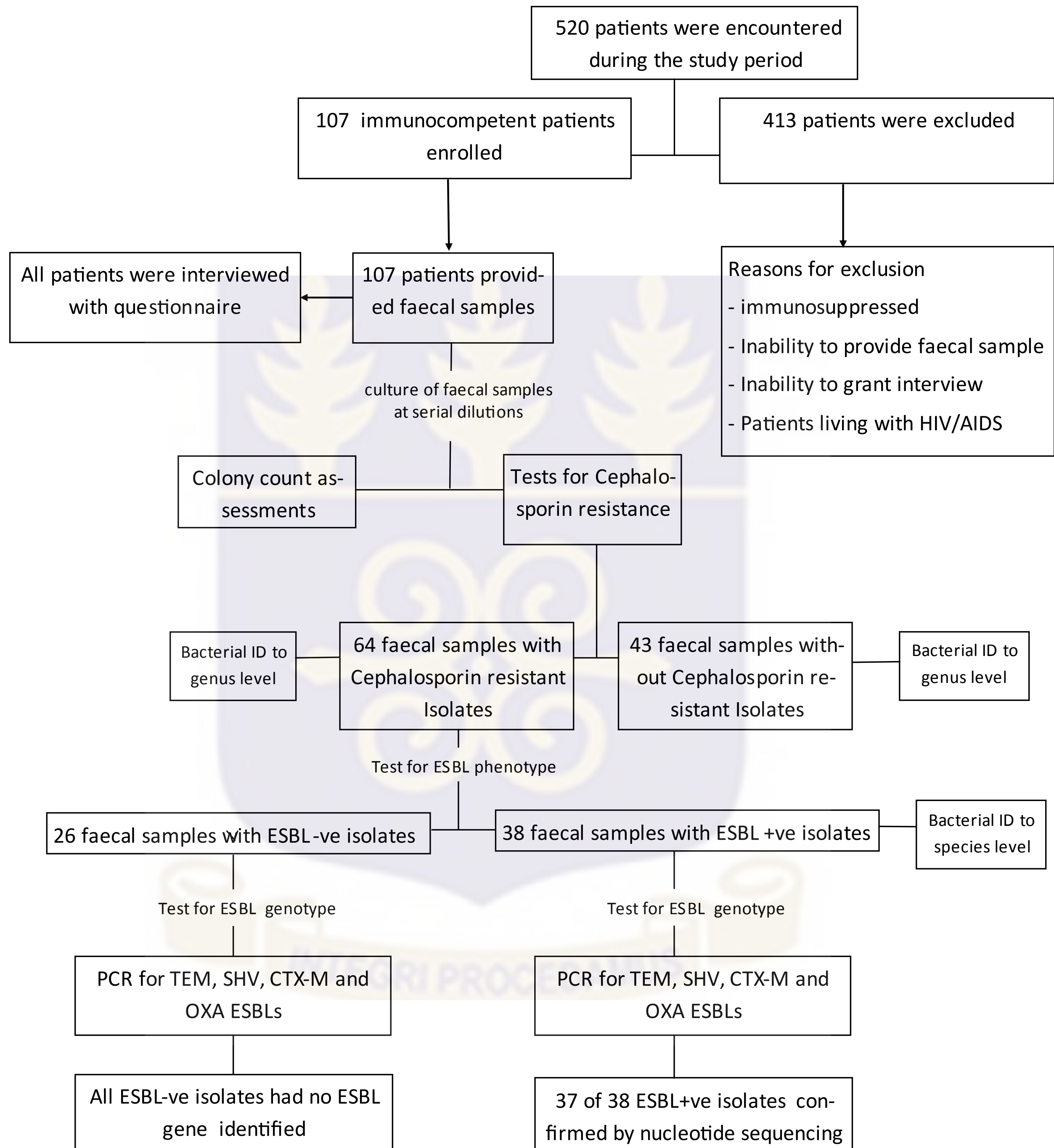

Figure4 1: Flowchart summary of results

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4.1 Demographics of immunocompetent patients enrolled in the study.

Between February and May 2017, 520 in-patients were encountered at the hospital. Of these, only 107 were enrolled in the study (Table 4.1). The excluded patients included those who refused participation and/or were immunosuppressed. Patients who consented participation but were unwilling to be questionnaire-interviewed or unable to provide faecal specimens were dismissed. The 107 study participants were from the pre- and post-natal ward (n = 27, 25.2 %), geriatric ward (n = 26, 24.3 %), male ward (n = 34, 31.8 %), and the gastrointestinal ward (n = 20, 18.7 %). They were (n = 72, 67.3 %) admitted from the home, or from another hospital [n = 35 (32.7 %)]. The mean number of patients in the wards was 8.5±3.0 [95 % confidence interval (CI):9-8] and ranged from 1-14. Forty–two (39.2 %) study patients were males and 65 (60.7 %) were females. The mean age of study patients was 39.4±13.8 [95 % confidence interval (.CI):42-37] and ranged from 13-83. Overall, 77(72.0 %) of the study patients had employment, 6 (5. 6 %) had no formal education, and 64 (59.8 %) had travelled outside Ghana in past year. Of the 107 patients, 75 (70.1 %) had the previous hospitalization in the past 1 year, 61 (57.0 %) no piped water in their household, and 72 (43.0 %) had no toilet facility in their household.

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Table 4.1 Patients Demographics

Description		Number (%)
Age (Mean ±SD)		39.4±13.8
Age group		
	<28 days	0(0.0)
	28 days- 1yr	0(0.0)
	>1yr -5yrs	0(0.0)
	6yrs-15yrs	2(1.9)
	16yrs-65yrs	98(91.6)
	>65yrs	7(6.5)
Gender		
	Male	42 (39.2)
	Female	65(60.7)
Number of persons (mean± SD)		8.5±3.0
Education		
	Primary	40(37)
	Secondary	37(34)
	Tertiary	24(22.4)
	None	6(5.4)
Employment		
	Yes	77(71)
	No	30(28)
Travelled out of Ghana		
	Yes	60(59.8)
	No	43(40.1)
Pipe water in household		
	Yes	61(57)
	No	46(43)
Toilet in household Home		
	Yes	35(32)
	No	72(67.2)
Hospitalization in past year		
	Yes	75(70)
	No	32(30)
Admitted from		
	Home	72(66.3)
	Another hospital	35(32.7)

* SD, standard deviation; %, percentage

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4.2*Enterobacteriaceae* isolates cultured from faecal samples.

In total, 107 faecal samples (1 per patient) were cultured (Table 4.2). A total of 676 *Enterobacteriaceae* isolates were recovered. The most frequently isolated *Enterobacteriaceae* was *Escherichia coli* (n=161), followed by *Klebseilla pneumonia* (n=111), *Citrobacter* species (n=64), Proteus species (n=64), *Providentia* species (n=42) and *Morganella* species (n=22) species. *Escherichia coli* was recovered from all 107 (100%) faecal samples, whiles *K. pneumoniae* was cultured from 71 (66.3%) of faecal specimens. Overall, 83 (77.5%) of 107 faecal samples harbored cephalosporin resistant isolates including *E. coli* (n=37), *K. pneumoniae* (n=12), other *Klebsiella* species (n=10), *Citrobacter* species (n=9), *Proteus* species (n=4), *Providentia* species (n=4) and *Morganella* species (n=4). Only *E. coli* and *K. pneumonia* were found to be ESBL-producing. Of the 83 cephalosporin resistant isolates, only 38 were ESBL-producing [*E. coli* (n=31, 44.5%); *K. pneumonia* (n=7, 8.4%)]. All the ESBL- producing isolates (except 1 *E. coli*) harboured a corresponding ESBL gene after PCR and sequencing. Overall, the prevalence of intestinal colonization with ESBL-producing *E. coli* or *Klebsiella pneumonia* was 35.5%. None of the ESBL-negative enterobacteria harboured an ESBL gene after PCR and sequencing.

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Table 4.2*Enterobacteriaceae* isolates cultured from faecal samples

Faecal enterobacteria	Number of faecal samples (n=107) with			Number of confirmed ESBL gene
	Isolates	Cephalosporin-resistant isolates	ESBL phenotype positive isolates	
Escherichia coli (n=161)	107	37	31	30
Klebseilla pneumoniae(n=111)	71	12	7	7
Other *Klebsiella* species (n=72)	21	10	0	0
Citrobacter species (n=64)	63	9	0	0
Enterobacter species (n=164)	32	4	0	0
Providencia species (n=42)	32	4	0	0
Morganella species (n=22)	11	4	0	0
Serratia species (n=12)	9	4	0	0
Proteus species (n=6)	6	1	0	0
Others (n=22)	8	0	0	0
Total (n=107)		83 (77.5%)	38 (35.5%)	37(34.6%)

* ESBL, extended-spectrum beta-lactamases; No. number; %, percentage

*Only ESBL-producing isolates were identified to the species level

*Other faecal enterobacteria encountered in smaller numbers included *Edwardsiella* species (n=6), *Erwinia* species (n=5), *Hafnia* species (n=4), *Shigella* species (n=4), *Salmonella* species (n=3).

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4.3 Specific type of ESBL gene sequences in *E. coli* and *K. pnuemoniae*

Nucleotide sequence analysis of CTX-M, SHV, TEM and OXA genes are shown in (Table 4.3). All the ESBL- producing isolates had at least one corresponding ESBL gene sequence type except 1 *E. coli* strain (which harboured a $bla_{\text{TEM-13}}$ but no identifiable TEM, CTX-M, SHV or OXA ESBL gene). Overall, 23 (54%) isolates harboured only one ESBL gene, 8 (25%) had 2 ESBL genes, whiles 6 (54%) combined an ESBL plus a broad-spectrum beta-lactamase gene. Eighteen different ESBL gene sequences (12 CTX-M types; 3 SHV types; 2 TEM types; and 1 OXA type) were identified either alone (n=12) or in various combinations (n=6). The most predominant ESBL gene type was $bla_{\text{CTX-M-15}}$(n=11), followed by $bla_{\text{CTX-M-2}}$ (n=5) and $bla_{\text{CTX-M-14}}$ (n=4). Overall, 34 (91.8%) of the ESBL sequenced genes were $bla_{\text{CTX-M}}$ (21.6%) of which were found in various combinations with bla_{SHV} or bla_{TEM} or bla_{OXA} ESBLS. The SHV ESBLs were $bla_{\text{SHV-40}}$, $bla_{\text{SHV-86}}$, and $bla_{\text{SHV-2A}}$; and these were identified among only *K. pneumoniae* isolates. The TEM ESBLs were $bla_{\text{TEM-3}}$ and $bla_{\text{TEM-15}}$. The only OXA ESBL identified was $bla_{\text{OXA-2}}$

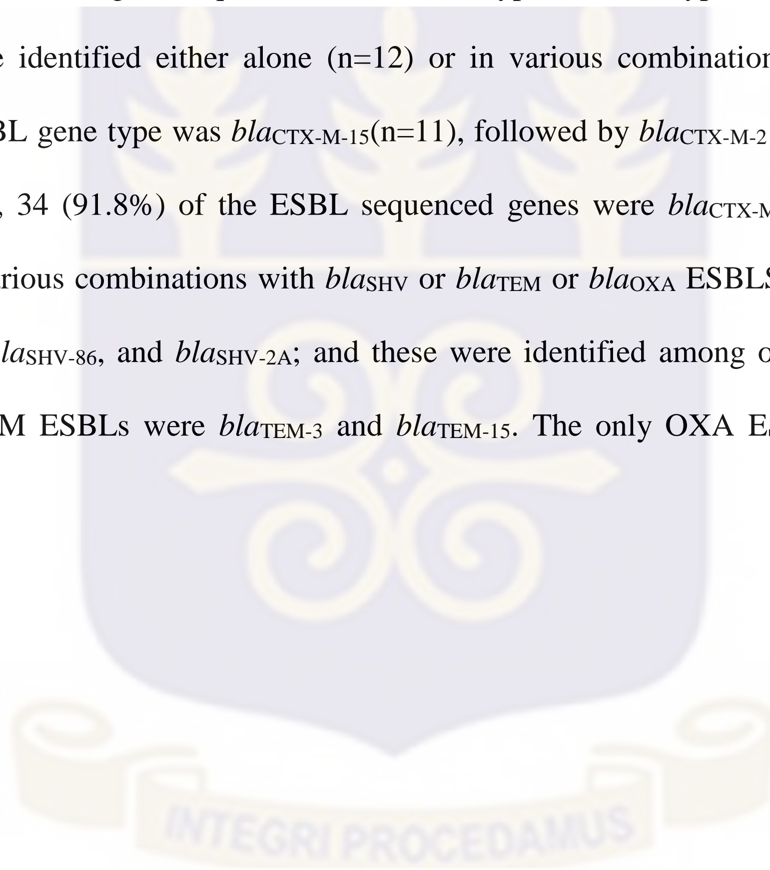

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Table 4.3 Specific type of ESBL gene sequences in *E. coli* and *K. pnuemoniae*

Type of Cephalosporin resistance	**ESBL gene**	***Escherichia coli***	***Klebseilla pneumoniae***	**Total**
ESBL only	SHV-40	0	1	1
	SHV-86	0	1	1
	TEM-3	1	0	1
	CTX-M-2	1	0	1
	CTX-M-3	1	-	1
	CTX-M-5	1	-	1
	CTX-M-14	4	-	4
	CTX-M-15	8	-	8
	CTX-M-20	1	-	1
	CTX-M-27	-	1	1
	CTX-M-31	1	1	2
	CTX-M-57	1	-	1
	CTX-M-1/SHV-40	-	1	1
	CTX-M-2/SHV-2A	-	1	1
	CTX-M-2/TEM-3	1	-	1
	CTX-M-3/TEM-15	1	-	1
	CTX-M-15/TEM-3	1	-	1
	CTX-M-15/TEM-15	1	-	1
	CTX-M-27/OXA-2	-	1	1
	CTX-M-57 ,TEM-3	1	-	1
ESBL +broad Beta-lactamases	CTX-M-2 ,TEM-1	1		1
	CTX-M-9 ,TEM-1	2	-	2
	CTX-M-12 ,TEM-1	1	-	1
	CTX-M-15,TEM-1	2	-	2
Only broad Beta-lactamases	TEM-13	1	-	1

* TEM-1 and TEM-13 are not ESBL gene sequence types

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4.4 Faecal concentration of ESBL-producing *E. coli* and *K. pnuemoniae* among the study patients.

The faecal concentration of ESBL-positive *E. coli*/*K. pneumonia* varied considerably by the type of ESBL gene present (Table 4.4). Among patients (n=11) colonized by a CTX-M-15 positive isolate, the predominant faecal enterobacteria were the ESBL-producers [mean ± SD, $190x10^4$ $\pm 27x10^4$ CFU/g; interquartile range (IQR), $175x10^4$ -$205x10^4$ CFU/g]. The CTX-M-15 positive *E. coli*/*K. pneumoniae* comprised >80.0% of the total enterobacteria counts per faecal samples for all 11 patients. The faecal concentration of all ESBL-negative *Enterobacteriaceae* for each CTX-M-15 colonized patient was $\leq 50x10^4$ CFU/kg (For all 11 patients: mean ± SD, $38x10^4 \pm 6x10^4$ CFU/g; IQR, $34x10^4$- $43x10^4$ CFU/g).

There were 27 patients with ESBL faecal carriage by a non-CTX-M-15 *E. coli*/*K. pneumoniae*. Within this cohort, total ESBL-negative bacteria per faecal sample constituted the predominant faecal isolates (For all 27 patients: mean ± SD, $134x10^4$ CFU/g; IQR, $46x10^4$-$200x10^4$ CFU/g). The mean faecal concentration of non-CTX-15 ESBL positive *E. coli*/*K. pneumoniae* in faecal samples of all 27 patients was $22x10^4 \pm 21x10^4$CFU/kg with an IQR of $10x10^4$- $24x10^4$ CFU/kg. In these faecal samples, the non-CTX-M-15 *E. coli*/*K. pneumonia* averaged about 16.4% of the total faecal enterobacteria.

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Table 4.4 Comparison of the faecal concentration (CFU/g) of ESBL-positive enterobacteria and all ESBL negative enterobacteria in faecal samples of 38 ESBL faecal carriers.

No.	Patients		Concentration x10^4 CFU/g of faecal sample (%)[a]			
			Type of ESBL faecal carriage			
		Type	Total	[b]Isolates with CTX-M-15 ESBL gene	Isolates with other ESBL gene but no CTX-M-15	ESBL negative isolates
1	*Patient 2*	*E.coli*CTX-15/TEM-1	183	150 (81.9)	-	33 (18.0)
2	*Patient 4*	*E.coli*CTX-15	197	162 (82.2)	-	35 (17.7)
3	*Patient 8*	*E.coli*CTX-15	212	171 (80.6)	-	41 (19.3)
4	*Patient 12*	*E.coli*CTX-15/TEM-1	222	178 (80.2)	-	44 (19.8)
5	*Patient 14*	*E.coli*CTX-15	216	181 (83.7)	-	35 (16.2)
6	*Patient 16*	*E.coli*CTX-15	229	189 (82.5)	-	40 (17.4)
7	*Patient 19*	*E.coli*CTX-15	224	190 (84.8)	-	34 (15.2)
8	*Patient 20*	*E.coli*CTX-15	236	191 (80.9)	-	45(19.1)
9	*Patient 24*	*E.coli*CTX-15	270	220 (81.5)	-	50 (18.5)
10	*Patient 26*	CTX-M15/TEM3	262	230 (87.7)	-	32 (12.2)
11	*Patient 29*	*E.coli*CTX-M15/TEM15	266	236 (88.7)	-	30 (11.3)
		Mean±SD; IQR		*190±27 ; 175-205*		*38±6 ;34-43*
12	*Patient 33*	*E.coli*CTX-M14	290	-	12 (4.1)	278 (95.9)
13	*Patient 37*	*K. pneumoniae*SHV86	280	-	10 (3.5)	270 (96.4)
14	*Patient 39*	*E.coli*TEM3	159	-	7 (4.4)	152 (95.6)
15	*Patient 41*	*E.coli*CTX-M-2	283	-	12 (4.2)	271 (95.7)
16	*Patient 42*	*E.coli*CTX-M3	183	-	21 (11.5)	162 (88.5)
17	*Patient 43*	*E.coli*CTX-M5	256	-	56 (21.9)	200 (78.1)
18	*Patient 47*	*E.coli*CTX-M14	223	-	12 (5.3)	211 (94.6)
19	*Patient 50*	*E.coli* CTX-M15/TEM3	181	-	10 (5.5)	171 (94.7)
20	*Patient 52*	*K. pneumoniae*CTX-M27/OXA-2	109	-	9 (8.3)	100 (91.7)
21	*Patient 54*	*E.coli* CTX-M15/TEM15	207	-	7 (3.3)	200 (96.6)
22	*Patient 58*	*K. pneumoniae* SHV40	88	-	10 (11.3)	78 (88.6)
23	*Patient 65*	*E.coli*CTX-M12 ,TEM1	103	-	11 (10.6)	92 (89.3)
24	*Patient 73*	*E.coli*CTX-M9 ,TEM1	230	-	21(9.1)	209 (90.8)
25	*Patient 77*	*E.coli*CTX-M14	79	-	23 (29.1)	56 (70.8)
26	*Patient 81*	*E.coli*CTX-M14	122	-	45 (36.9)	77 (63.1)
27	*Patient 85*	*E.coli*CTX-M20	147	-	67 (45.6)	80 (54.4)
28	*Patient 91*	*K. pneumoniae* CTX-M27	301	-	100 (33.2)	201 (66.7)
29	*Patient 93*	*K. pneumoniae* CTX-M31	294	-	7 (2.3)	287 (97.6)
30	*Patient 97*	*E.coli*CTX-M57	170	-	10 (5.8)	160 (94.1)
31	*Patient 98*	*E.coli*CTX-M20	145	-	24 (16.5)	121 (83.4)
32	*Patient 100*	*E.coli* CTX-M57 ,TEM-3	189	-	33 (17.4)	156 (82.5)
33	*Patient 101*	*K. pneumoniae* CTX-M-1/SHV40	123	-	20 (16.3)	103 (83.7)
34	*Patient 103*	*E.coli* CTX-M3/TEM15	140	-	34 (24.3)	106 (75.7)
35	*Patient 104*	*E.coli*CTX-M9 ,TEM1	297	-	10 (3.4)	287(96.6)
36	*Patient 105*	*E.coli* CTX-M2/TEM3	261	-	11 (4.2)	250 (95.7)
37	*Patient 106*	*E.coli* CTX-M2 ,TEM-1	86	-	9 (10.5)	77 (89.5)
38	*Patient 107*	*K. pneumoniae* CTX-M2/SHV2A	309	-	9 (2.9)	300 (97.1)
		Mean±SD ; IQR			*22±21;10-24*	*134±89 ; 46-200*

* CFU, colony forming units; SD, standard deviation; IQR, interquartile range

[a] %, percent of the total faecal enterobacteria colony counts; [b] for only *E. coli* and *K. pneumoniae*

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4.5 Univariate comparison of risk factors exposition in the study population with and without ESBL-positive faecal carriage.

The results of the univariate analyses on risk factors are presented in Table 4.5. Patients who were admitted from the hospital, compared to home, were significantly more likely to have faecal carriage with ESBL-producing *E. coli* or *Klebsiella pneumoniae* [OR, 3.5; 95%CI, 1.5-8.4; p-value, 0.003]. Patients with a history of hospitalization in the past 1 year were frequently ESBL faecal carriers [OR, 3.6; 95%CI, 1.5-8.6; p-value, 0.003]. Similarly, ESBL faecal carriage was significantly associated with patients who have had infections since admission [OR, 5.2; 95%CI, 2.2-12.3; p-value, <0.001]. Patients' lifestyle practices significantly associated with ESBL faecal carriage included chronic alcohol use [OR, 12.7; 95%CI, 4.9-32.8; p-value, <0.001], and animal contact in past 1 year [OR, 3.7; 95%CI, 1.5-8.5; p-value, <0.001]. Interestingly, diarrhoea [OR, 0.1; 95%CI, 0.02-0.16; p-value, <0.001] and the use of hand hygiene sanitizer in past 3 months [OR, 0.4; 95%CI, 0.1-2.0; p-value, <0.001]seemed to have a protective effect.

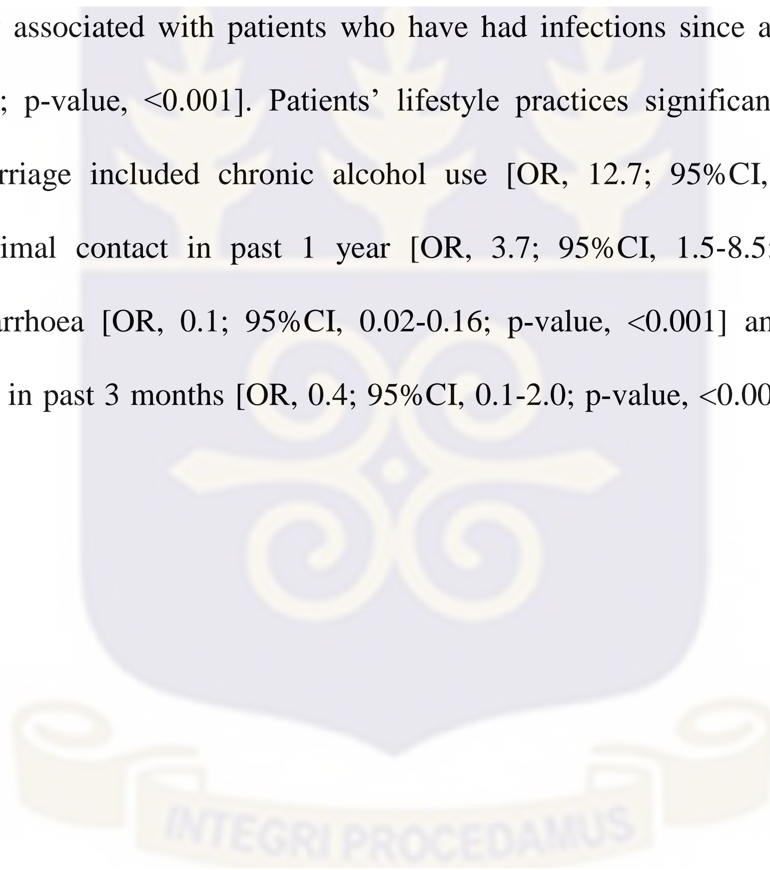

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Table 4.5 Univariate comparison of the risk factors exposition in the study population with and without ESBL-positive faecal carriage

Descriptions	Patients with ESBL faecal carriage (n=38)	Patients without ESBL faecal carriage (n=69)	Crude Odds Ratio (95%CI)	P - Value.
Demography				
Age (Mean ± SD)	39.6±13.8	39.5±13.8		0.1
Age group				
Neonates (<28days)	0	0	0	-
Infants (28days-5yrs)	0	0	0	-
Paediatric (>5yrs-18yrs)	1	1	1.8(0.1-30.2)	1
Adults(>18yrs-65yrs)	36	62	1.9(0.3-10.0)	0.5
Elderly (>65yrs)	1	6	0.3(0.3-2.5)	0.4
Male gender	15	27	1.0(0.4-2.3)	1
No of persons in the ward (Mean ±SD)	8.3±2.8	8.5±2.5		0.7
The total duration of hospital stay (Mean ±SD)	2.9±1.4	3.7±8.6		0.6
Admitted from				
Hospital	18	14	3.5 (1.5-8.4)	0.003
Home	20	55		
Employed	30	47	1.7(0.2-1.4)	0.2
Formal education	36	65	0.4(0.3-0.5)	0.13
Type of formal education				
Primary	10	30	0.4(0.1-1.1)	0.08
Secondary	14	23	1.2(0.5-2.7)	0.7
Tertiary	12	12	2.1(0.8-5.5)	0.09
Patient's lifestyle				
Used hand sanitizer in past 3 months	6	24	0.4(0.1-2.0)	0.04
Frequency of hand sanitizer use per day				
1	0	11	-	0.06
2	2	4	1.6(0.2-11.4)	0.5
3	1	5	0.8(0.1-8.1)	1
4	2	3	3.5(0.4-28.1)	0.5
>4	1	1	4.6(0.2-86.6)	0.3
Daily hand-washing in past 1 month	38	43		
Frequency of hand washing per day				
1	6	13	0.8(0.3-2.3)	0.7
2	9	5	4.0(1.2-13.0)	0.02f
3	8	11	1.4(0.5-4.0)	0.5
4	11	6	0.7(0.3-1.6)	0.4
>4	4	14	0.5(0.1-1.5)	0.2
Chronic smoking	2	4	0.9(0.2-5.2)	1f
Chronic alcohol use	29	14	12.7(4.9-32.8)	<.0001
Travelled overnight outside home in past 1year	26	43	1.3(0.5-3.0)	0.5
Travelled outside |Ghana in past year	10	12	1.6(0.6-4.4)	0.3
Number of patients in ward (Mean ±SD)	8.3±2.8	8.5±2.5	0.7	
Animal contact in past 3 months	20	16	3.7(1.5-8.5)	0.002
Pipe water in household	19	30	1.3(0.5-2.8)	0.5
Toilet in household	30	54	1.0(0.3-2.7)	0.9
Hospitalization history				

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Infection since admission	24	17	5.2(2.2-12.25)	<0.0001
Surgery since admission	11	14	1.6(0.6-4.0)	0.3
Functional status: need help of any sort	9	9	2.1(0.7-5.7)	0.2
Co-morbidities				
Respiratory infections	5	3	3.3(0.7-14.8)	0.1
Diarrhoea	8	56	0.1 (0.02-0.16)	<0.001
Diabetes	7	12	1.1 (0.38-3.0	0.88
Hospitalized in past 1 year	28	30	3.6(1.5-8.6)	0.003
Invasive procedure of any type in past 1year	4	3	2.6(0.5-12.2)	0.2f
Presence of indwelling catheter	24	26	2.8(1.2-6.5)	0.01
Use of medications that affect intestinal flora (stomach acids neutralizer, proton pump inhibitor,H2 blockers)	18	22	1.9(0.8-4.3)	0.1
Used antibiotic in last 6 month	24	23	3.4(1.5-7.8)	0.002
Used antibiotic without prescription	17	19	2.1(0.9-4.9)	0.07
Current antibiotic use	27	47	1.1 (0.4-2.7)	0.75
Specified antibiotics				
aminoglycosides	4	10	0.7(0.2-2.4)	0.76
Beta-lactam	14	27	0.9(0.4-2.1)	0.82
Beta-lactam/aminoglycosides	2	4	0.9 (0.2-5.2)	1
Beta-lactam/fluoroquinolones	6	5	2.4 (0.7-8.5)	0.19
Beta-lactam /macrolides	2	2	1.9(0.3-13.8)	0.61
fluoroquinolones	2	8	0.4 (0.08-2.1)	0.32
Lincosamides	3	3	1.8 (0.4-9.6)	0.67
Macrolides	4	4	1.8(0.4-8.0)	0.45
Metronidazole	2	2	1.8(0.3-13.8)	0.61
Phenols	0	1	-	-
None	1	3	0.6(0.06-5.9)	1

*N, number; CI, confidence interval; ESBL, extended-spectrum beta-lactamases; SD, standard deviation; Risk ratios and Fisher's exact probability tests P-values were calculated for variables with cell counts <5.

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4.6 Independent risk factors of ESBL positive faecal carriage identified using multivariate logistic regression analysis.

The results of the multivariate analyses are presented in Table 4.5. Patients with faecal carriage by ESBL-producing *E. coli* or *Klebsiella pneumonia* had used antibiotics 3.5 times more during the past 6 weeks than patients with a non-ESBL faecal carriage, and this was the strongest predictor for ESBL faecal carriage (AOR, 3.4; 95% CI: 1.5–10.5; p-value =0.0001). Infections since admission was to a lesser degree associated with ESBL faecal carriage (AOR, 3.2; 95% CI: 1.9–7.8; p-value =0.002). Hospitalization in the past 1 year (AOR, 1.6; 95% CI: 1.2–3.6; p-value =0.04) the hospital as source of current admission (AOR, 1.6; 95% CI: 1.1–4.3; p-value =0.003) were also identified as independent risk factors.

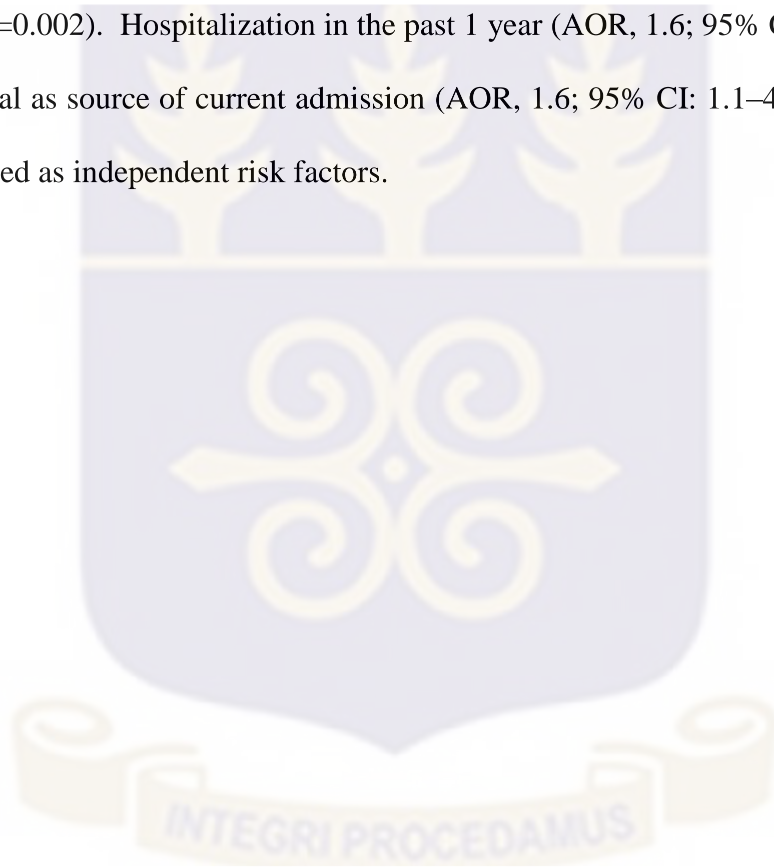

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Table 4.6 Independent risk factors of ESBL positive faecal carriage using multivariate logistic regression analysis.

Variable	Level	Adjusted OR	95% CI	P-value
Admitted from a hospital	Yes/No	1.6	1.1-4.3	0.003
Chronic alcohol use	Yes/No	2.4	0.9-5.3	0.08
Used hand sanitizer in past 3 months	Yes/No	0.7	0.1-2.0	0.07
Diarrhoea	Yes/No	0.9	0.3-1.6	0.11
Animal contact in past 3 months	Yes/No	1.7	0.8-3.5	0.09
Hospitalized in past 1 year	Yes/No	1.5	1.2-3.6	0.04
Presence of indwelling catheter	Yes/No	1.5	0.7-5.5	0.2
Used antibiotic in last 6 month	Yes/No	3.4	1.5-10.5	<0.0001
Infection since admission	Yes/No	3.2	1.9-7.8	0.002

* OR, adjusted Odds ratio; CI, confidence interval

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CHAPTER 5

5.0 DISCUSSION

In recent years, faecal carriage with ESBL-producing *enterobacteria* has been increasingly reported around the world (Karanika *et al.*, 2016). The hospital is widely documented to be a repository of resistant bacteria especially ESBL-producers. This pose a significant health threat to hospitalized patients (Isendahl *et al.*, 2012; Andriatahina *et al.*, 2010). In the present study, we examined the occurrence of faecal carriage with ESBL-producing *enterobacteria* recovered from immunocompetent patients in a district hospital setting in Ghana.

In this study, the overall proportion of faecal carriage of ESBL-producing isolates was 35.5%. The CTX-M genes, mostly *bla*$_{CTX\text{-}M\text{-}15}$ were the predominant ESBL genotype. When CTX-M-15-producing isolates occurred in faecal samples, these isolates were the predominant faecal enterobacteria. In this study, independent risk factors for ESBL faecal carriage included previous hospitalization for past 1 year, admission from another hospital, infections since admission and use of antibiotics during the past 6 weeks.

5.1 Occurrence of ESBL faecal carriage immunocompetent patients

Studies on ESBL faecal carriage among hospitalized patients is globally prevalent but there is little data on the role of immunity on this subject matter. What is not clear in literature is whether ESBL intestinal colonization patterns differ in patients with varying immune status. Although answers to this conundrum are beyond the purview of this study, the findings of this work may offer some preliminary insights. The proportion of patients with faecal carriage (n=38/107) is significantly lower than the ESBL levels reported for another patient host in Ghana by Obeng-Nkrumah *et.al.*, (n=148/300), Hackman *et al.*, (n=202/400), Feglo *et al.*, (n=77/159) and Sarkodie *et al.*, (n=149/300) (Hackman *et al.*, 2016; Hackman *et al.*, 2014; Obeng-Nkrumah *et*

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al., 2013). Although these other studies were conducted among clinical isolates, and without clear distinction between immune-competent or –suppressed patients, perhaps the considerably lower ESBL levels in the present study may point to the protective role of an active immune system.

The proportion of ESBL faecal carriage reported in this study is lower than that cited in Cameroon (55.3%), Morroco (42.9%), and Central African Republic (59%) Lonchel *et al*, 2013;Farra *et al*., 2016). The ESBL level in this study is, however, higher than that reported in Guinea-Bissau (32.5%), Gabon (33.6%), Tanzania (34.3%), America (2%), South Europe (6%) Japan (6.4%) (Tellevik *et al.,* 2016; Karanika *et al*., 2016b; Girlich *et al*., 2014; Schaumburg *et al*., 2013; Isendahl *et al*., 2012; Luvsansharav *et al*., 2011). In all these, the reports were silent on whether the study patients were immune-competent or –suppressed. Indeed, there is limited data on ESBL faecal carriage in immunocompetent patients to compare study findings.

In this study, several enterobacteria isolates were resistant to 3rd generation cephalosporins but were ESBL negative. These isolates perhaps harboured other beta-lactamase types, such as the AmpCs, which are able to mediate resistance to cephalosporins. Indeed AmpC-producing enterobacteria that are resistant to beta-lactams antimicrobials, particularly 3rd generation cephalosporins, are widespread in literature (Gonggrijp *et al*., 2016; Hammerum *et al*., 2010). The global expansion of other beta-lactamase types including AmpCs is considered as a significant contributor to the emergence of multidrug-resistant enterobacteria and a potential threat to the limited antibiotic options globally. Cephalosporins are possibly the most widely used antibiotics in Ghana for empirical therapy in patients with suspected Gram-negative infections. Resistance to these drugs leaves physicians with very limited empiric options for

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treating patients. Carbapenems are considered the treatment of choice. Carbapenem therapy is however very expensive and not many patients may be able to afford it.

In this study, only TEM, SHV, CTX-M, and OXA ESBL genes were amplified. Other less reported ESBLs such as the bla_{PER}, bla_{VER} and bla_{BER} were not sought for in this study. These ESBL types have been reported few and far between in literature. These minority ESBL types have all been reported in the South Americas and Asia, and the possibility of identifying them in this study was rather slim (Naas, Poirel & Nordmann, 2008).

5.2 Characterization of the ESBL gene sequences.

Reports on CTX-M ESBLs, predominantly CTX-M-15, are gradually becoming common worldwide. Literature document CTXMs as the dominant gene in ESBL-producing enterobacteria (Rossolini, Andrea & Mugnaioli, 2008). Cefotaxime plays an important role in the selection for CTX-M ESBLs (Lewis *et al.*, 2007). Overall, CTX-M enzymes represented 91.8% in this study. This situation mirrors the current trend observed across Africa (Storberg, 2014). For its broad-spectrum antibacterial activity, less toxic side effects, and high efficacy, hospitalized patients are most often given cefotaxime leading to extensive and irrational prescription by clinicians. A possible explanation for the dominance of CTX-M genes could be its ability to localize on large plasmids and co-harbor other resistant genes such as bla_{AmpC}, quinolone resistance genes or methylase affecting aminoglycosides (Lahlaoui, Khalifa & Moussa, 2014). The CTX-M-15 borne plasmids, in particular, have been reported to have a high conjugation frequency and are thus more frequently disseminated to other enterobacteria species (Coque *et al.*, 2008). The CTX-M genes are transmissible by conjugation with high transfer frequencies of 10^{-7} to 10^{-2} per donor cell. Perhaps the few TEM, SHV and OXA ESBL types

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identified in this study point to the fact that CTX-M genes are fast replacing other ESBL types (Livermore *et al.*, 2007).

5.3 Faecal concentration of ESBL-producing E. coli /K. pneunoniae.

A noteworthy finding in this study was the fact that whenever CTX-M-15 ESBL-positive isolates occurred in a faecal sample, these isolates constituted the predominant faecal bacteria compared to all other enterobacteria. In such instances, the CTX-M-15-producing isolates constituted over 80% of the total faecal enterobacteria. These patients with CTX-M-15 faecal carriage comprise high-density ESBL shedders and may be of public health significance in the dissemination of multidrug-resistant bacteria. The CTX-M-15 genes spread rapidly among bacteria and also are significantly resistance to different classes of of antibiotics. They frequently carry genes that mediate resistance to aminoglycoside, tetracycline, sulfonamide and fluoroquinolones. Thus, CTX-M-15 producers are often multi-drug resistant (Cantón, González-Alba & Galán, 2012).

5.4 Risk factors of the ESBL-faecal carriage.

In the absence of identifiable risk factors, ESBL faecal carriers may be undetected at hospital admission, resulting in a steady increase in the number of ESBL-producing isolates brought into the hospitals. In multivariate analysis, this study identified antibiotic use in the past 6 months as the highest predictor of the ESBL faecal carriage. Indeed several other studies have linked prior antibiotic use within the past 4 or 12 months to an increased possibility of faecal ESBL colonization. In fact, some observers have even suggested that the more current the antibiotic use, the greater the risk of faecal carriage with ESBL-positive enterobacteria. This is not surprising given that ESBLs and many other antibiotic-resistant mechanisms evolved as a consequence of misuse and abuse of antibiotics.

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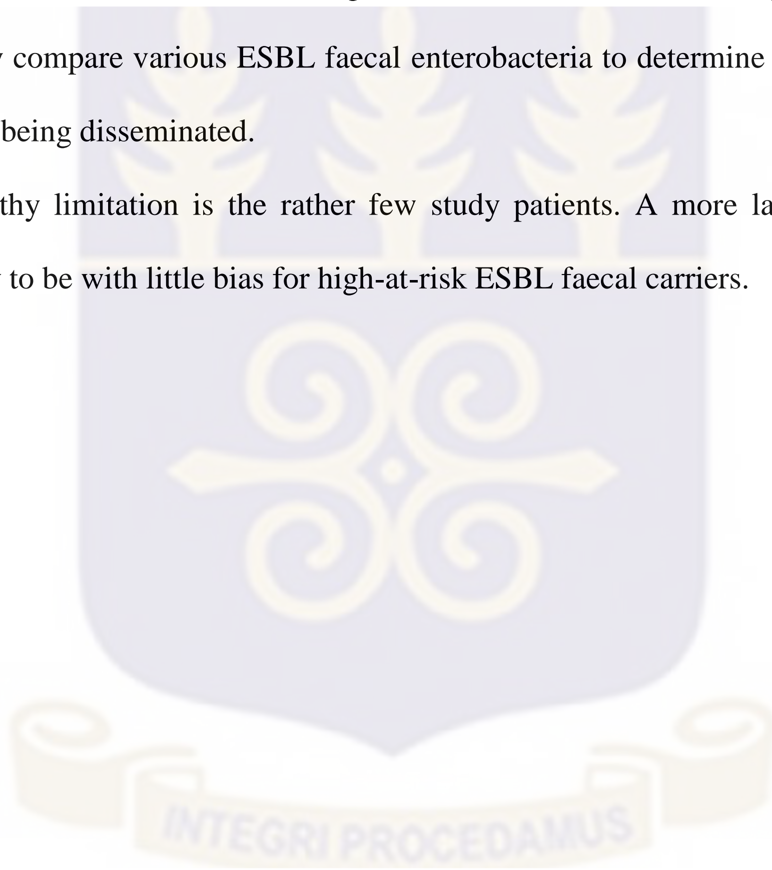
5.4 Limitations of the study

There are some potential limitations of this study that should be discussed briefly.

It should be noted that analysis of specific antibiotic resistance were not a focus of this study. However, Antibiogram of ESBLs versus non-ESBL-producing faecal enterobacteria may perhaps have provided data on the efficacy of some selective antibiotics that could help to maximize clinical outcome of empiric antibiotic therapy.

Bacterial clonal relatedness was not investigated in this work. Such investigations would have enabled the study compare various ESBL faecal enterobacteria to determine the extent to which these isolates are being disseminated.

Another noteworthy limitation is the rather few study patients. A more large-scale survey is much more likely to be with little bias for high-at-risk ESBL faecal carriers.



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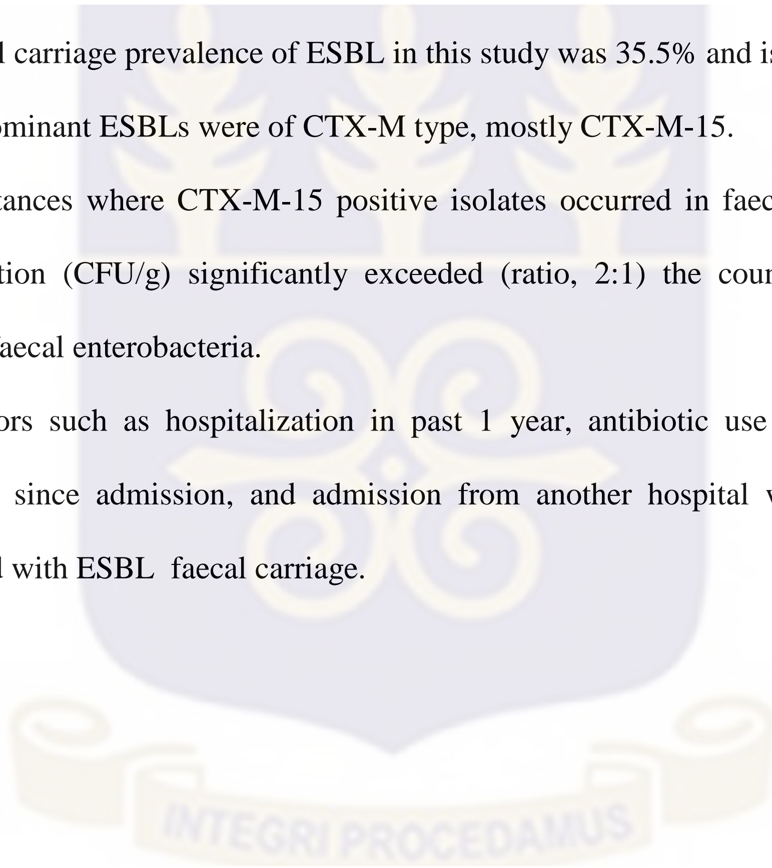
CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

The aim of this study was to examine immunocompetent inpatients for faecal carriage with extended-spectrum beta-lactamase producing enterobacteria at a Achimota District Hospital in Ghana. The following are the conclusions based on the study findings and the overall objectives.



(1) The faecal carriage prevalence of ESBL in this study was 35.5% and is considered high.

(2) The predominant ESBLs were of CTX-M type, mostly CTX-M-15.

(3) In all instances where CTX-M-15 positive isolates occurred in faecal specimens, their concentration (CFU/g) significantly exceeded (ratio, 2:1) the counts of total ESBL-negative faecal enterobacteria.

(4) Risk factors such as hospitalization in past 1 year, antibiotic use in past 6 months, infections since admission, and admission from another hospital were independently associated with ESBL faecal carriage.

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6.2 RECOMMENDATIONS

(1) The proportion of patients with ESBL faecal carriage in this study was high (35.5%). However, this is significantly lower than other figures (> 49.0%) reported for ESBL prevalence in clinical isolates across Ghana. Routine laboratory screening for ESBLs may help reduce the menace.

(2) The significance of CTX-M-15-producing isolates as predominant faecal enterobacteria in this study is worrying. This has implications for rapid ESBL spread. Further investigations on these isolates to ascertain the reasons behind such dominance is warranted.

(3) In order to reduce the spread of ESBLs and avoid compromising patient care, there is the need to perform regular surveillance of ESBL producing bacteria in the hospitals. This information is critical to the appropriate use of antimicrobials for empirical treatment of hospital and community-acquired infections. Additionally, it is important to reinforce strict infection control measures in order to prevent further spread within the hospital and community and from the community into hospital settings.

(3) Immediate implementation of antibiotic stewardship and other preventive strategies are necessary to stem the tide of dangerous spread of ESBL-positive enterobacteria in Ghana.

(4) Public health efforts on antibiotic resistance should include teaching the population and healthcare personnel on the significance of ESBLs.

(6) Hospitalization in past 1 year, antibiotic use in past 6 months, infections since admission, and admission from another hospital were independently associated with ESBL faecal carriage. These risk factors point to the hospital as a major repository of ESBL-producing organisms. There is the need for firm adherence to contact precautions, including simple hand washing, in

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order to prevent the spread of these organisms. Proper antibiotic stewardship policies may also help control ESBL spread.

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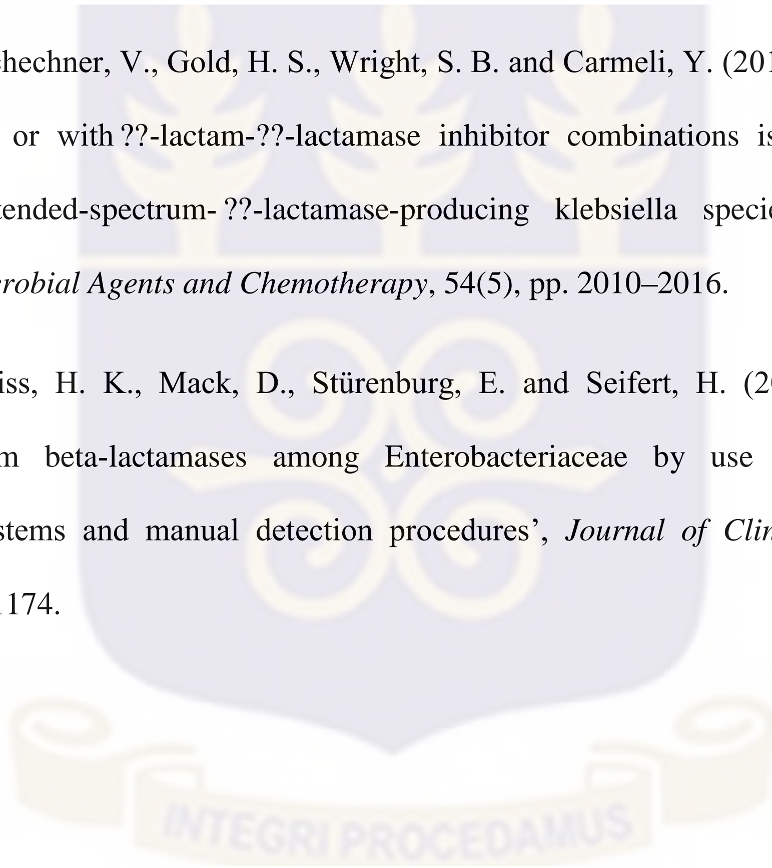

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APPENDIX

Appendix 1: Identification of immunosuppression/immunocompetent patient

- *Criteria for immunosuppression will be as defined by Salisbury et al., 2006*

Immunocompromised patients will be defined as patients with suppression (as by drugs or disease) of the immune response.The following will be regarded as causes of immunosuppression.

Systemic illnesses:

- Diabetes mellitus
- Chronic alcoholism
- Renal or hepatic failure
- Autoimmune disorders such as systemic lupus erythematosus or rheumatoid arthritis
- CNS infection

Immunosuppressive treatment

- Corticosteroids
- Polyclonal immunoglobulins such as antilymphocyte globulin, and monoclonal immunoglobulins such as daclizumab (both monoclonal and polyclonal immunoglobulins target cellular immunity alone by depleting lymphocytes)n

Antimetabolites:Calcineurin inhibitors which prevent T cell transcription, such as cyclosporine. Rapamycin which block the mTOR kinase in lymphocytes, such as everolimus. Mitosis inhibitors which block purine metabolism, such as azathioprine

- Ionizing radiation
- Biological alkylating agents such as cyclophosphamide and chlorambucil

Immunosuppression is clinically indicated in three distinct situations:

- The post-transplant period, to prevent graft rejection and graft-versus-host reactions

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· . The presence of an autoimmune or hypersensitivity disorder which causes self-antigens to be identified as foreign targets of immune attack, and leads to tissue and organ damage, and the occurrence of lymphoproliferative disorders

- *At the hospital, immunocompetent patients will be patients….*

(1) with no major organ or bone marrow transplant, or a diagnosis of HIV, severe combined immunodeficiency, or Wiskott-Aldrich syndrome before the baseline date;

(2) without any record of prescription of an immunosuppressive drug (azathioprine, sulfasalazine, methotrexate, cyclosporine, and leflunomide) in the 1 months before baseline date; and

(3) without any record of a steroid prescription at a defined dose or higher (dexamethasone 3 mg daily, hydrocortisone 80 mg daily, prednisolone 40 mg daily for >1 week, and cortisone 100 mg daily) in the 1 month before baseline date.

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Appendix2

INFORMATION SHEET

PROJECT TOPIC: Intestinal carriage with extended-spectrum Beta-lactamase producing enterobacteria in immunocompetent patients .

PURPOSE OF STUDY:Towards the Control of Antibiotic Drug Resistance in Ghana

COLLABORATING INSTITUTIONS:University of Ghana School of Biomedical and Allied Health Sciences, Department of Medical Laboratory Sciences; University of Ghana Hospital, Legon).

Invitation:This is an invitation for you to participate in a study which aims to improve our knowledge on infections and antibiotic resistance in the hospital. This study will last from March to May 2017. **Your participation in this study will involve your approval to provide us with a faecal specimen during this period of hospitalization. You will also be required to help us fill a simple questionnaire with the help of an assistant.**

***Benefits*:** There will be no financial remuneration for your participation. Your participation will be of no cost to you. Your participation in this study will provide data that will inform policies and measures aimed at controlling the spread of bacterial infections and antibiotic resistance in the hospital. Any important results during the course of study that will be of benefit to your health will be made available to you or your physician for appropriate medication. The study will also help us document the extent of the antibiotic resistance problem in the hospital, as well as implement control and monitoring efforts in reducing this menace in Ghana.

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Hazard of study: There is no harm or discomfort associated with your participation in this study. Some of the questions in the questionnaire may, however, prove embarrassing.

Procedure for sample collection: The procedure to be used for stool collection in this study will be the same as those used in the routine laboratory stool collection. This will be done by qualified attending nurses or physicians on duty.

Use of patient's sample: Bacteria will be isolated from the faecal specimens. The bacteria will be studied for antibiotic resistance and preserved for future investigation. The stool material will be preserved for as long as possible for further investigations.

Subjects right to refuse or withdraw: Your participation in the study is completely voluntary. All information related to your participation would be kept strictly confidential. Stool material will be number coded. You are free to refuse permission to participate and this will in no way affect how you will be treated at this hospital. If at any point in time during the study you take a decision not to participate any further, you are at liberty to do so immediately without any further discussion.

If you have any problems or questions about this study, feel free to contact the following:

1. Obeng Nkrumah Noah - Department of Medical Laboratory Science, University of Ghana School of Biomedical and Allied Health Sciences. Tel : 0548394763
2. Gloria Dela Tawiah - Department of Medical Laboratory Science, University of Ghana School of Biomedical and Allied Health Sciences. 0542701668

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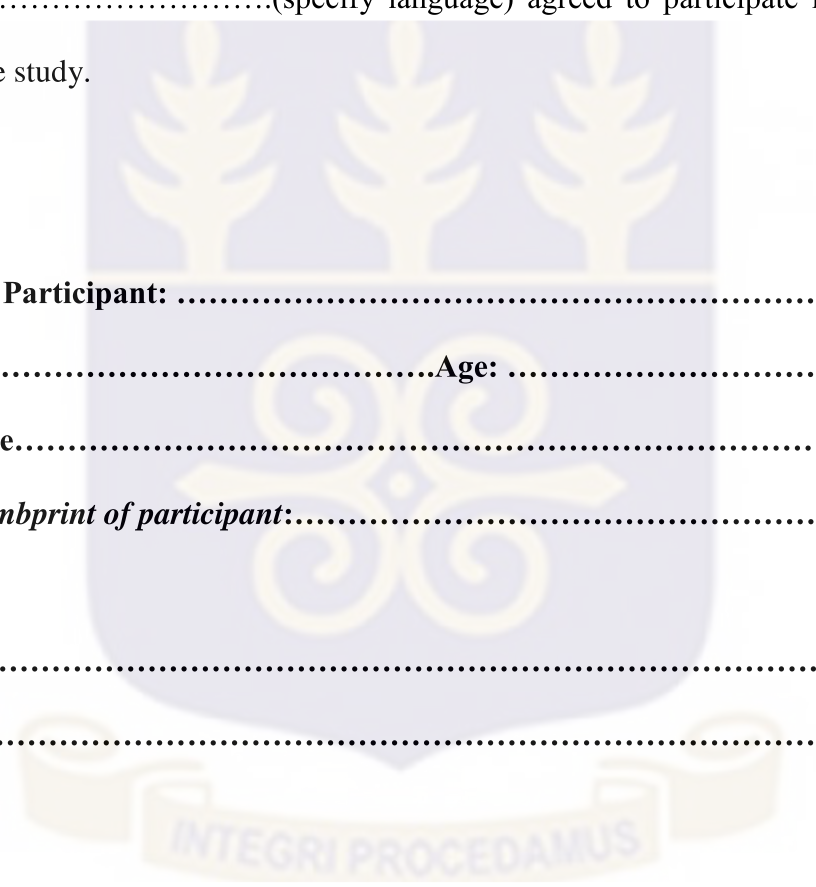
PARTICIPANTS CONSENT

Participant Declaration:

I,………………………………………………………………………..of……………
…………………….., having understood the contents of the attached sheet, after it has been thoroughly explained together with this consent form to me in ……………………………….(specify language) agreed to participate in the Antibiotic resistance study.



Name of Participant: ………………………………………………………………..

Sex: ……………………………………….Age: ………………………………………

Signature………………………………………………………………………………..

OR *Thumbprint of participant*:…………………………………………………………...

Witness:

……………………………………………………………………………………………...

Date: ……………………………………………………………………………………….

Date:

Signature:

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Appendix 3

Structured Questionnaire for Interviews

Towards the determination of risk factors for ESBL faecal carriage

Intestinal carriage with extended-spectrum beta-lactamase producing enterobacteria inimmunocompetent patients

Patient code:

Q 1. Personal information:

Q1.1	Name:	
Q1.2	Date of birth:	Date of admission:
Q1.3	Gender:	Female:___ Male:____
Q1.4	Ward name: Number of beds: Number of patients in ward: Number of healthcare staff: Flush toilet in the ward: Indicate number Source of water:	…………………………… …………………………… …………………………… …………………………… Yes ☐ No ☐: if 'No' indicate type…………………… ……………… Running tap ☐; Veronica buckets ☐ Others (specify)………….

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Q1.5	Admitted from home ☐ current hospital ☐ another healthcare facility ☐

Q2. Questions of underlying diseases:

Do you suffer from any of the following diseases?

Admitting diagnosis:		Yes:	No:	I do not know:
Q2.1	Haematological disorders:			
Q2.2	Respiratory infections:			
Q2.3	Diarrhoea:			
Q2.4	Diabetes:			
Q2.5	Cancer:			
Q2.6	HIV/AIDS:			
Q2.7	Liver cirrhosis:			
Q2.8	Alcoholism:			
Q2.9	Infections since admission			
Q2.10	Surgery since admission			
Q2.11	Functional status: Need help of any sort			

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Q2.12	Immunosuppression (of any type) Steroids Chemotherapy in past 3 months Radiation in past 3 months Others (specify):……………………………………..			
Q2.13	Gastro-oesophageal disease…………………………….			

Q3. Questions of underlying patient's lifestyle:

Q3.1	Alcohol hand rubs/sanitizer	Yes	No
	If yes, indicate how often you use this in a day: Once ☐ ; Twice ☐ ; Thrice ☐ ; > Thrice ☐ ; as often as possible ☐		
Q3.2	How often do you wash your hands?	Yes	No
	once ☐ ☐ ☐ ☐ ☐		
Q3.3	Employment	Yes	No
Q3.4	Current smoking	Yes	No

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Q3.5	Alcoholism	Yes	No
Q3.6	Education Indicate status: Primary ☐; Secondary ☐; Tertiary ☐ None ☐	Yes	No
Q3.6	Profession/Occupation: …………………………………………..		
Q3.6 **Q3.7** **Q3.8** **Q3.9** **Q3.10**	Have travel overnight outside your home in past one year……………….. Have travel outside Ghana in the past year………………………………………………….. Number of person in the household Toilet facility in household Pipe water in household……………………………….		

Q4.History of hospitalization

Q.4.1	Hospitalization in the past 1 year Number of hospitalization………………………………………………. Total duration hospital stay ……………………………….	Yes:	No:
Q.4.2		Yes:	No:
Q.4.3	Invasive procedure of any type in past 1 year (endoscopy, gastroscopy, sigmoidoscopy, colonoscopy, etc.)	Yes	No
Q.4.4		Yes	No

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	Presence of central vascular catheter		
	Presence of peripheral vascular catheter		
	Presence of urinary catheter		
	Presence of intubation		
	Use of medications that affect intestinal flora	Yes	No
	Drugs that neutralize stomach acids		
	Proton pump inhibitors		
Q.4.5	H2 blockers		
		Yes	No
	Used antibiotics in last 3 months		
	Used antibiotics without prescription		
Q.4.6	Indicate type of antibiotics used in last 3 months……………………..		
Q.4.7	…………………………………………………………………		
	Current antibiotics used………………………………………………		
	Specify antibiotics……………………………………………………………. Animal contact in the past 6months………………………………………………………………		
Q4.8