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INTESTINAL CARRIAGE OF EXTENDED-SPECTRUM BETALACTAMASE PRODUCING ENTEROBACTERIA IN HIV/AIDS PATIENT

BY

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DECLARATION

The work described in the thesis was carried out at the School of Biomedical and Allied Health Sciences Laboratory (Microbiology laboratory department), Korle-Bu Teaching Hospital under the supervision of Dr Noah Obeng Nkrumah and Mrs Harriet Blankson.

"I hereby declare that, except for references to other people's work which have been duly acknowledged, everything in this thesis is as a result of my original investigation. Further I certify that, this work has not been presented to this university or elsewhere.

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"We hereby declare that the preparation and presentation of this thesis were done inaccordance with the guidelines on supervision of thesis laid down by the University of Ghana."

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Mrs Harriet Blankson)

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ABSTRACT

Background: Routine empiric antibiotic therapy is common in patients living with HIV/AIDS. Such routine antibiotic regimen could lead to selection of multidrug resistance especially ESBLs in hitherto drug sensitive intestinal colonizing flora. There is paucity of data to describe intestinal carriage of ESBL-producing enterobacteria in HIV/AIDS patients.

Aim: This study was designed to examine HIV/AIDS patients for faecal carriage with extendedspectrum beta-lactamase producing enterobacteria at a district care hospital setting in Ghana**.**

Methods: A cross-sectional study design was conducted to recruit HIV/AIDS patients at the Achimota District Hospital in Accra from March through May 2017. Questionnaire-administered interviews were conducted to determine independent risk factors for ESBL faecal carriage. Faecal samples were examined for ESBL-producing enterobacteria by serial dilution colony counts, polymerase chain reaction (PCR) and nucleotide sequencing.

Results: Overall, 43 of 100 patients living with HIV/AIDS had faecal carriage with ESBLproducing enterobacteria. The predominant ESBL type was CTX-M, mostly $bla_{CTX\text{-}M\text{-}15}$. Among patients colonized with CTX-M-15 positive enterobacteria, these isolates were the predominant faecal enterobacteria. In such patients, the total faecal ESBL-negative enterobacteria were the sub-dominant colonies. Two patients were each colonized by isolates that carried the complex mutant TEM beta-lactamase, $bla_{TEM\text{-}121.}$ SHV and OXA type ESBLs were not identified. In a multivariate logistic regression, the number of persons living with an HIV/AIDS patient in a household was an independent risk factor for ESBL faecal colonization.

Conclusion. Patients living with HIV/AIDS patients may constitute a significant reservoir of ESBLs in the hospital and community. The results highlight the need for better surveillance of ESBLs in the hospital, and Ghana as a whole.

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DEDICATION

This work is dedicated to 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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LIST OF ABBREVIATIONS

Amp C Ampicillin hydrolyzing Cephalosporinase

AIDS- Acquired immunodeficiency syndrome

ATCC American Typed Culture collection

CRE Cephalosporin resistance enterobacteria

Cfu Colony forming units

CLSI Clinical and Laboratory Standards Institute

CTX-M Cefotaximase

DNAse Deoxyribonucleases

EDTA Ethylenediaminetetraacetic acid

ESBLs Extended-Spectrum β-Lactamses

HIV-Human immunodeficiency virus

HPA Health Protection Agency

MDR Multidrug resistant

MIC Minimum inhibitory concentration

OXA oxacillinase

TAE Tris acetic EDTA buffer

PCR Polymerase Chain Reaction

PER *Pseudomonas* Extended Resistances

RNAse Ribonuclease

SHV- Sulfhydryl variable active sites

TAB Total aerobic bacteria

TEM Temoniera

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µg Microgram

VEB Vietnamese extended-spectrum β-lactamase

SSI Statens serum institute enteric agar

SD Standard deviation

OR Odds ratio

IQR Interquartile range

SPSS-Statistical package for social sciences

UV Ultraviolet

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CHAPTER ONE

INTRODUCTION

1.0 GENERAL BACKGROUND

Members of the family *Enterobacteriaceae* are found in the environment and make up normal microbiota of the intestines (Fam *et al*., 2015). They can be divided into 51 genera; some of which are causes of intestinal infections. *Enterobacteriaceae* are Gram negative rods, ferments glucose into acid, reduce nitrates to nitrites, and oxidase negative and facultative anaerobes (Fam *et al*., 2015). Some members are clinically significant and comprise of the species *Escherichia, Klebsiella, Enterobacter, Salmonella, Shigella, Proteus, Citrobacter, Serratia, Yersinia and Morganella.* Frequently, the risk factors of acquiring infection with Extended Spectrum Beta-lactamase (ESBL)-producing enterobacteria are prolonged intake of antibiotic such as the beta-lactam antibiotics. Beta-lactam antimicrobials are the most common treatment for bacterial infection (Shaikh *et al*., 2015).

Favoured by their broad spectra and minimal side effects, beta-lactams remain the most widely used antimicrobial agent. However the rates of bacterial resistance to beta-lactam agents are increasing worldwide, including Ghana (Obeng-Nkrumah *et al*., 2013). Three mechanisms are involved in bacteria resistance to beta-lactam antibiotics: Ability to produce beta-lactamase enzymes, utilization of beta-lactam insensitive cell wall transpeptidase and the active expulsion of beta-lactam molecules by efflux pumps (Isendahl *et al.* 2012). Nevertheless, the variety of beta-lactamase enzymes, with their increasing substrate diversity and wide dissemination,

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constitute the most important antimicrobial resistant mechanism in gram negative bacteria (Kumar 2013) . Generally, three broad groups of beta-lactamases are differentiated: class C cephalosporinases (AmpC), extended-spectrum beta-lactamases (ESBLs) and ß -lactamases with carbepenamase activity (Girlich *et al.*,2013). The ESBLs with its increasing potential for associations with plasmid-borne mobile genetic elements, constitute the most versatile cause of antimicrobial resistance among the enterobacteria.

1.1 Extended Spectrum Beta-lactamases (ESBLs) in the *family Enterobacteriaceae*

Generally, extended spectrum beta lactamase (ESBLs) are enzymes that are produced by aerobic and anaerobic Gram-negative bacteria. These group of bacteria hydrolysis the beta-lactam ring of penicillin, first-, second- and third-generation cephalosporins and monobactams; consequently, inactivating these antibiotics (Girlich *et al.*, 2013; Kumar., 2013). The extended beta-lactamase are enzymes inhibited by clavulanate and have been reported to be an important cause of nosocomial infection worldwide (Brolund *et al .,*2016; Uemura *et al*., 2017). Encoded on a transferable conjugative plasmids are the ESBLs enzymes which facilitate widespread dissemination between species and across different species. Furthermore, the bacteria plasmids code for resistance to many classes of antibiotics such as aminoglycosides and fluoroquinolones, (Brolund *et al.,* 2016; Uemura *et al*., 2017). However the treatment of choice for serious infections with ESBLs is Carbapenems with Fluoroquinolones and aminoglycosides being the alternative choice, if organism is shown to be susceptible. One of the most important resistance mechanisms by enterobacteria that prevent the progress of antimicrobial treatment of infections is the production of ESBL enzymes (Cassir *et al*.,2014; Fam *et a*l, 2017).

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1.2 Recent concerns over ESBL-producing enterobacteria

In recent times, the dynamic spread of ESBL-producing enterobacteria has placed tremendous pressure on the limited therapeutic options and raises concerns about the epidemiologic problem of their resistance (Cassir *et al.*, 2014; Brolund *et al*., 2016; Fam *et al*., 2017; Uemura *et al.*, 2017). A threatening epidemiological concern is the increasing dissemination of ESBL-producing isolates as intestinal flora of hospital patients (Reuland *et al.* 2016; Marit G. Tellevik et al. 2016; Farra *et al.* 2016). Although this threat underscores the importance of epidemiological surveys to infection control, research conducted to date on carriage of ESBLs has focused more on Europe, Asia and the Americas (Dhillon *et al*.,2012; Ghafourian *et al.,* 2015; Shaikh *et al*., 2015). In contrast little research has been conducted in Africa, where there appears to be a massive spread of intestinal ESBL carriage (Storberg, 2014; Tansarli *et al*.,2014). Extended spectrum producing enterobacteria are resistant to penicillins, narrow and extended spectrum betalactams. Moreover, ESBLs presence in bacterial cells do not always produce phenotypic resistance and results in some ESBL-producing isolates appearing susceptible to beta-lactams in vitro, although treatments with such antimicrobials are ineffective.

The ESBL-producing enterobacteria, with their potential for plasmid-mediated resistance to quinolones, aminoglycosides, tetracyclines, sulphurmethoxazoles and other non-beta-lactam antibiotics, are generating tremendous public health concerns (Cassir *et al.*, 2014; Fan *et al*.,*,* 2016; Uemura *et al*., 2017). This is illustrated by their widening epidemiological patterns; the increasing global selection of their multidrug resistant phenotypes and the severe implications of their infections. Indeed, surveillance studies for ESBLs are important for infection control. In Ghana, routine laboratory detection of ESBLs is absent. Moreover, no systematic survey of

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ESBL-producing enterobacteria has been conducted, and the extent of the problem remains unclear (Obeng-Nkrumah *et al*. 2013).

1.3 The ESBLs in patients with human immunodeficiency virus (HIV)

Advances in the treatment of human immunodeficiency virus with effective antiretroviral therapy in the last 20 years have dramatically changed the face of HIV infection.

This has led to a sharp reduction in both opportunistic infection and death rates (Cutland *et al*., 2012; Lozupone *et al.*, 2013; Albarillo *et al.*, 2016.). In spite of this, bacterial infections continue to cause significant morbidity and mortality in patients with HIV, especially in sub-sahara Africa. Persons with chronic immunosuppression including persons living with HIV/AIDs are more susceptible to bacterial infection because of defects in both the cell-mediated and humoral immunity and this can lead to serious bacterial infection which may cause morbidity and mortality (Cutland *et al*., 2012; Lozupone *et al.*, 2013; Albarillo *et al.,* 2016.). There is frequent use of empirical antibiotic within this patient group. The HIV-infected patients may have increased faecal colonization with ESBLs due to frequent antibiotics use. As colonization by ESBL-producing enterobacteria is associated with increased risk of infection by ESBL-positive isolates, individuals at risk for both the colonization and infection by ESBL-producers may serve as sources of outbreaks in both hospital and community settings.

Generally, ESBL colonization is said to occur in individuals who have frequent exposure to healthcare settings and in those with frequent antibiotic usage as well as immune suppression (Kuenzli et al. 2014; Nakai et al. 2016). Other factors listed in the literature as risk for health facility associated ESBL are age, duration and place of hospitalization, underlying disease, invasive procedures or devices, previous hospitalization, intensity of care, proximity to an

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ESBL-colonized patient, and housing conditions. (Kuenzli *et al.*, 2014; Nakai *et al.*, 2016., Fouda *et al.,* 2016). Infections by ESBL-producing enterobacteria are a challenge for physicians in developing countries to treat because of the limited choice of therapeutic options available and due to the possibility of concomitant drug resistance of the isolate to other antimicrobials. ESBL-producers are also a challenge to patients in developing settings due to increased cost of care.

The financial burden of ESBLs in care in regions of limited resource, such as Ghana, is not expected to be easy because there are other priorities such as TB, HIV and malaria. Infection prevention and control measures have been the primary means to attempt to limit the spread of ESBLs globally. For instance, patient isolation and antibiotic regimen based on knowledge of ESBL faecal carriage are increasingly being practiced in Europe and across the Americas (Bar-Yoseph *et al.*,2016) .

This is because previous colonization with an ESBL-positive bacterium is a reported risk factor for subsequent clinical infections with ESBL, empiric antibiotic treatment for a patient with ESBL faecal carriage usually must exclude most beta-lactam antibiotics (as ESBLs confer resistance to these drugs). To this study's knowledge, there are no reports on the epidemiology and microbiology of ESBL faecal colonization among HIV infected patients in Ghana and Africa in General.

1.4 PROBLEM STATEMENT

In Ghana, the prevalence of ESBL-producing enterobacteria causing infections in hospitals is high (about 50%)(Obeng-Nkrumah *et al*. 2013). Also, the ESBL-producing enterobacteria are increasingly occurring as intestinal flora in apparently healthy community persons. The ESBL-producers are a known cause of community-acquired bacterial infections and are an important

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hospital-acquired pathogen (Birgy *et al.* 2012). They are resistant to many clinically useful antibiotics and therefore infections by these bacteria results in increased morbidity and mortality, and longer hospital stays.

The prevalence and burden of such pathogens in a hospital setting is likely to be a reflection of the sanitary conditions of these areas (Kuenzli *et al*. 2014; Nakai *et al*. 2016; Fouda *et al.* 2016; Karanika *et al*. 2016). Since the 1990s, hospital acquired infections due to ESBL-producing *Enterobacteriaceae* have been increasingly reported worldwide, especially in developed countries. In hospital settings, intestinal carriage is the main reservoir of these organisms (Smith *et al*., 2004). The gut colonization of inpatients is associated with a high risk for developing self and cross infections due to ESBL-producers, especially in long term care units (FAM *et al*. 2015). Clinical conditions such as HIV/AIDs may produce one of the most severe form of immunosuppression. In such patients, routine empiric antibiotic therapy is common to prevent the emergence of opportunistic infections. Although it is common knowledge that such routine antibiotic regimen could lead to selection of multidrug resistance especially ESBLs in hitherto drug sensitive intestinal colonizing flora, there is paucity of data to describe associations between the occurrence of HIV/AIDS and ESBL intestinal colonization (FAM *et al*. 2015). This project is part of a larger study on the occurrence of ESBL intestinal colonization in a district care hospital setting in Ghana. One of the objectives of the parent project is a comparative study to examine ESBL intestinal colonization in different patient cohorts that are age- and time- matched. This current study was designed to describe the occurrence of intestinal colonization with ESBL-producing *Enterobacteriaceae* among patients living with HIV — with particular focus on quantification of ESBL-producing isolates and on genotypes of ESBLs. Furthermore, risk factors for intestinal colonization with these resistant bacteria was examined.

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1.5 JUSTIFICATION

The epidemiological importance of faecal carriage in hospitalized patients is rather substantial but hugely under-reported in Africa and in Ghana in particular. The primary objective of this study is to examine the occurrence of intestinal colonization with ESBL-producing enterobacteria in a hospital setting and determine factors predictive of this phenomenon among patients living with HIV/AIDS.

Detection of ESBL-producing enterobacteria and their pattern of antimicrobial resistance can provide useful information about the local epidemiology of and risk factors associated with such bacterial infections within a geographic setting. This is important in understanding, among patients with HIV/AIDS, the (i) prevalent ESBL-producing members of the family *Enterobacteriaceae* being disseminated in a hospital care setting; (ii) antibiotic options effective against such ESBL-producing bacteria; and (iii) comparisons between ESBL genotypes of bacteria from the different patient populations. Such knowledge is critical to the success of infection control policies particularly in low income countries where there is limited financial tolerance for various illnesses.

The availability of such local epidemiological data will prove indispensable to hospital infection management whilst creating awareness on the clinical implications of ESBL-producing organisms in Ghanaian hospitals. The findings of the study will inform effective public health interventions in hospitals including proper management of patients with infections.

1.6 HYPOTHESIS

This study hypothesized that the predominant ESBL gene type in faecal enterobacteria of HIV patients is CTX-M-15

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1.7 AIM

This study aimed to determine the faecal carriage with extended-spectrum beta-lactamase producing enterobacteria in patients living with HIV/AIDS at a district care hospital in Ghana.

1.8 SPECIFIC OBJECTIVES

The specific objectives of the study were to determine the

a) prevalence of ESBL-producing enterobacteria and the specific type of ESBL gene sequences involved;

b) faecal concentration of ESBL-positive versus ESBL-negative faecal enterobacteria; and

c) risk factors that may predispose HIV patients to intestinal colonization with ESBL- producing enterobacteria

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CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

This section reviews work on intestinal carriage of extended spectrum betalactamase producing-enterobacteria in the HIV/AIDS

This is discussed under the following sub headings:

- The family *Enterobacteriaceae*; This section reviews the classification of ESBLs and highlights on some pathogenic strains, their site, beta-lactamase enzyme, their classification, and other types of ESBLs.
- Epidermiology and risk factor of colonization; It reviews areas of incidence, distribution and possible control transmission and acquisition.
- Faecal carriage of ESBLs constitute a hidden threat; This elaborates the epidermiological concern of ESBL producing - enterobacteria infection.
- Extended spectrum beta-lactamases producing-enterobacteria in the HIV/AIDs; This highlights the occurrence of ESBLs in the HIV/AIDS.
- Clinical significance of ESBL detection in immunosuppressed patients; This explains how ESBLs infection leads to selection of multidrug resistance strains.

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2.2 THE FAMILY ENTEROBACTERIACEAE

Enterobacteriaceae also refered to as enterics are large group of Gram- negative bacteria found in the environment, water, soil and plants and they are causes of nosocomial infections. They make up part of the normal flora of human intestines, however some of them are human intestinal pathogens (Fam *et al*., 2014).

Escherichia coli and *K. pneumoniae* are the most frequently isolated in clinical samples and cause urinary tract infections, septicaemia, pneumonia and gastrointestinal problems. However, most *E. coli* strains in the gut are non-pathogenic commensals but some strains carry combinations of virulence genes which enable them to cause intestinal infections. Further, some work on the distribution targets genes and multi-locus sequencing typing indicated that, most *E.coli* isolates can be divided into eight phylogenetic groups: A, B1, B2, C, D, E, F. Group A, B1, C and E comprise mostly commensal strains, whereas B2, D and F are mainly associated with the virulent extraintestinal strains (Luvsansharav *et al*., 2012). Pathogenic *E.coli* strains can be grouped into intestinal and extraintestinal pathogens. Furthermore intestinal pathogenic strains of E.coli can be sub-grouped based on their virulence factors into enteropathogenic, enterohemorrhagic, enterotoxigenic, entero-invasive, entero-agggregative and diffusely adherent *E.coli*. Frequently, *K. pneumoniae* isolates are found in human gastrointestinal tract and urinary tract infections and in septicaemia (Eibach *et al*., 2015).

2.3 BETA -LACTAMASES AND THEIR CLASSIFICATION

Members of the family *Enterobacteriaceae* produce the beta-lactamases which hydrolysis the beta-lactam rings of antibiotics such as penicillins, monobactam.

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The extended spectrum beta-lactamases (ESBLs) has generally been defined as a transmissible beta-lactamases that can be inhibited by clavulanic acid, tazobactam or sulbactam, and which are encoded by genes that can be exchanged between bacteria. In addition, the current most common genetic variant of ESBL is CTX-M, (Paterson *et al* .,2005). Further, the extended spectrum beta-lactamases (ESBLs) are often encoded on plasmids that are transferable from strain to strain between bacterial species (Ruppé *et al.,* 2011). However ESBLs gene can also be located on integrons which are mobile genetic elements and allows integration of antimicrobial drugs resistance genes via site specific recombination events. Therefore integrons play an important role in dissemination of antimicrobial resistance (Hawkey., 2008). Beta–lactams are broad class of antibiotics that are widely prescribed and frequently used in treating bacterial infections. They include the penicillins, cephamycins, cephalosporins, monobactams, carbapenems and beta lactamases inhibitors. However, beta-lactamases can be classified according two schemes:

1. The Ambler Molecular classification which classifies beta-lactamase enzyme based on the protein homology into group A to D. The class A, C and D are phylogenetically different serine betalactamase and class B is the metallobetalactamase group (Shaikh *et al*., 2015).

2. The second scheme of extended spectrum classification is the Bush-Jacob-Medeiros scheme which classifies beta-lactamases according to their functional similarity and lactamase inhibitor susceptibility (Andriatahina *et al.,* 2010).

3. Moreover, Extended spectrum betalactamases (ESBLs) were first defined as plasmid –mediated beta-lactamases, and these plasmid carried on them different genes such as genes conferring resistance to other classes of antibiotics such as cephalosporins and

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monobactams, and inhibited invitro by beta-lactamase inhibitors (Tärnberg, 2012). Hence, extended spectrum beta-lactamases (ESBLs) provide resistance to penicillins, cephalosporins and carbapenems and inhibited by cloxacillin or boronic acid. The ESBLs are coded by four main gene types: TEM, SHV and CTX-M and OXA genes and have the ability to transfer a replica of themselves to other bacteria (Tärnberg, 2012).

2.4 GENE TYPES OF BETA-LACTAMASES

2.4.1 SHV-TYPE ESBLs

The SHV extended spectrum beta-lactamases comprises of variants. In addition majority of the SHV variant showing ESBL phenotype are identified by substituition of serine for glycerine at position 238 but more than 40 SHV–type ESBLs has been identified. The first emergence of the SHV–ESBL was the SHV-2 type and it however differs from SHVI-1 by replacement of glycine with serine at position 238 and some SHV variants have substituition of lysine for glutamate at position 240. SHV-1 betalactamase is encoded on the plasmid or chromosome and confers resistance to penicillins and first generation cephalosporins mostly produced *by K. pneumonae* (Paterson *et al* .,2005). Moreover, the SHV-type ESBLs are mostly found in *K. pneumoniae* but could also be seen in the *Citrobacter diversus* and *Pseudomonas aeruginosa* (Toth *et al.*, 2005). Some group of researchers indicated that, the SHV family of beta-lactamases appears to be derived from *Klebsiella spp*, SHV-1 universally found in *Klebsiella pneunoniae* (Shaikh *et al.*, 2015).

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2.4.2 TEM-TYPE ESBLs

The TEM–type of ESBL gene comprise of TEM-1, TEM-2 and TEM-3. TEM-2 is the first variant described and it differ from TEM-1 through the substitution of lysine for glutamine at position 39. Although TEM-2 is not ESBL, it can hydrolysed penicillin and first generation cephalosporin's, but enable to hydrolysed monobactam. TEM3 was the first TEM–type beta-lactamase that showed the ESBL phenotype and since, the number and different type of extended spectrum TEM-type has increased (**http://www.lahey.org/studies).**

TEM-type extended spectrum beta-lactamase (ESBLs) are most frequently found in *E.coli* and *K. pnemoniae* (Bradford., 2001). TEM-1 is capable of hydrolyzing penicillins and first generation cephalosporins but it is unables to attack the oxyimino cephalosporins. In addition some work has indicated that, the TEM-type (TEM-42) of Gram negative non–enterobacreriaceae bacteria are found in *Pseudomonas aeruginosa* (Rupp *et al.,* 2003).

2.4.3 CTX-M TYPE ESBLs

This type of ESBL was first recognized in 1989 and is found exclusively in the functional group 2 (Andriatahina *et al.,* 2010). However, this family of enzymes are thought to derived from initial transfer of chromosomal beta-lactamase from *Kluyvera species* to conjugative plasmids that are already disseminated among different, members of enterobacteriaece. Further, the CTX-M proteins can be subgrouped into CTX-M 1, 2, 8, 9 and 25 (Tobergte *et al* ., 2013).

The origin of the CTX-M enzymes is different from that TEM and SHV ESBLs. Whereas SHV-ESBLs and TEM–ESBLs were generated by amino acid substitutions of their parent enzymes, CTX-M ESBLs were acquired by horizontal gene transfer from other bacteria using genetic

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apparatus such as conjugative plasmid transposons (Kumar., 2013). Though there is some hydrolysis of ceftazidime by these enzymes, it is usually not enough to provide clinical resistance to organisms in which they reside (Shaikh *et al.,* 2015).

2.4.4 OTHER ESBLS

Some work have describe a few ESBLs that are not closely related to the SHV-type, CTX-M type-family and the TEM-type ESBLs, although they are plasmid–related, they are not derivatives of any known Beta-lactamase. An example is the OXA-type Beta-lactamases which hydrolysis oxacillin (Bush *et al* ,1995). They predorminantely occur in *P. aeruginosa* and most discovered are OXA-10 and 13(Weldagen *et al*, 2003.;D. Girlich *et al*., 2014;). OXA-1 is the most common discovered in 1-10% of *E.coli* isolates (Livermore,1995). Another ESBL-type is PER which shares about 25% homology with the TEM and SHV-type ESBLs (Delphine Girlich *et al*., 2014) . PER-1 Beta-lactamases hydrolyzes penicillins and cephalosporins and is susceptible to clavulanic acid inhibitors and PER-1 beta-lactamase was first detected in strains of *Pseudomonas aeroginosa* isolated in Turkey. The PER-2 which shares 86% homology to PER-1 was detected more frequently in South America (Bradford, 2001). Another rare ESBL type is the VEB-1 which was first found in isolate of *E.coli* from Vietnam (Kiremitçi *et al*., 2011). In addition, other VEB enzymes have been detected in Kuwait and China (Kumar, 2013).

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2.5 EPIDEMIOLOGY OF FAECAL CARRIAGE AND RISK FACTOR OF COLONIZATION

The human faecal carriage with ESBLs was first reported from spain in outpatients, in 2001 (Valverde *et al.,* 2004). Since then, there has been asymptomatic faecal carriage rate of ESBL in outpatients in Spain from 0.7% to 5.5% (Valverde *et al*., 2004). Several studies have reported wide difference in carriage rates between geographical areas.The highest prevalence rates have been reported from Thailand 69.3%, Egypt 63.3%, and China 50.5% (Woerther *et al*., 2013).

In addition, one crucial factor that determines the epidemiology of ESBLs at the hospital settings is the long period of intestinal carriage. However, some risk factors of acquisition of ESBL in the hospital are history of extended antibiotic courses, especially, cephalosporins and fluoroquinolones (Andriatahina *et al.,* 2010), prolong hospital stay and admission to intensive care unit (Kumar, 2013). In addition host factors such as a neutropenic condition, transplant recipient or age of patients (the old and neonates) predispose the individual to ESBL (Kiremitçi et al. 2011). The history of travel is also recognized as a risk factor of becoming colonized with ESBL producers (Jallad *et al*. 2015). In addition patient to patient transmission of ESBL producers occurs frequently but *K. pneumoniae* have a higher chance to be transmitted than *E.coli* (Harris *et al*. 2007). The transmission of ESBL producers usually occurs through faecal oral route, either directly or by hand contact with fomites, sinks in a hospital setting *(Paterson et al*, 2005).

2.5.1 Faecal Carriage of ESBLS Constitute a Hidden Threat

Although ESBL-producing *enterobacteria* constitute an apparent clinical threat because of their drug resistance phenotypes, the spread of these pathogens in the population as gut colonizing flora and the potential for wider dissemination present a much greater concern (Toth *et al*. 2013).

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Several ESBLs particularly the *bla*CTX-M genes, migrated from the chromosomes of environmental bacteria onto highly transmissible plasmids that are capable of continually maintaining their circulation in the human population (Hawkey, 2008).

However, the population (either hospital or community) thus represent a reservoir for isolates that produce ESBLs and even those yet to be detected. Though intestinal carriage is a key factor in the epidemiology of ESBLs, faecal flora of colonized persons represent a reservoir for ESBL genes and increases the risk that other population dwellers will become carriers (Chandel *et al*., 2011). Gastrointestinal colonization by ESBL-producing bacteria has been associated with subsequent clinical infections. Admission of patients with faecal carriage by ESBL-producers therefore increases the risk of other hospitalized patients acquiring the organisms as gut flora or as an infection (Chandel *et al*., 2011). Surveillance of ESBL faecal carriage is thus warranted. Early identification of ESBL-producers at the carriage state for patients at high risk of developing infections may prove useful for selecting effective empiric therapy (Shaikh *et al.* 2015) .

2.6 EXTENDED SPECTRUM BETALACTAMASES PRODUCING *ENTEROBACTERIA* IN THE HIV/AIDS

Population mobility is the main factor in globalization of public health threats and spread of antimicrobial drug–resistant organisms. There is increasing evidence that travelers contribute to the spread of antimicrobial drug resistance pathogens (Rymdutredningen, 2015). In a cross-sectional study design to compare the magnitude of bacterial resistance in patients on cotrimoxazole and other antimicrobials prophylaxis and those that are not on prophylaxis

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among HIV/AIDs isolates in Tanzania have revealed that, of the bacteria isolates used, ESBL producers were higher for isolates from HIV/AIDs positive patients than non-ESBL producers. Furthermore, the prevalence of more than one ESBL-producing isolate was highest among the screened positive HIV/AIDs children who were admitted due to diarrhoea, followed by those admitted due to other diseases (Marwa *et al*., 2015).

In addition, proportion of isolates resistant to cotrimoxazole and ciprofloxacin were significantly higher in ESBL producing bacteria compared to the non-ESBL producing isolates (Marwa *et al.,* 2015). Some group of researchers investigated the prevalence risk factors of acquisition of ESBL-Producing Enterobacteriaece among young children below 2years of age in Tanzani and documented a similar high ESBL isolates in the HIV/AIDs children (Tellevik *et al*., 2016).

2.7 CLINICAL SIGNIFICANCE OF ESBL DETECTION IN IMMUNOSUPPRESSED PATIENTS

In the HIV/AIDs infected individual, multidrugs resistance (MDR) bacterial infections are important cause of life threating illness which require careful selection of antimicrobial treatment (Tumbarello *et al.,* 2011).

Indeed, the emergence of multidrug resistance bacterial and the frequent transfer of resistance strains from one individual to the other, makes bacteria infection in the HIV/AIDs more difficult to treat since the treatment option in this situation is limited. More frequently, persons living with HIV/AIDS are more susceptible to bacterial infection because of the defects in both the cell-mediated and humoral immunity and hence makes the management of bacterial infection in HIV/AIDS infected individual of public health concern as the HIV disrupt the body's defense mechamism (Tumbarello *et al.,* 2011). Furthermore, this can lead to serious bacterial infection

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which may cause morbidity and mortality in the individual. The ESBL–producing *Enterobacteriaceae* are one of the most frequent resistance strains (Obeng-Nkrumah *et al.,* 2013). Most often, ESBL-producing *Enterobacteriaceae* pose clinical threat due to their drug resistance phenotypes. More so, the spread of these pathogens as a colonizing flora present a higher risk for developing an infection due to these pathogens (Isendahl *et al*. 2012).

2.8 COMPARATIVE LITERATURE OF ESBL DETECTION METHODS

There are various methods for screening extended spectrum Beta-lactamase (Bassyouni *et a*l. 2015). However these methods could differ between countries and laboratories. Moreover, to overcome these defects, it is mandatory to standardize cephalosporins breakpoints and develop ESBL detection methods, sensitive enough to detect resistance levels achieved by ESBL enzymes. Several researchers proposed different techniques for ESBL detection but the various test required different interpretations. In response to these ambiguous interpretations, the United States Clinical and Laboratory Standard Institute (CLSI) and United Kingdom Health Protection Agency (HPA) have published standard guidelines for extended spectrum Beta-lactamase (ESBL) in *enterobacteriaceae* (Sun *et al*. 2014).

2.8.1 Standard Guidelines for ESBL Detection

The two approved standard guidelines for ESBL detection are the Clinical Laboratory and Standard Institute (CLSI) and the Health Protection Agency (HPA).

These two methods are used in the initial screening and confirmatory test. The broth dilution and the disk diffusion methods are both advised in CLSI and HPA, and they yield similar results (Sun *et al*., 2014). The CLSI also proposed disk diffusion methods for screening ESBLs producing *Escherichia coli* and *Klebsiella pneumonia.*However cefpodoxime ,ceftazidime,

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aztreonam, cefotaxime or ceftriaxone could be used and the use of more than one of these disc increase sensitivity of ESBL detection (Magoue *et al*., 2013). In addition, 10ug cefpodoxime has been found to be more sensitive than other cephalosporins for screening extended spectrum Beta-lactamase production and clinical laboratory standard recommends, the isolates with zone of diameter less than 17mm should be confirmed for ESBL. Furthermore in broth dilution test, the minimum inhibition concentration (MIC) of ≥ 2µg/ml for cepodoxime, cefotaxime and aztreonam is an indication of phenotypic confirmation of ESBL production.

2.8.1.1 Methods For Screening Esbl Producers

2.8.1.1.1 Broth-dilution method

In MICs, the presence of clavulante compared to other confirmatory methods, makes it a bit laborious. This method include dilution methods for screening ESBL production in *Klebsiella Escherichia coli* and *Proteus species* recommended by the CLSI guidelines (M100-S17, 2007). However a ceftazidime, aztreonam, cefotaxime or ceftriaxone minimum inhibitory concentration (MIC) of ≥2µg/ml and cefpodoxime MIC of ≥8µg/ml is suggestive of ESBL expression.

The CLSI guidelines also have standardized broth dilution protocols for *Proteus mirabilis with a* MIC of ≥2µg/ml, whereas the United Kingdom HPA (QSOP 51, 2008) recommends that cefotxime and cefpodoxime of MICs of ≥2µg/ml, and ceftazidime MIC of ≥4µg/ml should be used for screening all *Enterobacteriaceae.*

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2 .8.1.2.Disk diffusion method

A CLSI guideline (M100-S17, 2007) recommends a confluent growth on Muller Hinton agar. In addition, it documents an initial screening of ESBL production in enterobacteriaceae with the various zones of inhibition as follows: Escherichia coli, Klebsiella species, and proteus species with the zone of inhibition diameters of ≤22mm for ceftazidime (30µg), ≤27mm for cefotaxime (30µg). In addition, CSLI recommends 10ug cefpodoxime disk for ESBL screening using a zone diameter of ≤22mm for *Proteus mirabilis* and ≤17mm for *Escherichia coli*, *Klebsiella oxytoca* and *Proteus mirabilis.* Isolates resistant at this breakpoint are selected and confirmed using other methods.

The United Kingdom HPA (QSOP51, 2008) also recommends similar screening of *enterobacteriaceace* for ESBL production with different zone of inhibition diameter breakpoints; ceftazidime (30µg) (zone size ≤21mm for *Escherichia coli* and *Klebiella* species, and ≤27mm for the other species), cefotaxime (30µg (zone size ≤29mm), or 10µg cefpodoxime (zone inhibition ≤19mm) and the guidelines requires the use of semiconfluent growth on Iso–sensitest agar.

2.8.1.3 Confirmatory test for ESBL –producing Enterobacteriaeceae

The confirmation protocols currently suggest a positive ESBL–Producer based on synergy between cefotaxime, ceftazidime or cefpodoxime with or without clavulanic acid. In addition, both CLSI (M100-S17) and HPA (QSOP 51, 2008) recommends a combined disk method and MIC method for ESBL confirmation.

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2.8.1.3.2 Broth dilution test

The standard broth dilution methods is also used as a confirmatory for ESBL. The clinical laboratory standard institute recommends the following antibiogram for ESBL detection ceftazidime(0-25-0.128ug/ml), ceftazidime plus clavulanat (0.25/4 to 128/4ug/ml), cefotaxime (0.25 to 64ugml), and cefotaxime plus clavulanate (0.25/4 to 64/4ug/ml). However the ESBL production results is a threefold serial dilution reductions.

2 .8.1.3.3 Combined Disk synergy test

The following antibiotics are recommended by the Clinical laboratory standard institute for antimicrobial susceptibility testing protocol; cefotaxime (30ug) and cefotaxime (30ug)/clavulanate (10ug), ceftazidime (30ug) and ceftazidime (30ug)/clavulanate (10ug). Furthermore, a zone inhibition diameter of 5mm or more, in the presence of clavulanate for any of the antibiotics disks mentioned signifies ESBL production, whereas HPA recommends a ratio greater than 1.5 between inhibition zones diameter of cephalosporins with or without clavulanate confirms ESBL production.

2.9 OTHER RECOMMENDED ESBL DETECTION METHODS

Several researchers have proposed different phenotypic methods for detecting ESBL enzymes invitro and molecular methods for ESBL gene detection and these are additional methods aside the CLSI and HPA approved guidelines.

2.9.1 Phenotypic methods

The principle behind this test is based on the ability of ESBLs enzymes to hydrolysed third generation cephalosporins antibiotics and whereas these enzymes are inhibited by *B-lactamase inhibitors.* Further this method utilizes the Kirby-Bauer disk diffusion methods. However

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several researchers have proposed different phenotypic methods that is based on the Kirby Bauer disk diffusion methods. These methods are:

2.9.1.1 Double Disk Approximation test

The first routine double disk diffusion approximation test method for ESBL detection was demonstrated by synergy between 30ug antibiotic disk of ceftazidime, ceftriaxone, cefotaxime and cefpodoxime placed 30mm (center to center) from amoxicillin/clavulanate (20*ug*/10*ug)* disk, (Drieux *et al.,* 2008). However ,this method is influenced by low ESBL activities which widen inhibition zones. Furtherther, interpretation of the test is quite subjective. Despite this, several researchers have done similar work on this method and indicated that, sensitivities and specificities may range from 79% to 97% and 97% to 100% respectively (Gangoué-Piéboji *et al*., 2005). The main advantage of this test is that, procedures are simple and also it remains one of the convenient method for screening ESBLs in the laboratory laboratory (Rupp *et al*. 2003).

2.9.1.2 Disk Replacement method

This method involves the replacement of three 6-mm sterile paper disk inoculated within 20ul of clavulanic acid (200ug) and at the same spots, after one hour, with cefotaxime, ceftazidime and aztreonam disk on media inoculated with a test organism. In addition, control disk are simultaneously placed at least 30mm from these locations.

However, a positive control test will be defined by a zone increase of over 5mm for the replaced disk compared to controls (Andriatahina *et al.,* 2010). Comparatively this method is sensitive as the double disk approximation method but the second step involve renders it unsuitable for routine microbial method.

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2.9.1.3 Modified Disk synergy approximation method

This method involves the use of disc of amoxicillin-clavulanate (20/10ug) placed at the centre of the plate along with four cephalosporins placed 15mm to 20mm apart; third generation cephalosporins, cefotaxime, ceftriaxone, cefpodoxime and a fourth generation cephalosporins-cefepime. After which test organism was innoculated onto Muller-Hinton Agar . A susceptible control of Klebsiella pneumonia ATCC 700603 and a resistance control of Escherichia coli ATCC 25922 inoculated onto Muller –Hinton agar. Hence any increase in the zone towards the disc of amoxicillin–clavulanate was considered a positive for ESBL producton (Kaur,*et al.* ,2013). Although the process involve does not make it straight forward and non–specific for a particular ESBL production, in situations, where the AMpC-lactamases can interfere with the clavulanate synergy, the application of the double disc synergy test that combine amoxicillin-clavulanate with cefepime, may increase the possibility of the ESBL detection.

2.9.1.4 Three –dimensional method

This method involves a susceptible control strain such as *Escherichia coli ATCC* inoculated onto Mulller–Hinton agar plate,after which a slit is cut into the media and filled with a heavy inoculum (109cfu/ml) of test organism. In addition, extended spectrum cephalosporins disk are subsequently placed on the surface of the plate 3mm from slit. Further, a production of discrete colonies in the vicinity of the inoculated slit is considered positive *(*Andriatahina *et al.,* 2010). Though this test seems not to be specicific for ESBLs, it is quite sensitive than the double-disk diffusion test. Moreover this method is technically challenging an labor intensive.

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2.9.2 E-test method

It is a commercially manufactured strip by the AB Biodisk in Sweden and has two sided plastic strip in which a fixed concentration (4ug/ml) is added to one side of the oxyimmino-B-lactam MIC gradient. A positive ESBL production is indicated in a greater than 8fold reduction in the MIC of cephalosporins with clavulanic acid (Tansarli *et al.,* 2014). Further, some researchers analysed this method and concluded the following; that, the E-test remains the easiest method for ESBL detection and suitable for routine work with reported sensitivities and specificities ranged from 87%-100% and 95%-100%, respectively. Nevertheless, it has the following limitations; results are indeterminate for weak enzyme, subtle zone of inhibition are difficult to identify and interpret, also expensive to be used as a routine microbial detection method (Tansarli *et al.,* 2014).

2.9.3 Automated ESBL detection systems

These are automated antimicrobial susceptibility test which perform analyses and interpretation of ESBL phenotype. Dade Behring Microscan (Sacramento, United States), developa microscan panels which uses dehydrated serial dilutions of cephalosporins and clavulante combination panels (Gangoué-Piéboji *et al*. 2005).

The Vitex ESBL test produced by BioMerieux (Missouri, United States), utilizes cephalosporins and cephalosporin- inhibitor combinations in wells on a card to detect ESBLs within 4 to 15hours (Sundsfjord *et al.,* 2017). Becton Dickinson Biosciences also produce the phoenix Automated Microbiology System which introduces a short incubation system of 6 hours to measure responses to ceplaosporins, with or without clavulanic acid (Sundsfjord *et al.,* 2017).

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Nevertheless some researchers have investigated some advantages of these automated detection methods inroutine laboratory work. Several researchers investigated the performance levels of some automated systems. Some group of workers concluded on the percentages of sensitivity and specificity of three automated systems. They however concluded that, the system with the highest sensitivity was Phoenix (99%), followed by Vitex2 (86%) and MicroScan (84%). Nevertheless their specificities were much lower ranging from 52% (Phoenix) to interpretation of ESBL phenotypes. Becton Dickson Biosciences also manufactured by the Phoenix Automated System introduces a short incubation system of 6 hours to measure growth responses to cephalosporins, with or without clavulanate (Sanguinetti *et. al*., 2003). Dade Behring MicroScan (Sacramento, United States) designed the Microscan panels which uses dehydrated serial dilutions of cephalosporins and clavulanate in panels (Paterson *et al.*,2005).

Finally, the Vitex ESBL test, produced by Bio Merieux Vitex (Missouri, United States) utilizes cephalosporins and cephalosporins–inhibitors combinations in wells on card to detect ESBLs within 4 to 15hours (Bar-Yoseph *et al.,* 2016). Some studies have also evaluated the advantages of these methods in routine microbial ESBL screening. In an earlier study, a group of researchers compared the potency of Vitek2 System and the Phoenix system to detect ESBLs. Whereas the Phoenix system showed 100% detection rate, the Vitex2 System misidentified 5% of the ESBL producing isolates due to susceptibilities to either cefotaxime or ceftazidime. These results are comparable to the conclusions made by Leverstein-van Hall and others (2002) who evaluated the various automated ESBL test and concluded that the automated system are capable of detecting ESBL positives with almost the same efficiency as the conventional techniques. Nonetheless, they can be complex in operation and expensive. The new Alifax HB&L ESBL/AmpC screening kit provides report of ESBL/AmpCproducing Enterobacteriaceae spp

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presence/absence in few hours thus supporting the active surveillance of Multi Drug Resistant Organisms (MDROs). The principle is based on light scattering systems are able to monitor the bacteria replication activity from the inoculum step into selective culture broth providing real time growth curves.

2.9.4 ESBL GENOTYPING

Genotypic detection is used to identify the specific ESBL genes that are present in each isolates. However, the most common and widely used genotypic method is the polymerase chain reaction(PCR).Further the first step is to extract DNA from each isolates and then it is screened for the presence TEM, SHV ,CTX-M, CTX, OXA by a polymerase chain reaction (PCR) amplification with oligonucleotide primers to determine genes of specific β-lactamase families (Abdallah *et al*., 2017) In addition, electrophoresis is used to show the presence or absence of amplicons and when present they can be subjected to DNA sequencing but this may also give variable results as difficulties in reading some sequencing autoradiographs introduce errors in establishing true differences amongst many sequences especially SHV genes (Rupp *et al*., 2003). The authors indicated in that the presence of multiple copies of ESBL genes in some clinical isolates may make the procedure uneasy and expensive. Nonetheless, nucleotide sequencing remains the gold standard for determination of specicific ESBL gene present in a strain (Sundsfjord *et al.,* 2017).

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CHAPTER THREE

3.0 METHODS

3.1 STUDY SETTINGS

The study was conducted at the Achimota District Hospital (ADH) in the Greater-Accra region of Ghana. The ADH is approximately a 100-bedded primary care government hospital that serves a pediatric and adult population of over 200,000 in the Achimota urban community within the Accra Metropolitan Area (Ghana Statistical Service & Ghana Demographic Health Survery 2008; Asante *et al*. 2015). The hospital has emergency wards but no intensive care units; and caters for medical and trauma cases. The ADH runs outpatient department clinics for patients with HIV/AIDS. Per clinic day, the Unit offers counselling and routine HIV/AIDS management for about 40 patients. The hospital has a ‘’Retro Ward’’ for admission of HIV/AID patients. Diagnosis of HIV at the ADH is by the INNO-LIA HIV-1/-2 line immunoassay. The ADH has a central laboratory that provides some microbiological services, including routine parasitological examinations, but does not perform bacterial culture and antibiotic sensitivity testing. The laboratory offers immunology, biochemistry, as well as haematology and transfusion services.

3.2 STUDY DESIGN

Overall, two sampling designs were used in parallel. First, a cross-sectional prospective sampling was conducted to recruit study participants. Second, recruited participants were interviewed with a structured questionnaire. Patient recruitment, faecal sampling, and questionnaire administration were conducted between February and May 2017.

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3.3 STUDY PARTICIPANTS

The study participants comprised patients living with HIV/AIDS.

3.3.1 Inclusion criteria.

Study participants were either patients on admission ≤48 hours at the "Retro ward" or attending the hospitals HIV/AIDS clinic during the survey period

3.3.2 Exclusion criteria.

Exclusion criteria included unresponsive patients with poor prognosis due to AIDS defining illness.

3.3.3 Minimum number of study participants

The minimum number of patients recruited for this study was determined using the statistical formula (Buderer 1996);

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

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

Using a 10% allowable ERROR (to compensate for minimum sample size)

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$$N = \frac{1.97^2 (0.5)(1-0.5)}{(10/100)^2}$$

$$=98$$

A minimum of 100 patients living with HIV/AIDS were recruited for study.

3.3.3.1 Study Samples

The study samples comprised faecal specimens collected from study participants

3.3.3.2 Study isolates

The following constituted the strain collection for the present study

a) *Enterobacteriaceae* isolates recovered from fecal samples of study patients.

b) Non-enterobacteria isolates recovered from faecal samples were excluded from further studies.

3.4 PROCEDURE FOR DATA COLLECTION

The exercise was grouped into four work packages:

a) Phase 1- specimen collection and faecal cultures

b) Phase 2- phenotypic ESBL assays

c) Phase 3- molecular investigation

3.4.1 Specimen collection

This phase included three sampling methods: selection of potential participants, administration of questionnaire-structured interviews, and collection of fecal samples. Appropriate permissions were sought from the authorities at Achimota District Hospital before commencement of study. On each sampling day, all patients with HIV/AIDS attending the OPD clinic or on ‘’Retro’’ at

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the ward were considered potential study participants. The first 100 randomly selected patients to provide consent of participation were enrolled into the study.

Prior to enrollment, study aim and objectives, risks and benefits as well as requirements from patients were explained to potential participants. They were also provided with copies of project's information sheet (Appendix 1). Patients were then requested to join the study. Those who provided informed consent and were willing to fill questionnaires and submit stool samples were enrolled. The HIV/AIDS status of all enrolled patients were confirmed in their folders with the help of attending doctors.

After informed consent from respondents, questionnaires were used for interviews. Study participants were interviewed using a questionnaire instrument (Appendix 2) to collect data regarding demography (age, gender, place of stay, number of persons in household, educational level, employment status). Underlying comorbidities of study patients, such as diabetes and malignancy, were collected. Information on patients' lifestyle characteristics were recorded.

These included the use of hand sanitizer at least once per day in the past 3 months, frequency of hand sanitizer uses per day, frequency of hand sanitizer per day, daily hand washing per day, travel overnight outside home in past one year, travel outside Ghana in past 1 year, pipe water in household, toilet facility in household, and animal contact in past 3 months. Patients were also interviewed on hospitalization history and the evidence corroborated with attending nurses and physicians. Here, patients were asked about hospitalization in the past 1 year, invasive hospital disease of any type in past 1 year, use of medication that affect intestinal flora stomach flora (e.g., stomach acids neutralizer, proton pump inhibitor or H_2 blockers) current antibiotic use, and specific current antibiotic use.After interviews, study participants were requested to provide

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faecal specimens for study. They were instructed on how to aseptically self-collect and submitted stool samples for laboratory investigations.

Faecal samples were transported on ice at $0^{o}C$ to the microbiology laboratory unit of the Department of Medical Laboratory Sciences, University of Ghana, where primary faecal cultures were prepared for enumeration of enterobacteria colony counts.

3.4.1.1 Primary faecal cultures

Viable bacteria count for total culturable enterobacteria was performed. Briefly, 1g of each faecal sample was suspended in 10ml 0.9% sterile 0.9% normal saline solution. 1 ml of the suspension was then spotted to prepare serial 10^{-1}-10^{-4} fold dilutions in 9ml sterile phosphate buffered saline. For each dilution, 1 ml was thoroughly mixed with 22ml of SSI agar plate (at $52^{o}C$) in a 90mm petri dish and allowed to solidify. The SSI Enteric agar is a selective for rapid differentiation of members of the family *Enterobacteriaceae*. Plates were incubated at 35 - 37°C for 24 hours. The serial dilution (10^{n}) with a total enterobacteria count between 30 to 300 colonies was selected for reporting. Here, all individual isolates with different colonial morphology were assigned numbers and subsequently countered. The total colony count per morphology type was recorded and the faecal concentration calculated as: $10_{\text{(for 1g in 10ml dilution)}}$ x colony count x 10^{n} $_{\text{(where n is the nth serial dilution factor)}}$ and the units stated as CFU/g of faecal sample. All enterobacteria isolates with different colonial morphologies from the colony count plates were subcultured on SSI agar for purity.

3.4.2 Phenotypic determination of ESBL

The pure enterobacteria cultures of various morphological types were then subjected to ESBL phenotypic screening tests.

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3.4.2.1 Screening test for ESBLs

Each entrobacteria morphotype was screened for the presumptive presence of ESBL production using the Kirby-Bauer's method of sensitivity testing according to protocols by the Clinical and Laboratory Standards Institute (CLSI, 2016). Briefly, from a pure subculture of each isolate, four morphologically similar colonies were touched with a sterile inoculating wire and transferred into 5ml of 0.9% normal saline solution. The test suspension was incubated at room temperature for 15 minutes. Density of the incubated inoculum was adjusted where necessary with sterile distilled water until it equalled that of 0.5McFarland standard [10^{7-8} Colony forming units (CFU/ml)]. A 5µm loopful of the standardized inoculum was dispensed to the canter of a 90mm Mueller-Hinton agar plate (Oxoid, United Kingdom). The inoculum on the agar plate was swabbed carefully 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 at least 15 minutes. The antibiotic disks ceftazidime (30µg) and cefotaxime (30µg) were 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 reference breakpoints. Enterobacteria with zone inhibition diameters of ≤27mm for ceftazidime and ≤29mm for cefotaxime were reported as cephalosporin resistant and positive for ESBL screening.

3.4.3 Bacterial identifications

Representative colonies of isolates were picked using sterile straight wires and inoculated into tubes of peptone broths (Sigma, UK), urea agar slants (Sigma,UK), citrate slants (Sigma, UK), Triple–Sugar-Iron agar slants (Sigma, UK) and motility test media.

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Inoculated biochemical media were incubated aerobically at 35-37 for 18 to 24 hours and examined for reactions suggestive of members of the family *Enterobacteriacea*. Drops of Kovac's reagent (Oxoid, UK) were added to peptone broth cultures for reactions suggestive of indole production. For each isolate, reactions of biochemical tests were compared to that of reference strains for presumptive species identification. All enterobacteria faecal colonies that were cephalosporin sensitive were identified to the genus level. Those previously determined to be cephalosporin resistant were identified to the species level. Definitive confirmations of isolates to the species level were performed with the MINIBACT-E ® (SSI Diagnostica, Denmark) according to manufacturer's guidelines.

3.4.4 Confirmation of ESBL production

All isolates positive for ESBL screening were subjected to ESBL confirmation test using the Kirby-Bauer's method of sensitivity testing according to protocols by the Clinical and Laboratory Standards Institute (CLSI ,2016). Detection of ESBL-production was done by the combination disk method with cefotaxime (30µg) and ceftazidime (30µg) alone and in combination with clavulanic acid (10µg) using NeoSensitabs (Rosco Diagnostica, Denmark). A zone difference of ≥5mm between the single and the combination disks for cefotaxime and/or ceftazidime was regarded as positive for ESBL production. Klebsiella pneumoniae ATCC 700603 was used as positive control for ESBL production. Escherichia coli ATCC 25922 was used as a negative control.

3.4.5 Phase 3 –Molecular investigations

All ESBL-producing enterobacteria were characterized by Polymerase Chain Reactions (PCR) to confirm the presence of gene families encoding the ESBLs

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.3.4.5.1 Bacterial DNA extraction.

Bacteria whole DNA template for PCR was extracted using the boiling suspension method. Briefly, 10µL of pure culture on Mueller Hinton agar was suspended in 300µL Milli-Q® water, heated for 10 minutes at 98ºC, and subsequently centrifuged for 5 minutes at 4ºC and 20.000g. The supernatant was transferred into sterile 1.5ml Eppendorf® tubes, and the DNA lysates kept at -5ºC until further work up.

3.4.5.2 Polymerase Chain Reactions

Polymerase chain reactions were performed for the genes (TEM, SHV, CTX-M, OXA) that code for the ESBLs. Table 3.1 shows the amplification primers and conditions. The PCR volumes for ESBL assays comprised 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). For all PCR processes, previously characterized strains positive for the specific genotypes under study 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. Standard PCR assay was performed for TEM gene at 94ºC for 15 minutes and then for 30 cycles of 94ºC for 30 seconds, 63ºC for 90 seconds, 72ºC for 60 seconds, and a final extension at 72ºC for 10 minutes, with a final hold at 6ºC in a thermal cycler. For CTX-M-9, PCR was conducted at 94ºC for 15minutes and then for 27 cycles of 94ºC for 30 seconds, 50ºC for 90 seconds, 72ºC for 60 seconds, and a final extension at 72ºC for 10 minutes, with a final hold at 6ºC in a thermal cycler. Multiplex PCR assay was performed for CTX-M-1 and CTX-M-2 genes at 94ºC for 15 minutes and then for 27cycles of 94ºC for 30 seconds, 50ºC for 90 seconds, 72ºC for 60 seconds, and a final extension at 72ºC for 10 minutes, with a final hold at 6ºC in a thermal cycler.

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3.1 PCR primers and Thermocycling conditions

Primer (target)	Primer sequences (5'-3')	T °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 °C at 90 seconds	30
CTX-M-2 (253 bp)	FP: ACAGTTGGTGACGTGGCTTAAGG RP: TCAGAAACCGTGGGTTACGA	50 °C for 90 seconds	30
CTX-M-8/25/26 (690/346bp)	FP1: ACATCGCGTTAAGCGGAT FP2: GCACGATGACATTCGGG RP: AACCCACGATGTGGGTAGC	50 °C for 90 seconds	30
CTX-M-9 (860bp)	FP: ATGGTGACAAAGAGAGTGCAACG RP: ATGATTCTCGCCGCTGAAGC Internal FP: CAAATTGATTGCCCAGCTCG Internal RP: AAACGTCTCATCGCCGATCG	50 °C for 90 seconds	30

T°C , activation temperature, PCR, polymerase chain reaction

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Isolates negative for CTX-M-1 and -2 genes were also subjected to PCR amplification using conditions as for CTX-M-1. Another multiplex PCR assay was also performed for SHV, OXA 2 and OXA 10 genes at 94ºC for 15 minutes and then for 30 cycles of 94ºC for 30 seconds, 63ºC for 90 seconds, 72ºC for 60 seconds, and a final extension at 72ºC for 10 minutes, with a final hold at 6ºC in a thermal cycler (Doosti et al. 2015).

3.4.5.3. Agarose gel electrophoresis

The amplified products was run on 2% (weight/volume) agarose gel tinged with 5µL of 0.5mg/mL ethidium bromide in a dark room. A constant voltage of 100V for 30 minutes was supplied for the separation process. A 1X Tris-acetate-EDTA (TAE) was used as buffer. A 100-bp ladder molecular weight marker will be used to measure the molecular weight of the amplified products. The images of ethidium bromide stained DNA bands was visualized using a UV trans-illuminator.

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3.4.5.4 ***Sequencing*****:** All PCR products with amplified genes for TEM, SHV, CTX-M-1, -2, and 9, as well as OXA-2, and -10 were sent to Macrogen (Seoul, Korea) for nucleotide sequencing. Additional internal primers were used for sequencing CTX-M-1, CTX-M-9, SHV and TEM genes. The contigs were examined using the ColonCode® analyzer (Codon Code cooperation, USA) and the aligned nucleotide sequences compared with sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). All 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) at the http://www.lahey.org/studies

3.5 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 col*i ATCC 25922 for every batch of new antibiotics and media and the results interpreted according to performance standards of CLSI guidelines. Student's *t*-test was used to analyze significant differences in inhibition zone sizes of the control strains. Briefly, no 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. Likewise, not more than 1 in 20 results was outside the stated accuracy limits by CLSI 2016 guidelines. Otherwise, the media or antibiotic is rejected

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3.6 DATA ANALYSIS

Study data was entered into a Microsoft Excel sheet for editing and results analyzed using Statistical Package for Social Sciences (SPSS) V16. The cephalosporin resistant isolates, ESBL-producing isolates and ESBL-negative isolates were described using relative numbers and, where appropriate, proportions or percentages. The PCR results were discussed using descriptive analysis with proportions and percentages. Overall, point estimates of statistical significance was determined at 2 tailed P-values <0.05. Continuous data was compared using student's t-test. Categorical data was compared across study parameters using $\chi 2$ or Fisher's exact tests where appropriate. Faecal colony counts were analyzed with box and whisker displays to illustrate the lower, upper, interquartile range, mean and median concentrations of the faecal enterobacteria isolates with or without ESBLs. The odds ratio (OR) was used to quantify the association between patients with and without faecal carriage of ESBL-producing enterobacteria and their independent predictor variables. From univariate analyses, predictor variables with P-value $<$ 0.05 were examined in multivariate logistic regression models to determine independent risk factors. Predictive accuracy of the models was evaluated by Hosmer and Lemeshow 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 analyse the discriminatory capability of ESBL faecal carriage versus their respective controls.

3.7 ETHICAL CONSIDERATION

Study approval was sought 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./10550562/AA/5A/2016-2017)..

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Informed written consent was obtained before subjects were enrolled in the study. Details of the study were clearly explained to participants before obtaining their consent and subsequent enrollment into the study. Information provided in this regard included the risk, benefits and the right to refuse participation or withdraw participation from the project at any time without any consequence (Information sheet in Appendix 1).

They were assured that enrollment into the study is wholly voluntary and that their clinical care and management will not be jeopardized by refusing to participate in the study. Participants were informed that there will be no financial incentives for participarting in this study.

3.8 FINANCIAL ACKNOWLEDGEMENT

This thesis was supported by a 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 (Danida) who financed the whole project.

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CHAPTER FOUR

RESULTS

4.1 PATIENT DEMOGRAPHIC CHARACTERISTICS

During the sampling period, a total of 430 patients living with HIV (including children and adults) were prospectively encountered and considered eligible for participation. Out of this population, 100 were recruited to the study. The others were either unresponsive, were unwilling to provide consent, fill a questionnaire or submit a faecal specimen for laboratory investigations.

They included 10 hospitalized patients and 90 outpatients, recruited respectively from the ''Retro-ward'' and the "HIV Outpatient Clinic'' of the study hospital. The hospitalized patients had been admitted ≤48 hours prior to start of enrollment. Eighty-seven study patients were females and 13 were males. Their ages ranged between 15-68 years with a mean±[standard deviation(SD) of 44.3±10.5 years]. Table 4.1 summarizes the demographic characteristics of study patients. The majority (53%) had primary education, 41% and 7% had respectively travelled outside their home and Ghana in the past 1 year, 56% had toilet facility in their household, 46% had pipe-borne water in their household, and 34% had previously been hospitalized in the past 1 year. All patients were on antiretroviral therapy and most (n=72) had lived with HIV (after first diagnosis) for 7 years (mean±SD, 7.4±5.2 years; range, 3 months to 13 years). In all, the mean number of household persons living with a study patient was 4.0±3.7 (range, 1- 8 persons per household).

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Table 4.1. Patients demographic characteristics 100 enrolled participants.

Description		Number (%)
Age (Mean ± SD)		44.3±10.5
Age groups		
	<28 days	-
	28 days-1 year	-
	1 -5 years	-
	5-15 years	-
	15-65 years	99
	>65 years	1
Years after first HIV infection diagnosis (mean±SD)		7.4± 5.2
Number of persons in household (mean±SD)		4.0± 3.7
Gender		
	Female	87
	Male	13
Education		
	None	12
	Primary	53
	Secondary	30
	Tertiary	5
Inpatient status		
	Yes	10
	No	90
Travel outside home in past 1 year		
	Yes	41
	No	59
Travel outside Ghana in past 1 year		
	Yes	7
	No	93
Toilet Facility in the household		
	Yes	63
	No	27
Pipe water in household		
	Yes	39
	No	61
Hospitalized in the past one year		
	Yes	62
	No	38

* SD, standard deviation; %, percentage; n, number.

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4.2 FAECAL SAMPLE CULTURES FOR CEPHALOSPORIN RESISTANT AND ESBL-POSITIVE *ENTEROBACTERIACEAE*

A 100 faecal samples were received, one per study patient. Of these, 100, 45, 24, 18 and 8 respectively grew *E. coli*, *klebsiellae*, *Citrobacter* species, *Enterobacter* species, and *Morganella* species (Table 4.2). *Proteus* and *Salmonella* species were each recovered from 7 and 5 feacal cultures respectively. Overall, 65 feacal cultures grew 89 cephalosporin resistance enterobacteria: 52 grew *E. coli* only; 5 had *E. coli*, *K. pneumoniae*, and *K. oxytoca*; 2 had *E. coli* and *Enterobacter cloacaea*; 2 had *E. coli*, *Citrobacter freundii*, and *Salmonella enteritidis*; 2 had *E. coli*, *K. pneumoniae*, and *Citrobacter koseri*; 1 had *E. coli*, *K. oxytoca,* and *Morganella morgani*; and 1 had *K. oxytoca* and *Proteus vulgaris*. When the 89 cephalosporin resistant enterobacteria were confirmed for ESBL activity, 41 isolates (1 per faecal sample) showed clear ESBL phenotype: 39 *E. coli*, 1 *Proteus vulgaris*, and 1 *Salmonella enteritidis*.

In this study, all the cephalosporin resistant enterobacteria (irrespective of ESBL phenotype) were subjected to PCR and nucleotide sequencing for TEM, SHV, CTX-M and OXA ESBL genes. All ESBL-producing isolates had a corresponding ESBL gene identified by PCR and sequencing. Two cephalosporin resistant isolates 1 *C. freundii* and 1 *M. morgani*) with a clear ESBL-negative phenotype were found to harbour ESBL genes. No ESBL genes were found in all the other ESBL-negative cephalosporin resistant isolates.In all 43 isolates were considered ESBL-positive and subsequently included in analysis [i.e., (a) 41 cephalosporin resistant isolates with ESBL phenotype and ESBL genes plus (b) 2 cephalosporin resistant isolates without ESBL phenotype but ESBL genes]

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Table 4.2: Faecal sample cultures for cephalosporin resistant and ESBL-positive *Enterobacteriaceae*

Bacteria isolates / Cephalosporin resistant species	Number of faecal samples (n=100)	Number of faecal samples with Cephalosporin resistance enterobacteria (n=65)	Number of faecal samples with ESBL phenotype positive	Number of faecal samples with ESBL gene identified by sequencing
Escherichia coli	100	65	39	39
Klebsiella species	45			
K. pneumoniae	-	7	0	-
K. oxytoca	-	7	0	-
***Citrobacter* species**	24			
Citrobacter freundii	-	2	0	0
Citrobacter Koseri	-	2	0	1[a]
***Enterobacter* species**	18			
Enterobacter cloacae	-	2	0	-
***Morganella* species**	8			
Morganella morgani	-	1	0	1[a]
***Proteus* species**	7			
Proteus mirabilis		1	1	1
***Salmonella* species**	5			
Salmonella enteritidis	-	2	1	1
Total isolates	207	89	41	43

* Bacteria species identification confirmed for cephalosporin resistant isolates; ESBL, extended-spectrum beta-lactamases; No., number.These isolates had clear ESBL- negative phenotype but harboured ESBL genes

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4.3 Molecular characterization of ESBL gene types

Although PCR for sequencing were performed for TEM, SHV, CTX-M and OXA genes, none of the ESBL-producing enterobacteria harboured a SHV or an OXA ESBL (Table 4.3). All isolates with ESBL genes had either bla_{TEM} or bla_{CTX-M} and their combinations thereof. The ESBL-positive *S. enteritidis* and *P. vulgaris* isolates respectively had $bla_{CTXM-13}$ and bla_{CTXM-3} genes only. The two isolates (*Citrobacter freundii* and *Morganella morgani*) with ESBL genes but no clear ESBL-phenotype respectively harboured $bla_{CTX-M-14}$ and $bla_{CTX-M-27}$ ESBL, each plus a complex mutant TEM $bla_{TEM-121}$ gene.

The most predominant ESBL gene type was bla_{CTM-15} (n=17), followed by bla_{TEM-3} (n=11), $bla_{CTXM-14}$ (n=6), and bla_{CTXM-3} (n=6). In total, 16 isolates harbored one ESBL gene (4 bla_{TEM-3}, 1 bla_{TEM-4}, 1 bla_{TEM-6}, 3 bla_{TEM-15}, 1 $bla_{CTX-M-3}$, 2 $bla_{CTX-M-9}$, 2 $bla_{CTX-M-13}$, 2 $bla_{CTX-M-14}$, 10 $bla_{CTX-M-15}$), 11 had 2 ESBL genes (2 $bla_{CTX-M-3/TEM-8}$, 1 $bla_{CTX-M-13/TEM-3}$, 6 $bla_{CTX-M-15/TEM-3}$, 1 $bla_{CTX-M-14/TEM-121}$, 1 $bla_{CTX-M-27/TEM-121}$) whiles 6 combined an ESBL (1 $bla_{CTX-M-3}$, 1 $bla_{CTX-M-13}$, 3 $bla_{CTX-M-14}$, 1 $bla_{CTX-M-15}$) plus a broad-spectrum beta-lactamase (bla_{TEM-1}) gene.

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Table 4.3 Molecular characterization of ESBL gene types

ESBL gene type	*E. coli*	*S. enteritidis*	*P.* vulgaris	*M. morgani*	*C. freundii*	Total
ESBL only						
SHV-type	0	0	0	0	0	0
OXA-type	0	0	0	0	0	0
TEM-3	4	0	0	0	0	4
TEM-4	1	0	0	0	0	1
TEM-6	1	0	0	0	0	1
TEM-15	3	0	0	0	0	3
CTX-M-3	0	0	1	0	0	1
CTX-M-9	2	0	0	0	0	2
CTX-M-13	1	1	0	0	0	2
CTX-M-14	2	0	0	0	0	2
CTX-M-15	10	0	0	0	0	10
CTX-M-3/TEM-8	2	0	0	0	0	2
CTX-M-13/TEM-3	1	0	0	0	0	1
CTX-M-15/TEM-3	6	0	0	0	0	6
ESBL+ broad spectrum beta-lactamases						
CTX-M-3/TEM-1	1	0	0	0	0	1
CTX-M-13/TEM-1	1	0	0	0	0	1
CTX-M-14/TEM-1	3	0	0	0	0	3
CTX-M-15/TEM-1	1	0	0	0	0	1
Isolates with Complex Mutant TEM (CMT genes)						
CTX-M-14/TEM-121	0	0	0	1	0	1
CTX-M-27/TEM-121	0	0	0	0	1	1

* Isolates with CMT phenotype are resistant cephalosporins and ESBL inhibitors; *E. coli, Escherichia coli; S. enteritidis, Salmonella enteritidis; P. mirabilis, Proteus mirabilis, M. morgani, Morganella morgani; C. freundii, Citrobacter freundii*

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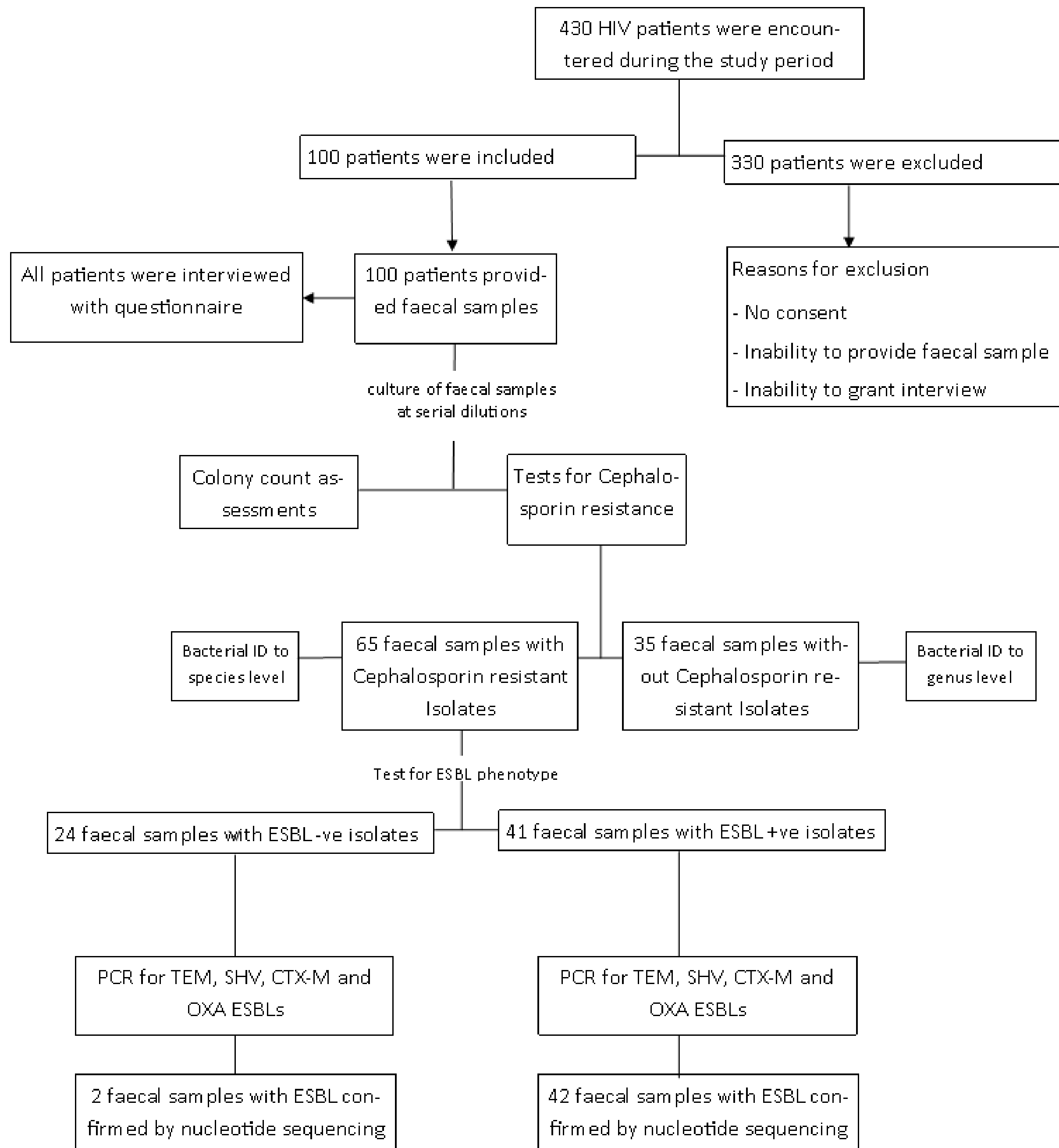

Figure 4.1 Flow chart for data collection and summary study outcome

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4.4 FAECAL CONCENTRATION OF ESBL-PRODUCING ENTEROBACTERIA

For patients found to be ESBL carriers, this study analyzed the faecal colony counts of ESBL-positive versus ESBL-negative enterobacteria to determine the predominant bacteria type. Overall, the colony counts of both bacteria groups varied markedly depending on the type of faecal carriage.

***Among all 43 ESBL carriers*:** In figure 4.4a, the mean (±SD) faecal concentration of all ESBL-positive isolates was $100x10^4$±$21x10^4$ CFU/g with an interquartile range (IQR) of $55x10^4$-$130x10^4$ CFU/g. That of ESBL-negative isolates was significantly higher (mean±SD, $126x10^4$±$81x10^4$ CFU/g) with a much wider IQR ($51x10^4$-$181x10^4$ CFU/g). The distribution changes with faecal carriage by ESBL gene type.

***By ESBL gene type*:** Given that bla_{CTM-15} is the most reported ESBL worldwide, this study sought to examine the faecal concentrations of $bla_{CTX-M-15}$-positive enterobacteria versus $bla_{CTX-M-15}$-negative-but-ESBL-positive enterobacteria; and how they compare to that of ESBL-negative enterobacteria within the same patient. Among the CTX-M-15 faecal carriers (Figure 4.4b), the predominant faecal isolates were the CTX-M-15 positive enterobacteria (mean±SD, $182x10^4$ ± $2.1x10^4$ CFU/g; IQR, $159x10^4$– $200x10^4$ CFU/g) compared to the fewer ESBL-negative enterobacteria (mean±SD, $75x10^4$ ± $2.5x10^3$ CFU/g; IQR, $55x10^4$-$121x10^4$CFU/g). Within the CTX-M-15 faecal carriers, one in every 1.6 faecal enterobacteria had ESBL. On the contrary, the predominant isolates in patients with non-CTX-M-15 ESBL faecal carriage (Figure 4.4c) were the ESBL-negative enterobacteria (mean ±SD, $134x10^4$±$71x10^4$ CFU/g of faecl sample; IQR, $49x10^4$-$161x10^4$ CFU/g). The non-CTX-M-15 ESBL-positive isolates had a significantly lower mean concentration of $81x10^4$ ±$1.1x10^3$CFU/kg (IQR, $32x10^4$-$1012x10^4$CFU/g. Here, one in every 2.1×10^2 faecal enterobacteria was ESBL-positive.

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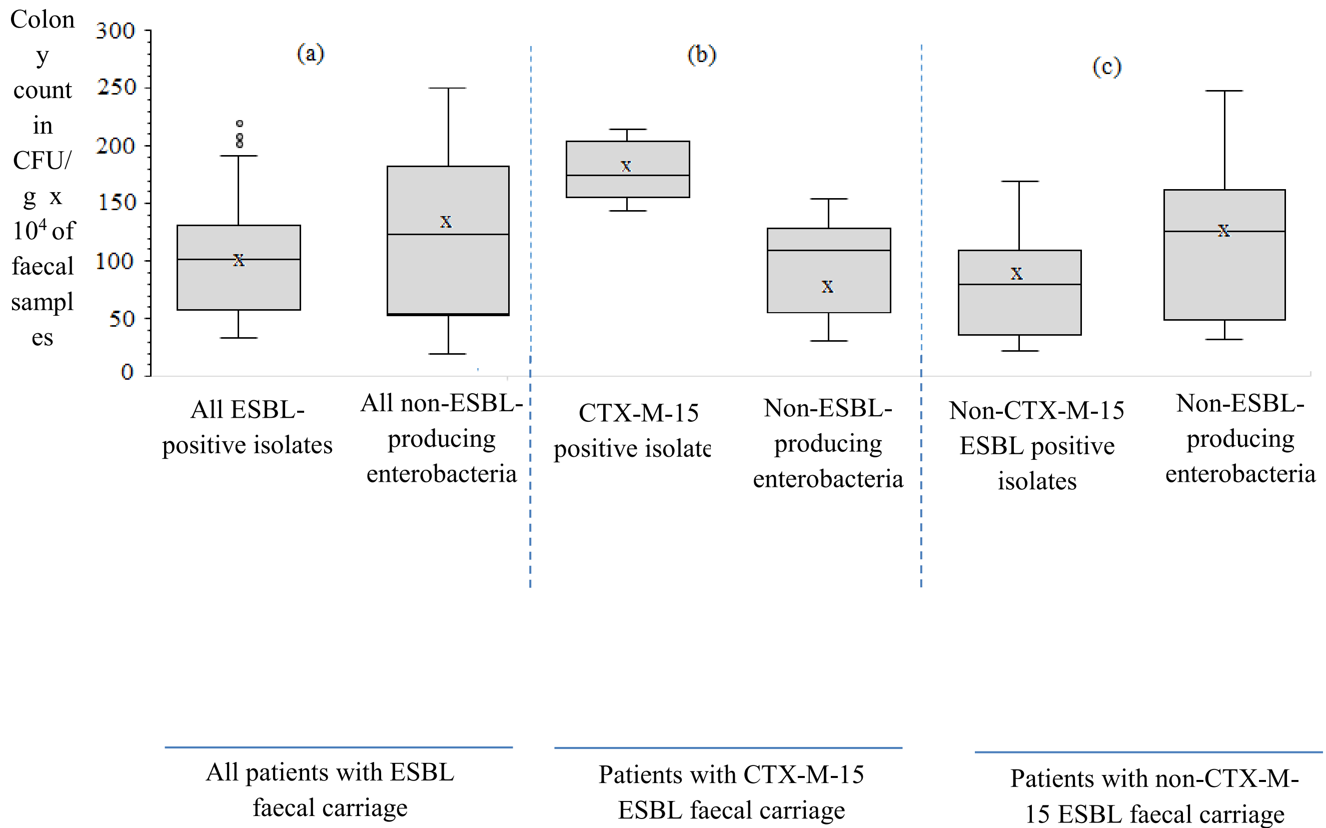

Figure 4.4a Faecal concentrations of ESBL-producing enterobacteria.Lower whisker, 25 percentile/lower quartile; Upper whisker, 75 percentile/upper quartile; Box, interquartile range; Horizontal line in box, median of the distribution; x, mean of distribution.

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4.5 A RISK FACTORS FOR ESBL FAECAL CARRIAGE

Univariate analysis: Analysis for potential risk factors for faecal carriage of ESBL-positive enterobacteria are shown in Table 4.5a. The following were significantly associated with ESBL faecal carriage: number of persons living with the study patient (p-value=0.008); current use of beta-lactam antibiotics [Odds Ratio (OR), 6.1; 95% Confidence Interval (CI), 2.4-14.9; p-value <0.001]; and mean faecal enterobacteria concentration (p-value=0.01). Study patients who used hand sanitizer at least once per day in past 3 months were less likely (OR, 0.3; 95%CI,0.1-0.9; p-value=0.03) to be colonized by ESBLs. Similarly, HIV outpatients who were currently on beta-lactam/fluoroquinolone antibiotic regimen were less often (OR, 0.4; 95%CI, 0.2-0.9; p-value=0.04) colonized by ESBL-positive enterobacteria. Variables that showed significant associations with ESBL faecal carriage in univariate analysis were regarded as potential risk factors and entered into stepwise logistic regression models.

Multivariate logistic regression: The results of the logistic regression to identify independent risk factors for faecal carriage with an ESBL-producing enterobacteria are presented in Table 4.5b. The only variable associated with an increased risk of ESBL faecal carriage was the number of persons living with the HIV patient in his or her household (Figure 4.5c). From the multivariate analysis, every 1 person increase in the number of household contacts was associated with a 10% risk of ESBL faecal carriage.

The Hosmer and Lemeshow goodness-of-fit test was not significant indicating a satisfactory fit of the multivariate logistic regression model (p = 0.69). The area under the ROC curve was 0.78 (95% CI: 0.69–0.89) indicating a good discriminative ability between ESBL faecal carriers and non-ESBL faecal carriers.

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Table 4. 4a Univariate comparison of risk factors exposition in the study population with and without ESBL-positive faecal carriage

Variables	Patients with ESBL fecal carriage (n=43)	Patients without ESBL fecal carriage (n=57)	Crude Odds Ratio(95%CI)	P-value
Age (mean ±SD)	44.4±10.5	43.8±9.9		0.8
Age group				
Neonate(<28days)	0	0	-	
Infants (<28days-5days)	0	0	-	
Paediatrics (5-18yrs)	0	0	-	
Adults (18-65yrs)	40	55	0.5(0/1-2.9)	0.6
Elderly(>65yrs)	3	2	20.(0.3-2-9)	0.6
Female gender	31	46	0.6 (0.2-1.6)	0.31
Number of persons in household (mean±SD)	6.0±2.1	5.0±1.6		0.008
Inpatients	10	0	-	-
Employed	34	51	0.4(0.1-1.4)	0.1
Education				
None	1	6	0.2(0.1-1.7)	0.1
Primary	8	18	0.4(0.2-1.3)	0.1
Secondary	25	23	1.0(0.5-2.5)	1.0
Tertiary	1	0	0	0.4
Underlining diseases				
Diabetes	10	9	1.6 (0.5-4.4)	0.3
Diarrhoae	8	7	1.6 (0.5-4.9)	0.4
Respiratory infection	0	2	-	-
Sickle cell	10	6	2.6(0.9-7.7)	0.08
Malignancy	1	1	1.3 (0.08-21.9)	1
Chronic alcoholic use	5	10	0.6(0.2-2.0)	0.6
Chronic smoker	7	9	1.0(0.3-3.0)	1
Patients life style characteristics				
Used hand sanitizer at least once per day in past 3 months	32	51	0.3 (0.1-0.9)	0.03
Frequency of hand sanitizer use per day				
2	8	11	0.9 (0.3-2.6)	0.9
3	8	10	1.1 (0.4-3.0)	0.8
4	10	9	1.6 (0.6-4.4)	0.3
5	5	12	0.4 (0.2-1.5)	0.2
>6	1	9	0.1 (0.01-1.04)	0.04
Daily hand washing in per day				
1	12	20	0.7 (0.5-1.4)	0.4
2	15	22	0.9 (0.4-1.9)	0.7

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3	3	4	0.9 (0.2-4.7)	1
4	1	1	1.3 (0.081-21.9)	1
>4	1	1	1.3 (0.081-21.9)	1
Travelled overnight outside home in past one year	19	22	1.3(0.6-2.8)	0.5
Travelled outside Ghana in past 1 year	14	13	1.6(0.3.97)	0.3
Mean (±SD) faecal enterobacteria concentration (CFU/kg)	$206x10^4 \pm 31x10^4$	$191x10^4 \pm 34x10^4$	1.5 (1.2-9.3)	0.01
Pipe water in the house	16	23	0.9(0.4-2.0)	0.8
Toilet facility in household	38	54	0.4(0.1- 1.9)	0.3
Animal contact in past 3 months	23	32	0.89 (0.4-1.9)	0.8
Hospitalization history				
Hospitalized in past 1 year	26	37	0.8 (0.3-1.8)	0.6
Invasive hospital procedures of any type in past 1year	11	9	1.5 (0.5-3.9)	0.5
Use of medications that affect intestinal flora stomach acids neutralizer, proton pump inhibitor,H_2 blockers)	12	19	0.8 (0.3-1.8)	0.6
Used antibiotics in last 6 months	40	55	0.4 (0.3-0.5)	0.08
Current antibiotic use	39	51	1.6 (0.4-5.5)	0.5
Specific antibiotics				
Aminoglycosides	23	31	9.9 (0.4-2.1)	0.9
Beta-lactams	34	22	6.1 (2.4-14.9)	<0.001
Beta-lactam/aminoglycosides	18	19	1.4 (0.6-3.2)	0.4
Beta-lactam/fluoroquinolones	20	43	0.4 (0.2-0.9)	0.04
Macrolides	10	6	1.6 (0.9-7.7)	0.08
Metronidazoles	7	7	1.4 (0.5-4.3)	0.6
Phenols	4	5	1.4 (0.4-5.7)	0.7
Fluoroquinolones	20	31	0.7 (0.3-1.6)	0.4
Lincosamides	18	33	0.5 (0.2-1.2)	0.11

SD, standard deviation; CFU/kg, colony forming units per g of faecal samples

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Table 4.4b. Independent risk factors for faecal carriage in patients living with HIV

Variable	Level	Adjusted Odds Ratio	95% Confidence Interval	P-value
Number of persons in household (mean±SD)	I person increase	1.1	1.1-2.6	<.001
Used hand sanitizer at least once per day in past 3 months	Yes/No	0.5	0.2 -0.0.98	0.19
Current use of beta-lactam antibiotics	Yes/No	4.1	3.2 -14.9	0.08
Current use of beta-lactam/fluoroquinolones	Yes/No	0.3	0.1 – 0.8	0.11
Mean (±SD)faecal enterobacteria concentration	10^2 increase	1.3	1.08 - 3.7	0.27

***SD, standard deviation**

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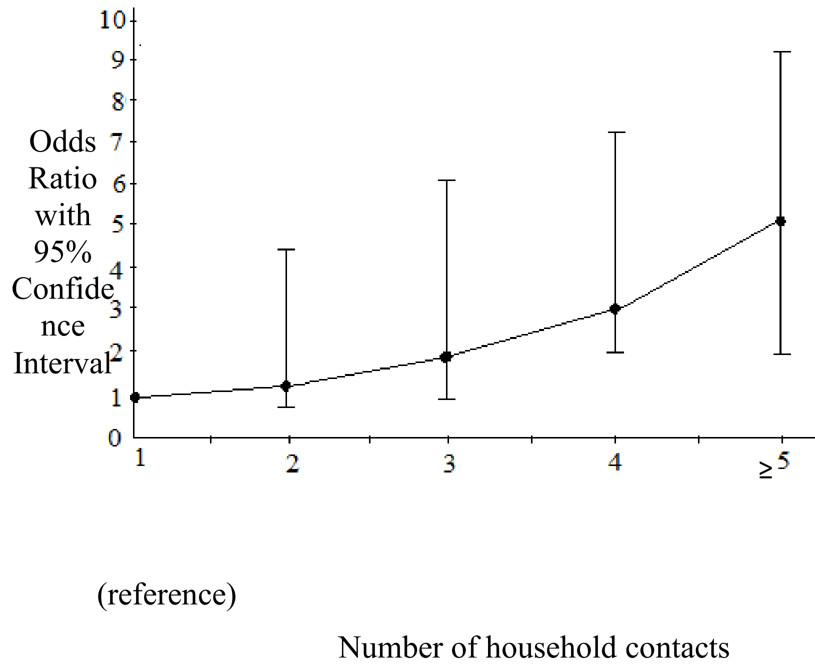

Figure 4.4 b. Increasing risk of ESBL-positive faecal carriage with increasing number of household contacts. Controlled for current use of beta-lactam/fluoroquinolone antibiotics; current use of beta-lactam antibiotics; use of hand sanitizer at least once per day for past 3 months; mean faecal enterobacteria concentration.

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CHAPTER FIVE

DISCUSSION

In recent times, the intensive use of the broad spectrum cephalosporins has led to the selection of multi-resistant strains of *enterobacteria* that produces extended spectrum beta-lactamase enzymes (Coque *et al,* 2006). HIV/AIDS produces one of the most severe form of immunosuppression. In HIV/AIDS patients, the need for routine antibiotic therapy to prevent emergence of opportunistic infectionsis heightened. However, the frequent use of these antibiotics particularly the beta-lactams leads to selection of multidrug resistant bacteria especially ESBLs (Marwa et al., 2015). This study was designed to determine the occurrence of intestinal carriage of ESBL-positive enterobacteria at a district care hospital.

5.1 Faecal carriage of ESBL Producing enterobacteria

In this study, nearly one of every two HIV/AIDS patient was colonized with an ESBL-producing enterobacteria. This high rate of colonization with ESBLs is most likely due to the misuse and abuse of antibiotics. In Ghana and other sub-Saharan countries, antimicrobial agents are readily purchased as commodity. What is more, many Ghanaian patients receive antibiotics without prescription from a physician. When antibiotics are prescribed, they are almost entirely empirical and not based on individual susceptibility results nor on surveillance data. Among the ESBL-producing isolates in this study, *E.coli* was the most prevalent. Indeed, *E. coli* is the most common ESBL-producer in Ghana, Africa and across Europe; and they have been on a continuous rise during the last decade (Livermore *et al.*, 2007; Storberg, 2014; Tansarli *et al.*, 2014; Ghafourian *et al.*, 2015). Fecal colonization by ESBL-producing enterobacteria has been previously detected in Egyptian patients with community-onset gastrointestinal complaints (68%)(Fam *et al.*, 2015). The percentage of ESBL faecal carriage reported in this study is

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however higher than those found in Libya (<13.5%), Nigeria (<40%), Central Africa Republic (<38.1%) and in many reports spanning across several African studies (Ahmed *et al.*, 2014; Ahmed *et al.*, 2014; Storberg, 2014).

5.2 Molecular Characterization of ESBL producing *enterobacteria* In this study, *bla*CTX-M gene was detected in over 80 of the isolates, with *bla*CTX-M-15 the most commonly identified resistance mechanism. Whenever *bla*CTX-M-15 positive isolates were encountered in a faecal sample, they comprised the dominant faecal enterobacteria outnumbering the colony counts of all other enterobacteria in the faecal sample by a ratio of 2:1. Overall, none of the ESBL-producing enterobacteria harboured a SHV or an OXA type ESBL. Perhaps, CTX-M-15 genes are fast replacing other ESBL types in Ghana. The findings support work by other workers indicating that a specific CTX-M enzyme can dominate in a geographical region (Livermore *et al.*, 2007; D'Andrea *et al.*, 2013; Stedt *et al.*, 2015). A possible explanation could be due to the dynamic nature of organisms harboring these enzymes. The CTX-M-15 genes have been reported worldwide to be associated with various dominant clonal groups. As the dominant clonal group changes, the dominant mechanism (CTX-M-15 in this case) will also change. Bacterial typing for clonal relatedness was not performed, and thus this study is unable to determine the clonal group that may be responsible for the dominance of CTX-M-15. It is important to note however that the dominance of CTX-M alleles may differ by geographical region. While CTX-M-15 is most common across many regions in Africa, other investigators in Europe and Asia have reported CTX-M-15 as sub-dominant genes among ESBL-producers in fecal isolates (Livermore *et al.*, 2007; Storberg, 2014; Tansarli *et al.*, 2014; Ghafourian *et al.*,

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2015). The distribution of CTX-M types has also been described by many workers to be dependent on the different sources of patients' intestinal flora (Livermore *et al.*, 2007; D'Andrea *et al.*, 2013; Stedt *et al.*, 2015). Some studies have suggested a possible transmission of ESBL-producing bacteria through the food chain. The proponents, indeed have shown that CTX-M-1 is the predominant CTX-M among food-producing animals.

Another study finding worthy of mentioning is the identification of the TEM-121 complex mutant TEM beta-lactamases in faecal samples of two study patients. TEM-121 has all the properties of ESBLs but resistant to ESBL inhibitors such as sulbactam, tazobactam, and clavulanic acid (Poirel, Mammeri and Nordmann, 2004; Robin *et al.*, 2007; Jacquier *et al.*, 2013). Isolates with complex mutant TEM beta-lactamases are thus resistant to all cephalosporin antibiotics with or without ESBL inhibitor combinations further limiting the therapeutic options for infections by these isolates. Faecal shedding of ESBLs in patient setting in Ghana is an emerging epidemiological problem that warrants application of stringent control measures such as routine rectal screening for faecal carriage of patients admitted from the community. Suffice to say that routine fecal examination is practically challenging and is not even required in most hospitals in the developed world if the patients were admitted to an intensive care unit (Paterson and R. a Bonomo, 2005; Malloy and Campos, 2011; Shaikh *et al.*, 2015).

5.3 Risk factors for ESBL faecal carriage

Risk factors for faecal carriage of ESBLs in Africa have been investigated especially in community settings (Bar-Yoseph *et al.*, 2016; Flokas *et al.*, 2016; Karanika *et al.*, 2016; Stapleton *et al.*, 2016). Because this study focuses on patients with HIV/AIDS, there are no literature from elsewhere to compare the findings. Thus the discussion will be limited to studies in hospital settings. Only a few European and African studies are available (Bar-Yoseph *et al.*,

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2016; Flokas *et al.*, 2016; Karanika *et al.*, 2016; Stapleton *et al.*, 2016). In one of such studies, Reuland *et. al*., described the risk associated with person-to-person transmission of resistant strains (Reuland *et al.*, 2016). In this study, ESBL-carrying *E. coli* strains from three households were genetically identical and carried the same plasmids and ESBL genes, pointing to person-to-person transmission. In that same study, different strains and plasmids were also identified in the same households. These findings suggest that acquisition of ESBLs within households may not be due only to strain transmission. In this current study, the number of persons living with HIV/AIDS patients in the household was determined to be an independent risk factor for ESBL faecal carriage. The dissemination of ESBL-producers may occur through faecal oral route, either directly or by hand contact with fomites , and sinks in the house or hospital setting (Paterson *et al.,*2005). Though intestinal carriage is a key factor in the epidemiology of ESBLs, faecal flora of colonized persons such as people living with HIV/AIDS represent a reservoir for ESBL genes; and increases the risk that other population dwellers will become carriers (Chandel *et al.*, 2011) .

In summary, the high proportion of ESBL-producers in human fecal flora represent an opportunity for these clones to become persistent. Because of the significant public health implications, the spread of organisms producing CTX-M-15 producing isolates merits close monitoring with enhanced surveillance efforts. The introduction of molecular diagnostic procedures in a clinical or reference laboratory to track their spread in the community and hospital settings is vital

5.4 Limitations

There are some limitations of this study worthy of mention. First, patients were recruited only from the Achimota District Hospital. Given that the work was performed in a single center, the

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results may not be generalizable to the entire patient population in Ghana. The findings should therefore be interpreted with caution. Second, the study could not determine cluster of differentiation 4 (CD4) T-cell count of study patients. Perhaps such data would have better help discussed study findings on ESBL faecal concentrations. Last, this work included 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 SIX

CONCLUSION AND RECOMMENDATION

6.1CONCLUSION

Based on the results, the following conclusions were made with regards to the study objectives:

(1) The prevalence of ESBL faecal carriage among study patient was high (43%). The predominant ESBL type were CTX-M 15. The complex mutant TEM beta-lactamase, $bla_{\text{TEM-121}}$, was identified in combination with $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-27}}$ in the faecal samples of two study participants. The clinical implications of these ESBL types pose significant public health threat antibiotic therapy.

(2) Among patients colonized with CTX-M-15 positive enterobacteria, these isolates were the predominant faecal enterobacteria. In such patients, the total ESBL-negative enterobacteria were sub-dominant colonies. The CTX-M-15 ESBLs may be fast overtaking all other ESBL types.

(3) In this study, the number of persons living with an HIV/AIDS patient in a household was an independent risk factor for ESBL faecal carriage. Personal hygiene and sanitation may be important to reducing the spread of ESBLs.

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6.2 RECOMMENDATION

(1) The high levels of ESBL faecal carriage in patients with HIV call for better surveillance of resistance in the hospital, and Ghana as a whole. The results also highlight the need for a more prudent antimicrobial use. Regulation of sales of antimicrobial agents, as recently introduced in India would be a large step forward.

(2) The link between ESBL faecal colonization and infection needs further elucidation. Indeed, ESBL intestinal colonization contributes to a substantial circulating pool of resistance genes. Stringent antibiotic stewardship including actions to prevent infections need immediate attention.

(3) Large-scale, population-based studies are warranted to better understand dissemination patterns of CTX-M- type ESBLs. Molecular studies to better understand the why CTX-M-15 producing isolates were the predominant faecal enterobacteria are warranted.

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APPENDIX

Appendix 1

Date:

INFORMATION SHEET

PROJECT TOPIC: Intestinal carriage with extended-spectrum beta-lactamase producing enterobacteria in patients living with HIV/AIDS at a district care hospital setting

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.

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.

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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. Glora Dela Tawiah - Department of Medical laboratory Science, University of Ghana School of Biomedical andAllied Health Sciences. 0542701668

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 2

Structured Questionnaire for Interviews
Towards the determination of risk factors for ESBL faecal carriage

Intestinal carriage with extended-spectrum beta-lactamase producing enterobacteria among patients living with HIV/AIDS in a district care hospital setting

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 ward: Indicate number Source of water:	 …………………………….. …………………………….. …………………………….. …………………………….. Yes ☐ No ☐ : if 'No' indicate type…………………... ……………… Running tap ☐; Veronica buckets ☐; Others (specify)…………..
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:			

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

Q3. Questions of underlying patient's lifestyle:

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

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Q4. History of hospitalization

		Yes:	No:
Q.4.1	Hospitalization in the past 1 year Number of hospitalisation…………………………………………………. Total duration hospital stay …………………………………..		
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	Presence of central vascular catheter Presence of peripheral vascular catheter Presence of urinary catheter Presence of intubation	Yes	No
Q.4.5	Use of medications that affect intestinal flora Drugs that neutralize stomach acids Proton pump inhibitors H2 blockers	Yes	No
Q.4.6 **Q.4.7** **Q4.8**	Used antibiotics in last 3 months Used antibiotics without prescription Indicate type of antibiotics used in last 3 months…………………….. ……………………………………………………………………… Current antibiotics used………………………………………………… Specify antibiotics………………………………………………………………… Animal contact in the past 6months………………………………………………………………………	Yes	No

Thank for helping fill this questionnaire