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**COLLEGE OF HEALTH SCIENCES**

**School of Medical Sciences**

**Department of Clinical Microbiology**



# Molecular Epidemiology of Extended Spectrum B-Lactamase- Producing *Enterobacteriaceae* at the Komfo Anokye Teaching Hospital

A thesis submitted to the School of Medical Sciences, Department of Clinical Microbiology, Kwame Nkrumah University of Science and Technology Kumasi in partial fulfilment of the requirements for the award of the degree (PHD IN CLINICAL MICROBIOLOGY), 2011

**By**

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**December, 2011**

## DECLARATION

This is to declare that that I Patrick Kwame Feglo undertook this project towards the award of a Ph D in Clinical Microbiology, and that to the best of my knowledge this work does not contain any material previously published by another person or accepted for the award of a degree in any university, except where due acknowledgement has been made in the text.



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**DEDICATION**



I DEDICATE THIS PROJECT TO GOD

## Abstract

Bacterial isolates of the *Enterobacteriaceae* at the Komfo Anokye Teaching Hospital (KATH) have become resistant to almost all the commonest antimicrobial drugs, including the third generation cephalosporins, but the cause of which is unknown. Worldwide reports however have it that many such resistances to the cefotaxime, ceftriaxone and caftazidime for example are often due to the production of extended spectrum β–lactamases (ESBLs) by the organisms. This study therefore was undertaken to determine the prevalence and the genotypes of ESBL isolates and the phylogenetic clones of clinical isolates of *E. coli*. A total of 405 isolates comprising E. *coli*, 156(38.5), *Klebsiella pneumoniae* 234(57.8%) and *Klebsiella oxytoca* 15(3.7%) were studied. These were non-duplicate isolates collected from February to April 2008 and again from March to July 2009. The isolates were obtained from blood, urine, sputum, wound and others (pus, aspirates etc). Antimicrobial susceptibility of the isolates was determined using Kirby-Bauer disc diffusion method. ESBL phenotypes were determined by the double disc synergy method using cefpodoxime with co-amoxiclav, and then confirmed by the combined disc method. The ESBL genotypes, $Bla_{\text{TEM}}$, $Bla_{\text{CTX-M}}$ and $Bla_{\text{SHV}}$ were determined using duplex PCR. ECOR type of ESBL positive *E. coli* isolates were determined. Out of the 405 isolates tested 234(57.8%) were ESBL producers. Among the *E. coli* were 77(49.4%) ESBL producers, 144(61.5%) were ESBL producers among *Klebsiella pneumoniae* and 13(86.7%) were among *Klebsiella oxytoca*. $Bla_{TEM}$ was the commonest, with prevalence of 96.2%, followed by $Bla_{CTX\text{-}M}$ (94.4%) and $Bla_{SHV}$ (32.5%) with as much as 151(64.5%) possessing two genes and 70(29.9%) of the isolates possessing three genes respectively. There were more ESBL producers among in-patients (64.4%) than there were in out-patients (39.7%), but the difference was not statistically significant (P= 0.2374). ECOR type of *E. coli* isolates belong to group B1 (74.0%) and group B2 (26.0%), no A or D genotypes were detected. A subset of 29 ESBL-producing isolates were further genotyped using multilocus sequence typing and all belonged to the same sequence type (ST88), which is a member of the B1 phylogroup. Representative ST88 isolates were further characterized for virulence factors that are associated with *E. coli* pathotypes (EAEC, EHEC, EPEC, ETEC, and ExPEC). Four fimbrial genes typically associated with ExPEC virulence were identified. Phylogenetic relationship among the isolates of ECOR collection and the ESBL ST88 producing clone from KATH was determined using a representative of the closest common ancestor as an outgroup for a dendogram. These data suggest that antibiotic use at KATH or in the community selected for the emergence and spread of a resistant ExPEC clone.

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## CHAPTER ONE

## 1.0 INTRODUCTION

### 1.1 *Historical perspective*

The discovery of antibiotics and their general use had transformed the patterns of disease and death in many countries (Rahal, 2000; Sekowska, *et al.,* 2002). Many diseases that once caused high mortality, such as tuberculosis, pneumonia and septicemia, became controllable and surgical procedures improved because high risks of post surgical infections reduced (Sekowska,

*et al*., 2002). These successes could hardly be measured when clinical antimicrobial resistance emerged (Knothe, 1983) leading to treatment failures. But this antimicrobial resistance was to a single antibiotic at the time, so many researchers (Al-Jasser, 2006; Canton, *et al*., 2008; Khan, 2010; Qin, *et al*., 2008; Sarma & Ahmed, 2010) are of the view that the appearance of multidrug-resistant pathogens is a recent phenomenon, which has become a worldwide problem.

Antimicrobial resistance occurs when the minimum inhibitory concentration (MIC) of a drug exceeds inhibitory concentration level, which is capable of being achieved for a particular strain of bacteria with safety *in vivo* (Hajjar, 2010; Vading, 2010). Resistance to an antimicrobial can arise either by mutation in the gene that determines sensitivity to the agent or by acquisition of extrachromosomal DNA (plasmid) carrying a resistance gene (Sarma & Ahmed, 2010). Antimicrobial resistance appears after the introduction of an antimicrobial agent into the environment (Meletis *et al.*, 2010; Pitout & Laupland, 2008). The introduction of the antimicrobial agent leads to a selective process whereby the agent selects for survival those strains possessing a resistance gene to the agent (Meletis, *et al.,* 2010). Bacteria that possess the resistant gene then multiply to become the disease causing agent. Such bacteria have become resistant to antimicrobial agents as a result of chromosomal changes caused by plasmids and transposons (Lee *et al*., 2010). As a result, organisms which cause diarrhoea, urinary tract infections and sepsis have become resistant to virtually all of the older antibiotics such as penicillin, ampicillin, tetracycline and chloramphenicol (Mshana *et al*., 2009; Sarma & Ahmed, 2010; Woodford *et al*., 2007). This antimicrobial resistance phenomenon has been blamed on the extensive use of antibiotics in the community and the hospitals resulting in a selective process for the bacterial strains that possess the resistance gene (Sharma *et al*., 2004).

At first, it was thought (Jacoby and Munoz-Price, 2005) that ESBL-producing organisms were only the *Klebsiella pneumoniae* and the *E. coli*, until it was found in other *Enterobacteriaceae*, including *Salmonella* and *Pseudomonas aeruginosa* (Bauerfeind *et al*., 1992). Prevalence of *Enterobacteriaceae* strains producing ESBLs vary widely internationally and as well as within the same country (Apisarnthanarak *et al*., 2007; Jacoby & Munoz-Price, 2005; Pfaller & Segreti, 2006; Pitout *et al*., 2005), depending on the degree of use of the antibiotic in that locality.



**1.2 *Extended spectrum β-lactamases* (ESBLs)**

Extended spectrum β-lactamases (ESBLs) are enzymes capable of hydrolyzing the β-lactam ring of penicillins, cephalosporins, cephamycins carbapenems (ertapenems), monobactams and related antimicrobial drugs (Queenan, 2007). The beta-lactamase enzymes are produced by some bacteria and are responsible for their resistance to the beta-lactam antibiotics (Pitout, 2005). These antibiotics have a common element in their molecular structure: a four-atom ring known as a β-lactam. The β-lactamase enzyme breaks that ring open, deactivating the molecule's antibacterial properties. Beta-lactamases have long been known, but the extended spectrum β-lactamses (ESBLs) have been suspected to be a recent phenomenon (Chaudhary & Aggarwal, 2004; Rahal, 2000). Since the discovery of the Extended Spectrum β-lactamases (ESBLs) among the *Klebsiella pneumoniae*, in 1983 (Knothe, 1983) there have been detections of ESBLs of various kinds among the family *Enterobacteria* (Miro *et al*., 2005). Drug resistance as a result of ESBL production has become so common that many researchers believe 'the 1990's was beginning to look like the pre-antibiotic era' (Canton, *et al*., 2008; Sarma & Ahmed, 2010). This crisis was envisaged due to the discovery of ESBLs (Chaudhary & Aggarwal, 2004). In the past it was believed that cephalosporins were relatively immune to attack by β-lactamase (Canton, 2008). It was surprising to find third generation cephalosporin resistant *Klebsiella sp* among

clinical isolates and the demonstrated ability of the *Klebsiella* sp to transfer the resistance determinants to *E. coli,* making the *E. coli* also resistant against various cephalosporins (Weill, *et al*., 2004). The resistance mechanism is now known to be plasmid mediated (Al-Jasser, 2006).

Classical ESBLs have evolved from widespread plasmid-encoded enzyme families Temoniera (TEM), Sufhydryl variable (SHV) and Oxacillin (OXA) (Sarma & Ahmed, 2010). These families and their variants have remained the most prevalent ESBL types. However, in recent years many ESBLs of non-TEM, non-SHV and non-OXA types especially the cefotaximase (CTX-M) have also been detected and reported worldwide(Sarma & Ahmed, 2010).

### 1.3 *Problems associated with ESBLs*

The emergence of these enzymes poses a serious threat to clinical therapeutics because:

1. Organisms producing these enzymes become very effective at inactivating various β-Lactam antibiotics (including even the 3$^{rd}$ generation cephalosporins)(Nordmann, 2009).

2. ESBLs induce resistance to many other classes of antibiotics which are non-β-lactam antibiotics such as the aminoglycosides, chloramphenicol, quinolones and sulphonamides resulting in difficult to treat infections (Lee *et al*., 2010; Nordmann *et al*., 2009; Rodriguez-Villalobos *et al*., 2010; Samaha-Kfoury & Araj, 2003).

3 The enzymes are easily and quickly inducible from one organism to another within the members of the family *Enterobacteriaceae* making them emerging pathogens (Lee *et al*., 2010; Rodriguez-Villalobos *et al*., 2010)

4 Some ESBL producers achieve outbreak status spreading among patients and locales, perhaps owing to particular pathogenicity traits (Woodford, 2007).

There are reports that ESBL-mediated resistance is not always obvious *in vitro* to all cephalosporins (Canton *et al*., 2008; Ramazanzadeh, 2010b).

ESBL bacteria have spread across the world but some Health Authorities are not aware of this problem especially in African countries; those who are aware are perhaps just complacent (Pitout *et al*., 2004). Some clinical laboratories are also not aware of screening for ESBLs, which is the case in many hospitals in Ghana (Adu-Sarkodie, 2010). So also many clinicians and health planners are not aware (Dancer, 2004). It is therefore important to:

- Create heightened awareness among clinicians,
- Enhance testing by clinical laboratories and
- Institute molecular surveillance studies

in order to determine the enormity of the problem, so as to attempt to limit the spread of ESBL-producing bacteria (Pfaller & Segreti, 2006; Woodford *et al*., 2007).

### 1.4 *Antimicrobial resistance in Ghana and reports of ESBLs*

A study at Komfo Anokye Teaching Hospital conducted by Adjei (1997) on Gram- negative pathogens in urinary isolates reported that more than 20% of *E. coli* were resistant to ampicillin and co-trimoxazole. Another study, also at KATH involving urinary tract infections among infants (Adjei and Opoku, 2004) found all the 150 isolates (comprising *E. coli, Klebsiella, Proteus*, and *Staphylococci*, the dominant flora) to be resistant to ampicillin. They found that 77.8% of the isolates were susceptible to co-amoxiclav and 67% to nitrofurantoin, and only 11% of the isolates were susceptible to Co-trimoxazole. Newman (1990) reported very high

proportions *E. coli* isolates from urine resistant to ampicillin (88.8%), tetracycline (88%) and co-trimoxazole (79.9%) at Korle-bu Teaching Hospital in Accra. At KATH, high proportions of resistance were also found in other disease conditions such as meningitis where *Streptococcus pneumoniae* isolated from cerebrospinal fluid with resistant were: 31.3% to penicillin, 12.6% to erythromycin and 18% to co-trimoxazole (Adjei and Agbemadzo, 1996). Multidrug resistant *Salmonella* sp has been reported in Accra (Mills-Roberson *et al.* (2003). Drug resistance is now so widespread that respiratory pathogens such as *Streptococcus pneumoniae*, cutaneous pathogens such as *Staphylococci* and members of the family of the *Enterobacteriaceae* and *Pseudomonas* sp have become resistant to many antibiotics (Mills-Roertson *et al*, 2003). Data from the KATH laboratory record books (Feglo, 2007) detected antimicrobial resistance among many urinary bacterial isolates of the *Enterobacteriaceae* to even the third generation cephalosporins from patients in the community. The only documented study on ESBLs was by Sarkodie (2010) when the ESBL phenotypes of *Klebsiella* and *E. coli* were studied. That study found out that 44% of *E. coli* and 55% of *Klebsiella* sp (57.8% *K. pneumoniae*) were ESBL producers. This ESBL production prevalence was found to be high among these enterobacteria and as a result the antibiotic spectrum from which to choose to treat severe infections was reduced, creating difficulties with therapeutic options.

Very few studies have reported on the problem of ESBL in Africa (Ndugulile *et al*., 2005) in general and Ghana in particular. There have been reports of ESBL genotypes in *Klebsiella pneumoniae* in Kenya (Kariuki *et al*., 2001), South Africa (Essack *et al*., 2001), and Tanzania (Blomberg *et al*., 2005). ESBL genotypes have been reported in *Salmonella enterica* and *S. enteritidis* from Mali (Weill *et al*., 2004) and *S typhimurium* in Morocco (AitMhand *et al*., 2002), but in Ghana information on ESBL genotypes types is scanty.

**1.4** *E. coli ECOR types*

The diversity of microorganisms producing ESBLs is highly complex and many of the members of enterobacteria have been described as commensals in the intestinal tract flora (Grimaldi, 2010) Two *E. coli* groups are known; the first group causes intestinal symptoms, while the other group causes diseases outside the intestinal tract. It is not yet known what ESBL types are responsible for the high levels of antimicrobial resistance in Ghana. It is prudent scientifically to establish the ESBL types and their prevalence levels as epidemiological data in Ghana. This will inform and guide policy makers and also be an advocacy tool for the formulation of infection control strategies.

**1.5 Problem statement and justification**

Data on ESBL phenotypes from the Konfo Anokye Teaching Hospital indicate high proportions of ESBL production (Adu-Sarkodie, 2010). It is not yet known which ESBL types are responsible for the high proportions of antimicrobial resistance in Ghana. Also ESBL are not tested for routinely at KATH. Though many reports (Adjei & Agbemadzo, 1996; P. Feglo, 2007) indicate high antimicrobial resistance levels in Ghana and world-wide reports show increasing prevalence of ESBLs the attention given to the problem in sub-Saharan Africa is scanty. This is perhaps due to lack of awareness and lack of laboratory expertise to detect and report the problem. It is the objective of this study to highlight the main ESBL types circulating both in the community and in our hospital, including the clinical samples mostly affected. *Klebsiella* sp and *E. coli* which are the main producers of these enzymes (Livermore & Brown, 2001; Romanus *et*

*al*, 2009) were selected for the study. It is anticipated that this study will give an understanding of the epidemiology of ESBL producing *Enterobacteriaceae* at KATH. It will elucidate the possible sources and spread direction of these resistant organisms. The results of the study will help create awareness about the problem of ESBL production. An immediate benefit will be to adopt the screening of *Enterobacteriaceae* for ESBL production in our laboratories, if the results indicate high ESBL prevalence in the Hospital. Screening for ESBLs may lead to the selection of appropriate antibiotics for the treatment of infections. This will lead to reduction in length of hospital stay and also reduce treatment costs for the patient.

This is perhaps, because health authorities, planners and clinicians are not aware of ESBLs. It is, therefore, important to create heightened ESBL awareness among clinicians, enhance laboratory ESBL testing and surveillance programmes in order to determine the enormity of the problem as a basis for initiation of preventive measures. A carbapenem agent is indicated for treatment of a serious infection caused by an ESBL isolate (Paterson and Bonono 2005). Multiple treatment regimes constitute unnecessary high treatment costs to the already poor patient (Projan, 2008). The key to preventing and controlling the spread of ESBLs is an effective and consistent surveillance which starts with the microbiology reporting on patient risk factors for ESBL colonization (Paterson, 2000). Then once ESBLs are identified protocols for the control of their spread are instituted. So far there are no ESBL data upon which to institute intervention strategies to limit the spread of ESBL-producing bacteria at the Komfo Anokye Teaching Hoapital. Other benefits this study would achieve would be to have an ESBL trained resource person available at KATH to train technicians and help institute ESBL testing at KATH to help select appropriate antibiotics to guide patient care. This may significantly lower the length of hospital stay by the patient care as these useful antibiotics are very expensive. It may also reduce

mortality rate by facilitating early treatment against ESBL-producing organisms. Also it is prudent to determine the ESBL types and their prevalence in Ghana, with the hope that the study results will inform and guide policy makers and also be an advocacy tool for the formulation of infection control strategies. This study will also serve as a guide in the empirical choice of antibiotics during treatment of infections caused by *Klebsiella pneumoniae* and *Escherichia coli*. The distribution of the phylogenetic clones in this study would enhance understanding of the bacterial diversity, and what clones there are circulating at KATH.



### 1.6 Aims of the study

The main aim of this study is to investigate the prevalence of ESBL producing *Enterobacteriaceae* at KATH and to establish the correlation of between ESBL production and the antimicrobial resistance by Polymerase Chain Reaction (PCR). This study also seeks to determine the phylogenetic grouping of the *E. coli* isolates so as to assess their pathogenic potential and/or virulence.

### 1.7 Working hypothesis

The hypothesis of this study is that the high proportions of *Klebsiellae* and *E. coli* resistance to commonly prescribed antimicrobials at KATH are as a result of ESBL production among these isolates.

### 1.8 The specific objectives of the study are to:

1. determine the antimicrobial resistance of *Klebsiella* and *E.coli* isolates at KATH.

2. Determine whether isolates of *Klebsiella pneumoniae* and *E. coli* from KATH produce ESBL.

3. Determine the genotypes of the ESBLs produced among the *Klebsiellae* and *E. coli* isolates at KATH.

4. Relate ESBL production to source of infection (i.e Community or Hospital),

5. Relate ESBL production to clinical specimen and patient socio-demographics.

6. Determine the clonal diversity (genotypes) of the *E. coli* isolates causing extrainstestinal infection at KATH.



# CHAPTER TWO

## 2.0 LITERATURE REVIEW

Humans have suffered through time in search of medicine to treat many ailments that afflicted society. Many approaches to treatment and cure of the diseased patients were practised, some with success, whilst others failed.

The history of chemotherapy dates back to folk medicine, in which moulds were often used to cure superficial skin infections, but the earliest evidence of successful chemotherapy, was traced to Peru where Indians used cinchona tree bark to treat malaria (Shaw, 2010). Other substances were used in ancient China (Shaw, 2010) and it is now known that poultices prepared and used by ancient peoples contained antibacterial and antifungal substances (Shaw, 2010). Modern chemotherapy has been traced to Paul Ehrlich in Germany, who sought systematically to discover effective agents to treat trypanosomiasis and syphilis (Corper, 1919; Donald, 1919). Ehrlich postulated that it was possible to find chemicals that were selectively toxic for parasites but not toxic for humans... "WE MUST SEARCH for magic bullets. We must strike the parasites and the parasites only, if possible, and to do this, we must learn to aim with chemical substances!"(Ehrlich, 1959; Ehrlich & Hata, 1910). This idea had little success until the 1930s when Gerhard Domagk discovered the protective effects of prontosil (Domagk, 1935), the forerunner of sulphonamide. This drug was used with somewhat success for a long time before newer and more potent sulphonamides were developed.

The first drug produced by microorganisms introduced into therapy was pyocyanase isolated from *Pseudomonas aeruginosa* (Emmerich, 1989). This was a chemically undefined preparation which caused lysis of *Bacillus anthracis in vitro*, and was active against *Salmonella typhi*, *Staphylococcus aureus* and *Vibrio cholera* (Kitasato, 1889) and some other bacteria. It produced no side effects when injected intravenously into animals and was considered safe (Kitasato, 1889). It was said to be used in clinical therapy for 25 years before it was found to be entirely an ineffective drug (Florey, 1946). Several other substances were discovered, extracted and tried but were found to be either too toxic or were just ineffective (Florey, 1946).

The turning point in the history of antibiotics was the isolation of penicillin by Florey and colleagues (Ligon, 2004) from cultures of *Penicillium chrysogenum*. This was based on the observation made 10 years previously (Florey, 1946) that growth of *Staphylococci* was inhibited by *Penicillium notatum*, and showed that filtrates produced by the *P. notatum* were not harmful to animal cells, but little did he appreciate the magnitude of his discovery (Florey, 1946). This ushered in the antibiotic era leading to the discovery of many antibiotics and the synthesis of many new chemotherapeutic agents by molecular modification of the naturally existing compounds as fermentation science and medicinal biochemistry improved. Some of these antimicrobial agents, their mode of action and resistance mechanisms are shown in Table 1, for revision purposes, but the emphasis of this study is on the β-lactam antibiotics, which are antibiotics that affect the cell wall of bacteria by disrupting the molecular configuration of the building subunits, leading to malformed peptidoglycan, which is unable to withstand the internal pressure of the bacterial cell causing the cell to burst.



**Table 1** Some common antimicrobial agents, their modes of action, and the corresponding mechanisms of bacterial resistance.

| Antimicrobial agents | Mode of action | Resistance mechanisms |
|---|---|---|
| B-lactams | Cell wall synthesis, cell division | β-lactamase, altered penicillin, binding proteins |
| Glycopeptides (azoles, cycloserine) | Cell wall division | Blocking of drug access to pentapeptide |
| Aminoglycosides (spectinomycin) | Inhibit protein synthesis (bind to 30S ribosome) | Enzymatic inactivation, altered target, impermeability |
| Macrolides | Inhibit protein synthesis (bind to 50S ribosome) | Altered target, enzymatic inactivation |

| | | |
|---|---|---|
| Tetracycline | Inhibit protein synthesis (affect t-RNA binding to 30S) | Efflux, altered target, impermeability, enzymatic inactivation |
| Chloramphenicol (lincosamides, streptogramin) | Inhibit protein synthesis (bind to 50S ribosome) | Enzymatic inactivation, impermeability |
| Quinolones | Replication: inhibit DNA gyrase | Altered target enzymes, impermeability |
| Rifampicin | Transcription: inhibit DNA dependent RNA polymerase | Altered target enzymes, impermeability |
| Sulfonamides | Folic acid synthesis | Altered target |
| Trimethoprim | Folic acid synthesis | Altered target, impermeability |
| Polyenes(nystatin, mphotericinB | Cell membrane permeability | Ergosterol deficient mutants |

(Neu, 1994)

### 2.1 β-lactam antibiotics

The beta-lactam antibiotics include the natural and semi synthetic penicillins, cephalosporins, carbapemes and monobactams. The chemical structure of these compounds is based on the two-ringed nucleus of 6-aminopenicilanic acid (6-APA) and 7-aminocephalosporanic acid (7-ACA) (Baron, 1996). Both compounds have a common unstable beta-lactam ring, and differ by the 6-APA containing thiazolidine ring and the 7-ACA containing delta-dihydrothiazine ring. These two acids are obtained by hydrolysis of benzyl penicillin and cephalosporin C respectively, which are starting products for the synthesis of new semi-synthetic derivatives when the side chain is modified (Baron, 1996; Csoregh & Palm, 1977). The basic structures of β-lactam antibiotics are shown in the Figures 1, 2, 3and 4 below.

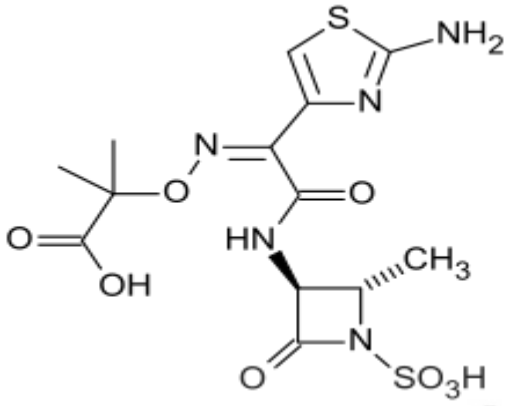




**Figure 4.** Basic structure of Aztreonam a β-lactam antibiotic. The square is the beta-lactam ring. There is a second thiazole ring, but it is not fused to the beta-lactam ring (Fuchs, 1988).

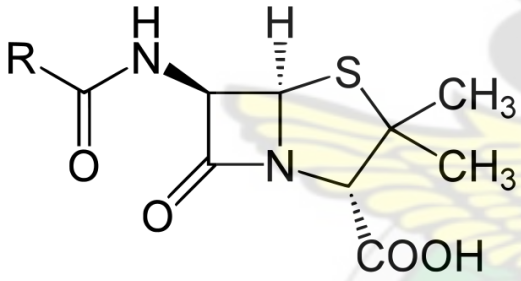


**Figure 5**: Penicillin

The chemical structure of penicillin core (R is the variable group)(Csoregh & Palm, 1977)

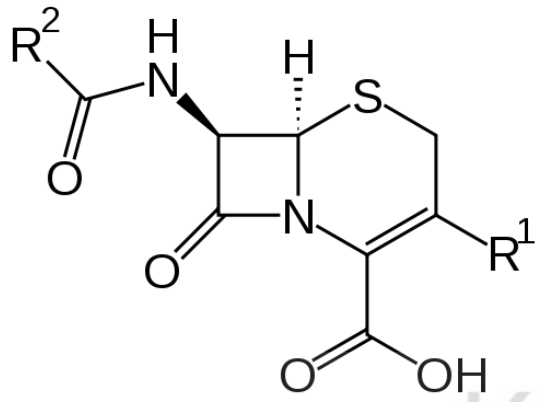



**Figure 6**: Cephalosporin: The chemical structure of cephalosporin core (both $R^1$ and $R^2$ are variable groups)(Csoregh & Palm, 1977).

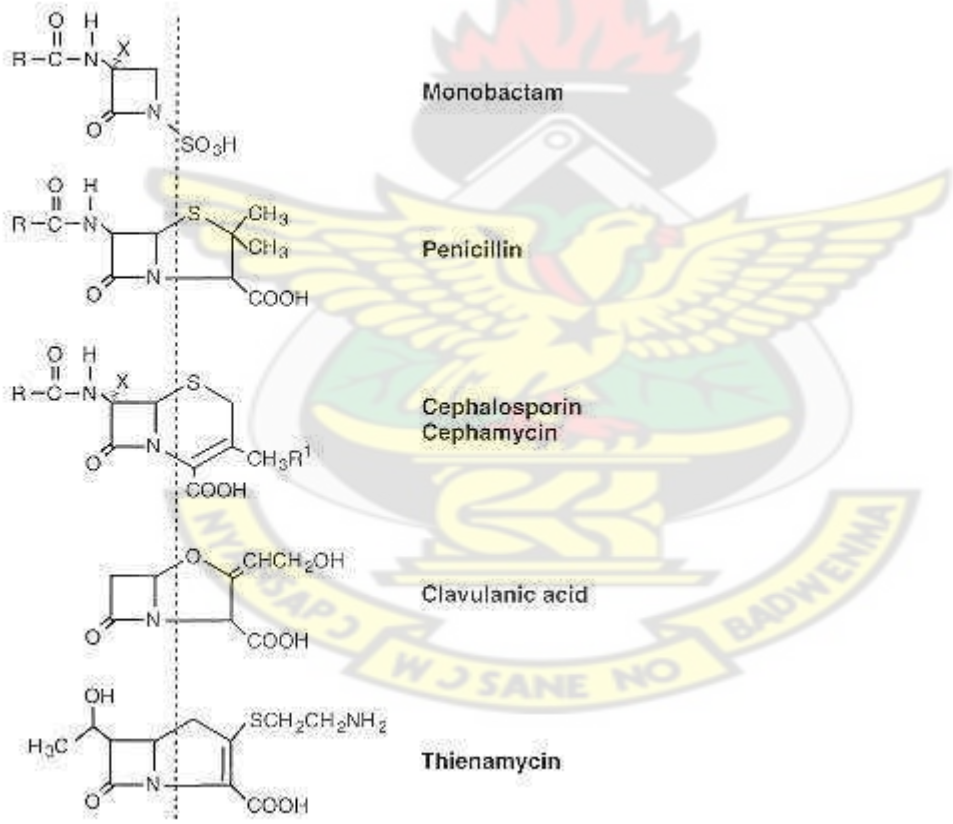


**Figure 4.** β-lactam antibiotic structures compared.

Penicillins and cephalosporins/cephamycins are widely used to inhibit both Gram positive and Gram negative bacilli. Monobactams inhibit only aerobic Gram-negative bacilli, clavulanic acid

acts as a b-lactamase inhibitor and thienamycin inhibits a wide range of aerobic and anaerobic species. R and R1 represent various carbon groups. X can be either hydrogen or a methoxy group (Baron, 1996) New semisynthetic drug variants of both penicillins and cephalosporins are obtained by modifying the side chain. All β-lactam antibiotics are inhibitors of bacterial cell wall biosynthesis (Baron, 1996). Differences in susceptibility to penicillin between Gram positive and Gram negative bacteria are due to different structure of the cell wall in the individual species (Baron, 1996)



The β-lactam antibiotics differ from one another by additional rings to the b-lactam ring: thiazolidine ring for penicillins, cephem nucleus for cephalosporins, none for monobactams and double structure for carbapenems (Samaha-Kfoury & Araj, 2003). These antibiotics in each group also differ by their side chains. The β-lactam antibiotics act on bacteria through two mechanisms targeting the inhibition of cell wall synthesis (Samaha-Kfoury & Araj, 2003). The first action is to incorporate into the bacterial cell wall and inhibit the action of the transpeptidase enzyme responsible for completion of the cell wall. In the second action they attach to the penicillin binding proteins that normally suppress cell wall hydrolases, thus making the hydrolases free, to act and lyse the bacterial cell wall. To bypass these mechanisms of antimicrobial action, bacteria resist by producing β-lactam inactivating enzymes such as the b-lactamases. There are many β-lactam drugs which are grouped according to the skeletal core of the antibiotic. The skeletal core and the synthetic components are as shown in Table 2.

**Table 2. Groups and examples of β-lactam antimicrobial agents**

| B-lactam groups | Examples of antimicrobial agents |
|---|---|
| Penicillins | i. Penicillin G, Penicillin.<br>ii. Penicillinase resistant penicillins: Methicillin, nafcillin, oxacillin, |

| | |
|---|---|
| | cloxacillin,cloxacillin.<br>iii. Aminopenicillins: ampicillin, amoxicillin.<br>iv. Carboxypenicillins: carbenicillin, ticarcillin<br>v. Ureidopenicillins: mezlocillin, piperacillin. |
| Cephalosporins | First generation: cefazolin, cephalotin, cephalexin.<br>Second generation: cefuroxime, cefaclor, cefamandole, cefamycins (cefotetan, cefoxitin).<br>Third generation: cefotaxime, ceftriaxone, cefpodoxime, ceftizoxime, cefperazone, ceftazidime.<br>Fourth generation: cefepime, cefpirome. |
| Carbapenems | Imipenem, meropenem, ertapenem. |
| Monobactams | Aztreonam |

(Samaha-Kfoury & Araj, 2003)

## 2.2 Composition and characteristics of bacterial cell wall and the mechanism of action of cell wall acting agents (also called β-lactam drugs)

The cell wall of the bacterial cell is a complex, semi-rigid structure responsible for the shape of the cell (Vollmer, 2008). The cell wall surrounds the underlying, fragile plasma (cytoplasmic) membrane and protects it (the plasma membrane) and all the other internal structures of the cell (Vollmer, 2008). This cell wall is composed of a macromolecular network called peptidoglycan (also called murein), which is present either alone or in combination with other substances (Vollmer & Seligman, 2010). Gram-positive bacteria cell wall contains peptidoglycan and teichoic or teichuronic acid, and the bacterium may or may not be surrounded by a protein or polysaccharide envelope (Vollmer and Seligman, 2010). The Gram-negative bacteria cell walls contain peptidoglycan, lipopolysaccharide, lipoprotein, phospholipid and protein (Volmer, 2008). The peptidoglycan is composed of repeating disaccharides attached by polypeptides. The

disaccharide portion is made of monosaccharides (sugars) called N-acetylglusamine (NAG) and N-acetylmuramic acid (NAM). The NAGs and NAMs molecules link alternately 10-65 sugars to form a carbohydrate "backbone"-the glycan portion of peptidoglycan (Vollmer & Seligman, 2010). The adjacent rows of the backbone are linked by polypeptides (the peptide portion of peptidoglycan). Though there are variations there are always tetrapeptide side chains, consisting of four amino acids, attached to NAMs in the backbone (Vollmer and Seligman, 2010). Parallel tetrapeptide side chains may be directly linked to each other or linked by peptide cross-bridge consisting of short chains of amino acids to produce the insoluble, strong mesh of peptidoglycan (Gitai, 2005; Vollmer & Seligman, 2010).

Peptidoglycan synthesis occurs in three stages. The first stage occurs in the cytoplasm where low molecular precursors are formed using the enzyme phosphoenolpyruvate transferase (Gitai, 2005). This stage of cell wall synthesis is blocked by a number of antimicrobials (MacLeod *et al*, 2009). Fosfomycin for example forms direct nucleophilic bond to the enzyme, destroying its configuration (MacLeod *et al*., 2009; Vollmer & Seligman, 2010). Also due to the structural similarity of the enzyme and the drug, cycloserine binds to alanine racemase the enzyme responsible for D-alanine synthesis thereby forming a peptidoglycan that is structurally defective because it lacks D-alanine (Baron, 1996). The second stage of cell wall synthesis is the polymerization of the preformed precursors on the cytoplasmic membrane (Baron, 1996). This stage is catalysed by membrane-bound enzymes, as the precursor molecules previously made are transferred sequentially to a carrier (which is a phosphorylated undecaprenyl alcohol) in the cytoplasmic membrane. The carrier functions as a point of attachment to the membrane for the precursors and allows the transport of subunits across the interior of the cytoplasmic membrane to the outside surface, where peptidoglycan is located (Baron, 1996). Bacitracin interacts with

the pyrophosphate derivative of the undecaprenyl alcohol preventing further transfer of muramylpentapeptide from the precursor nucleotide to the peptidoglycan (Baron, 1996; Gitai, 2005). The third stage of the cell wall synthesis involves polymerization of the subunits and the attachment of the peptidoglycan to the cell wall (Gitai, 2005). This action involves the enzyme transpeptidase, which catalyzes the peptide bond between two D-alanyl residues, a process called transpeptidation (Baron, 1996; Gitai, 2005). This final reaction is inhibited by β-lactam antibiotics (Gitai, 2005). The β-lactam antibiotics undergo acylation reaction with the transpeptidases that cross-link the polymers (Gitai, 2005). In other words β-lactam antibiotics inhibit the reaction of transpeptidation, and cell wall synthesis is blocked. They bind to the enzymes (also called penicillin binding proteins) involved in this final process of cell wall formation preventing the cell wall from functioning properly. The enzymes involved in these reactions differ in gram–positive and Gram-negative bacteria and also in anaerobes (Baron, 1996; Gitai, 2005). The differences account, to some extent, differences in antibacterial activity of the β-lactam antibiotics, because the different receptors of the enzymes have different affinities for a drug i.e. a β-lactam antibiotic (Gitai, 2005). For example attachment of penicillin to one type of penicillin binding protein results in abnormal elongation of the cell, whereas the attachment to another type of penicillin binding protein may result in the defect of the cell wall, with resulting cell lysis (Gitai, 2005). The inhibition of transpeptidation enzymes by penicillins and cephalosporins may be due to structural similarity of these drugs to acyl-D-alanyl-D-alamine, a structural element during peptidoglycan formation (Vollmer, 2008.; Vollmer & Bertsche, 2008; Vollmer & Seligman, 2010). The cell wall building actions by β-lactamases are rendered ineffective by many Gram negative enteric bacteria (also many other types of bacteria)

when they produce hydrolysing enzymes, which destroy the β-lactam antibiotics (Gitai, 2005; Madigan, 2005).

### 2.3 General mechanisms of antimicrobial resistance

The basic mechanisms by which a microorganism can resist an antimicrobial agent are:

1) to alter the receptor for the drug i.e. the molecule on which they exert their effect

2) to decrease the amount of the drug that reaches the receptor by altering entry or increasing removal of the drug

3) to destroy or inactivate the drug

4) to develop resistant metabolic pathways. Bacteria can possess one or all of these mechanisms simultaneously (Kern, *et al*., 2008; Vollmer, 2008; Vollmer & Bertsche, 2008).

The main resistance mechanism against β-lactam drugs is the production of β-lactamase enzymes which inhibit the drugs from binding to the penicillin-binding proteins (James *et al*., 2009). Introduction of these antibiotics in any population results in the evolution of bacterial strains capable of producing new penicillin-binding proteins, to which no β-lactam antibiotic can bind. The main mechanism is the production of enzymes called penicillinase. Penicillinase has the ability to attack other β-lactam drugs such as the cephalosporins, carbarpenems and monobactams, and so they are more appropriately designated β-lactamases (Rastogi, 2010; Sridhar Rao, 2008). The most important activity of these β-lactamase enzymes is the alteration of the β-lactam ring of the drug (Samaha-Kfoury and Araj, 2003). The introduction of newer classes of β-lactam antibiotics such as the third generation cephalosporins and aztreonam and their widespread use, have co-evolved with them new enzymes that have led to the Extended

Spectrum β-lactamases (ESBLs) among the *Enterobacteriaceae* (Samaha-Kfoury & Araj, 2003; Stratton, 2000b; Sturenburg *et al*., 2004; Thomson & Smith, 2000). These enzymes have an extended substrate among the cephalosporin and monobactam antibiotics together with the ability to confer resistance to other non-β-lactam antibiotics so these enzymes were designated ESBLs i.e. Extended Spectrum β-lactamases (Chaudhary & Aggarwal, 2004). The spread of bacterial resistance is mainly due to plasmids because they can be transferred between Gram negative bacteria by conjugation and between Gram positive organisms by transducing phages (Thomson and Smith, 2000). This transferability is responsible for outbreaks of resistance (Dbaibo, 2000; Sarma & Ahmed, 2010).



**2.4 Synthesis and mechanism of action of ß-Lactamases**

The secretion of β-lactamases in Gram positive bacteria occurs towards the outside of the cell membrane environment as coenzymes (Dbaibo, 2000). In the Gram negative bacteria they remain in the periplasmic space, where they attack the antibiotic before it can reach its receptor site (Stratton, 2000a). The destruction of the β-lactam ring occurs in two ways. The first mechanism is based on serine action, upon which the action is divided into three major classes (A, C and D) on the basis of amino acid sequences. The β-lactamase enzyme contains an active site with conformational flexibility for substrate binding (Arias *et al*., 1999). Close to this site lies the serine residue that irreversibly reacts with carbonyl carbon of the β-lactam ring resulting in an open ring which is an inactive β-lactam. These enzymes are active against penicillins, cephalosporins, and monobactams (Ambler, 1980). Secondly a less commonly encountered group of β-lactamases is the metallo-β-lactamases or class B β-lactamases. This group of enzymes uses divalent transition metal ion, most often zinc, linked to histidine or cysteine residue or both (Ambler, 1980; Queenan & Bush, 2007), to react with the carbonyl group of the

amide bond of most penicillins, cephalosporins and carbapenems, but it does not affect monobactams (Queenan & Bush, 2007). Ambler classification of β-lactamases is based on these reactions.

**2.5 Classification of β-Lactamases**

β-Lactamase production is the most common mechanism of enteric bacterial resistance (Coque *et al*., 2008). The enzymes are numerous, and they mutate continuously in response to the heavy pressure of antibiotic use, leading to the development of extended spectrum β-Lactamases (Bonnet, 2004; Bradford, *et al*., 2004; Canton, *et al*., 2008; Coque *et al*., 2008). Most ESBLs were derived from TEM (Temoniera) or SHV (Sulphydryl variable) expressed predominantly by *K. pneumoniae* and *E. coli* (Canton *et al*., 2008). In 1982, when ceftazidime first became available for clinical use, only TEM-1, TEM-2 and SHV-1 were known (Bonnet, 2004; Bush, 2001). Now more than 130 TEM-type β-Lactamases are known and more than 50 SHV-type β-Lactamases have been reported (Bonnet, 2004). Later, a non-TEM, non-SHV ESBL was isolated and designated CTX-M-1 (cefotaxime as main substrate) in reference to its hydrolytic activity against cefotaxime (Bonnet, 2004). The CTX-M enzymes were simultaneously observed in nosocomial and community strain isolates and from multiple geographic locations (Pattarachai Kiratisin, 2008). This observation led to the notion that the $Bla_{CTX\text{-}M}$ emergence is from the chromosomes of environmental *Kluyvera* bacteria, from which genes similar to $Bla_{\text{CTX-M}}$ have been identified. At present, the CTX-M family comprises over 40 enzymes and the list continues to grow (Bonnet, 2004; Khan, 2010; Pattarachai *et al*., 2008). OXA-type ESBLs are also many. There are many OXA-type derived ESBLs from OXA-10, OXA-1 and OXA-2 (Jacoby & Munoz-Price, 2005). Other ESBL enzyme-types are being found such as the Toho, PER, VIM, VEB, BES, GES, TLA, SFO, and IBC (Jacoby & Munoz-Price, 2005). This large diversity of the

β-lactamase enzymes drew the needed attention for their classification. The first attempt at classifying the β-lactamase enzymes was done using their substrates, but these attempts were found to be inadequate as it could not explain the molecular and biochemical properties of the enzymes (Kiratisin, 2008). Also most of the enzymes in those classifications were later found to belong to the Ambler's Molecular class A (Jacoby & Munoz-Price, 2005).

Richmond & Sykes classification was a scheme based on substrate profile and the location of the gene encoding the β-lactamase (Sykes, 2002). This classification scheme could also not differentiate the TEM and SHV enzymes and their derivatives (Jacoby & Munoz-Price, 2005; Samaha-Kfoury & Araj, 2003).

Several criteria were used in the functional classification of the β-lactamase enzymes leading to the evolution of many functional schemes. These classification criteria were based on the spectrum of antimicrobial substrate profile, enzyme inhibition profile, enzyme net charge (PI), hydrolysis rate (Vmax), binding affinity (Km), isoelectric focussing, protein molecular weight, and amino acid composition (Jacoby & Munoz-Price, 2005; Samaha-Kfoury & Araj, 2003).The evolution of functional classification scheme of the β-Lactamase enzymes is shown in Table 3.

**Table 3. Evolution of functional classification of β-lactamases.**

| Year | Author | Basis of classification of β-lactamases |
|---|---|---|
| 1968 | Sawai et al | Used cephalosporins versus penicillins as substrates |
| 1973 | Richmond and Sykes | Expanded substrate profile and suggested five major groups (Ia-d, II, III, IV, V) |
| 1976 | Sykes and Mathew | Differentiated the plasmid mediated β-lactamases on the basis of isoelectric focussing. |
| 1980 | Ambler | Biochemical and functional characteristics and molecular |

| | | |
|---|---|---|
| | | structure of the enzyme. |
| 1981 | Mitsuhachi and Inoue | Added the category "cefuroxime hydrolysing β-lactamase" |
| 1989 | Bush | Expanded further the substrate profile, added the reaction with EDTA, correlated between functional and molecular classification |
| 1995 | Bush, Jacoby and Medeiros | Expanded the Bush scheme and used biochemical properties, molecular structure and molecular sequence. Suggested classification into four groups (1-4) on the basis of the spectrum of activity and other functional characteristics. |

(Samaha-Kfoury & Araj, 2003)

Other classification systems based on molecular structure and nucleotide sequence of genes have made the classification easier in placing β-lactamase enzymes into functional groups. Two classification schemes were most popular. First was the molecular classification according to Ambler based on the nucleotide and amino acid sequences in these enzymes. Four classes (A-D) are recognized in the Ambler scheme. Classes A, C and D enzymes act by a serine based mechanism whereas enzymes in class B also known as metallo-β-lactamases need zinc for their action (Ambler, 1980). The second was the Bush–Jacoby-Medeiros classification (Samaha-Kfoury & Araj, 2003) based on the biochemical properties, molecular structure and molecular sequence, leading to an expansion of the Ambler classification scheme. This approach made it possible to define ESBLs as β-lactamases (enzymes) capable of hydrolysing oxyimino-cephalosporins that can be inhibited by clavulanic acid (Bush, 1995). Current classification of β-Lactamases by Ambler on one hand and Bush-Jacoby-Medeiros classification on the other hand as shown in Table 4 below.

**Table 4.β-lactamase classification schemes**

1. **Ambler classification Scheme**

| | | |
|---|---|---|
| Class A | Penicillinases | TEMs, SHVs, PCI, CTX-Ms, SME-I, KPC-I |
| Class B | Metallo-beta-lactamases (Zinc) | IMP-I, VIM-I, cer A |

| | | |
|---|---|---|
| Class C | Cephalosporinases | AmpCs, CMY-2, ACT-I |
| Class D | Oxacillinases | OXA-I. |

**2. Bush-Jacoby-Medeiros classification**

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

(Pfaller & Segreti, 2006)

The Bush-Jacoby-Medeiros classification is the latest classification of β-lactamases, in which the enzymes are divided into four groups (1-4) and subgroups (a-f) (Pfaller & Segreti, 2006; Samaha-Kfoury & Araj, 2003) as follows:

Group 1 are cephalosporinases not inhibited by clavulanate, belonging to the Ambler (molecular) class C.

Group 2 are penicillinases, cephalosporinases or both inhibited by clavulanic acid, belonging to the Ambler (molecular) classes A and D, reflecting the original TEM and SHV genes. Due to the

large number of TEM and SHV derived β-lactamases, they were divided into two subclasses, 2a and 2b. The subgroup 2a contains just penicillinases, whereas 2b are broad spectrum β-lactamases capable of inactivating penicillins and cephalosporins at the same rate. Furthermore new subgroups were segregated from subgroup 2b:

- Subgroup 2be with the letter "e" for extended spectrum of activity represents the ESBLs, which are capable of inactivating third generation cephalosporins (ceftazidime, cefotaxime and cefpodoxime) as well as monobactams (aztreonam).

- The 2br subgroup of enzymes with "r" denotes reduced binding to clavulanic acid and sulbactam. They are also called inhibitor-resistant TEM derivative enzyme but are rather sensitive to tazobactam.

- Subgroup 2c was segregated from group 2 because subgroup 2c enzymes inactivate carbanicillin more than benzylpenicillin, and also have some effect on cloxacillin.

- Subgroup 2d enzymes inactivate cloxacillin more than benzylpenicillin, with some activity against carbenicillin; these enzymes are poorly inhibited by clavulanic acid and some of them are ESBLs.

- Subgroup 2e enzymes are cephalosporinases that can also hydrolyze monobactams and they are inhibited by clavulanic acid.

- Subgroup 2f was added because these are serine-based carbapenemases, in contrast to the zinc based carbapenemases in group 3.

Group 3 are the zinc-based or metallo-β-lactamases, corresponding to the molecular class B, which are the only enzymes acting by the metal ion zinc. They have the ability to hydrolyze

penicillins, cephalosporins, and carbapenems. Thus carbapenems are inhibited by both group 2f (serine-based mechanism) and group 3 (zinc-based mechanism).

Group 4 are penicillinases that are not inhibited by clavulanic acid, and they do not yet have a corresponding molecular class (Samaha-Kfoury & Araj, 2003).

**2.6 Epidemiology of ESBL-producing organisms**



β-lactam antibiotics are used all over the world, but the distribution of the enzymes responsible for resistance to oxyimino-cephalosporins and carbapenems is not uniform (Miro *et al*., 2005; Nordmann *et al*., 2009). In the United States of America, some hospitals do not have ESBls at all, whereas in other hospitals as high as 40 percent of *Klebsiella pneumoniae* isolates have been reported to be ceftazidime –resistant as a result of ESBL production (Coque, et al., 2008). ESBLs have been reported in *K. pneumoniae, K. oxytoca* and *E. coli* especially, but it is not limited to them only (Apisarnthanarak *et al*., 2007; Coque *et al*., 2008). ESBLs have been reported in *Citrobacter*, *Enterobacter, Proteus, Serratia* and other genera of enteric organisms (Apisarnthanarak *et al*., 2007; Blomberg *et al*., 2005; Bonnet, 2004; Canton, *et al*., 2008; Chaudhary & Aggarwal, 2004; Coque, *et al*., 2008; Hajjar 2010; Jain *et al*., 2003; Lavigne, *et al*., 2004; Nordmann *et al*., 2009) and non-enteric organisms such as *Acinetobacter baumanii* (Song, *et al*., 2006; Yong, *et al*., 2006) and *Pseudomonas aeruginosa* (Weldhagen, 2003). Of greater concern is the appearance of ESBLs in *Salmonella species*, which are mostly community-acquired pathogens where *S. typhimurium* (Canton *et al*., 2008; Coque *et al*., 2008) *S. enteritidis* (Gonzalez-Sanz *et al*., 2009; Oteo *et al*., 2009) and *S. enterica* (Hasman *et al*., 2005) and other *Salmonellae* have been reported to produce ESBLs (Makanera *et al*., 2003). Also importantly, is the notion that ESBLs would spread from *Salmonella* sp to other community-acquired

pathogens, as ESBL producing *Shigella* sp was reported (Hrabak *et al*., 2008). In the hospital setting ESBL prevalence is reported to be higher in intensive care units than other places (sites) in the hospital (Ramazanzadeh, 2010a; Rodriguez-Villalobos, *et al*., 2010).

Globally the percentages of *K. pneumoniae* expressing an ESBL phenotype is more in isolates from Latin America (45.4%), the Western pacific (24.6%) and Europe (22.6%) than from the United States (7.6%) and in Canada(4.9%) (Weldhagen, 2003). In more than 13,000 isolates of *E. coli*, the percentages expressing the ESBL phenotype were again higher in Latin America (8.5%), Western pacific (7.9%), Europe (5.3%) than in the United States (3.3% ) and in Canada (4.2%) (Weldhagen, 2003).

There has been considerable geographical difference in the occurrence of ESBLs in Europe (Coque, *et al*., 2008) with increasing reports of extended-spectrum beta-lactamases (ESBLs) prevalence since the extended β-lactamases were first described in 1983. During the 1990s, the ESBLs types were mainly the TEM- and SHV-beta-lactamase families and the ESBLs were found mainly in *Klebsiella pneumoniae* in nosocomial outbreaks (Coque *et al*., 2008). Nowadays, it is the CTX-M ESBLs which are mostly found in *Escherichia coli* isolated from community-acquired infections that are increasingly reported (Coque *et al*., 2008). This phenomenon has been attributed to dissemination of specific clones or clonal groups and epidemic plasmids in community and nosocomial settings. The most widespread ESBLs reported in increasing order belong to the TEM (TEM-24, TEM-4, TEM-52); SHV (SHV-5, SHV-12) and CTX-M (CTX-M-9, CTX-M-3, CTX-M-14 or CTX-M-15) families in Europe (Coque *et al*., 2008). Co-evolution or co-selection with other resistance mechanisms, especially to fluoroquinolones, aminoglycosides and sulfonamides, (Coque *et al*., 2008; Song *et al*., 2006) and the spread to other members of the *Enterobacteriaceae* including the *Salmonellae* (Makanera *et*

*al*., 2003; Yong *et al*., 2006), have compounded the problem which continue to increase. In the United Kingdom *E. coli* resistance to third generation cephalosporins has increased yearly from about 2% in 2001 to approximately 11% in 2006( Coque *et al*., 2008)Also resistance of *Klebsiella* isolates to third generation cephalosporins has been 15-17% in 2006 as a result of ESBL production ( Coque *et al*., 2008). A survey of 1610 *E. coli* isolates and 785 *K. pneumoniae* isolates from 31 centre's in 10 European countries in year 2000 found that the prevalence of ESBLs in *Enterobacteriaceae* ranged from as low as 1–5% in Germany to as high as 39–47% in Eastern European countries such as Russia, Poland and Turkey (Goosens, 2001). A study in Belgium found the proportion of CTX-M-type enzymes increase significantly from 23% in 2006 to 54% in 2008 (Rodriguez-Villalobos *et al*., 2010). This was mostly linked to a rising proportion of CTX-M-15-producing *E. coli* (Rodriguez-Villalobos *et al*., 2010). TEM-24 however decreased from 43% in 2006 to 19%in 2008 during the same period, while the prevalence of TEM-52 remained the same (Rodriguez-Villalobos *et al*., 2010). Other European countries like Sweden and the Netherlands also had a relatively low prevalence of ESBL producing *Enterobacteriaceae*, registering 3% and 1% respectively (Rodriguez-Villalobos *et al*., 2010). In a Sweden hospital a study found colonization rate of ESBL-producing *Enterobacteriacae* in 1999 to range from 14% (n=73) in medical ward to the highest 41% (n=29) in orthopaedic ward with an intermediate 23% (n=80) in surgical ward due to stringent infection control measures the values reported by 2007 were about 1.5% (Soderblom & Struwe, 2007) a value considered to be low as compared to Italy, where ESBL prevalence values of 18.4% among *K. pneumoniae* isolates and 1.3% among *E. coli* isolates, 12.6% of *K. oxytoca* and 5.3% of *Proteus mirabilis* isolates have been reported (Nijssen *et al*., 2004). A study in the Czech republic on 913 *K.*

*pneumoniae* strains had 234 (25.6 %) to be ESBL-positive strains, but the prevalence of ESBL-positive strains was 38.5 % in ICUs and 15.8 % in standard wards (Kolar *et al*., 2006).

In United Arab Emirates130 isolates were tested for ESBL production and 53 (41%) were identified as ESBL phenotypes; of these, 31(60%) were *E. coli,* 20 (36%) *K. pneumoniae* and 2 (4%) *K. oxytoca* (Al-Zarouni *et al*., 2008). ESBL phenotype was seen in 100% of endotracheal tube isolates, 20 (31%) from urine, 7 (58%) from blood and 4 (80%) from catheter tips. Amikacin susceptibility was 100%. Over 90% of ESBL isolates showed resistance to aztreonam and cephalosporins (Al-Zarouni *et al*., 2008). In Saudi Arabia phenotypic characterization of enterobacteria identified a high ESBL rate of 55% among *K pneumoniae* isolates (Al-Agamy *et al*., 2009). ESBL-producing *K pneumoniae* were PCR positive for SHV, TEM and CTX-M β-lactamase genes with prevalence levels of 97.3%, 84.1% and 34.1%, respectively (Al-Agamy *et al.*, 2009).

In Africa prevalent values are scanty but reports from Tanzania quoted ESBLs values of 25% for *E. coli* and 17% for *K. pneumoniae* isolates causing pediatric septicemia at a tertiary hospital in Dar es Salaam (Blomberg *et al*, 2005). In Ile Ife (Nigeria), 86.6% was reported for *Klebsiella* sp, 73.4 % for *Enterobacter* sp and 63.6% for *E. coli* strains (Jain *et al*., 2003). Similarly high results were obtained from Enugu State metropolis, Nigeria, where ESBL production was determined among 300 isolates of *K. pneumoniae* and 186 (62%) of them expressed ESBL (Olonitola *et al*., 2007). ESBL producing *K. pneumoniae* were most frequently isolated from blood 76 (40.0%) followed by urine 66 (30.5%) and sputum 44 (23.6%) (Romanus *et al*., 2009). Similarly out of a total of 65 urine specimens studied at the Ahmadu Bello University Teaching Hospital, Shika-Zaria, Nigeria, 50 isolates consisting of 33 (66%) *E. coli* and 17 (34%) strains of *K. pneumoniae*

were recovered from the patients with Urinary Tract Infections (UTIs). Out of these 50 isolates, 15(30%) were ESBL producers. ESBL-positive *K. pneumoniae* isolates made up of 6/17 (35.3%) while 9/33 (27.3%) were ESBL-positive *E. coli* isolates (Olonitola *et al*., 2007). The susceptibility of the ESBL-positive *K. pneumoniae* isolates to ciprofloxacin, ofloxacin and amikacin were 64.7%, 82.4% and 82.4%, respectively, while the susceptibility of the ESBL-positive *E. coli* isolates were: ciprofloxacin (57.6%), ofloxacin (48.5%) and amikacin (84.8%) as reported by other workers (Olonitola *et al*., 2007). A similar study in Tanzania had found 29.2% of the gram negative bacilli isolate to be ESBL producers (Mshana *et al*., 2009). Proportions of species specific ESBLs among *K. pneumoniae, E. coli, Acinetobacter sp, Proteus sp* and other enterobacteria were 63.7%, 24.4%, 17.7%, 6.4% and 27.9% respectively. A statistically significant higher number of in-patients 100/283 (35.3%) compared to 10/94 (10.6%) of out-patients had ESBL-producing organisms in Tanzania (Mshana *et al*., 2009). In a study in Egypt Gram negative isolates tested for the production of ESBL enzymes had 64.7% (33/51) of the

isolates from in-patients and 20/135 (14.8%) environmental isolates confirmed to be ESBL producers (Ahmed *et al*., 2009). The type of beta-lactamase gene was determined by PCR and shown to be SHV-type-ESBL mainly (Ahmed *et al*., 2009).

According to Gniadkouski, (2001), one ESBL producing microbial clone (e.g. *K. pneumoniae*) may be present in different locations within a hospital, or even several different medical institutions as demonstrated in France where SHV-4-producing *K. pneuminiae* was found in 14 hospitals and TEM-24-producing *Enterobacter aerogenes* clone spreading to 21 hospitals in Greece. A single ESBL variant may be found in several bacterial types or species in many locations in different countries as plasmid TEM-3 found in France was the same plasmid borne TEM-3, which was responsible for ESBL spread in ten different microbial species in 26 hospitals in Poland (Gniadkouski, 2001)

## 2.7 Laboratory Detection of ESBL

The ability of a laboratory to identify organisms that produce ESBL is a major challenge, because the ESBL enzymes have variable affinity for different substrates (HPA, 2004). This substrate variability and inoculums effect make some ESBL producing organisms difficult to detect (HPA, 2004). ESBLs are most prevalent among the *Klebsiellae* so most detection methods were developed for the *Klebsiellae*, but are also used for the *E. coli*, so the methods may be applicable to other *Enterobacteriaceae (*Johnson *et al*., 2004)

### *2.7.1 Screening methods for ESBL detection*

Various detection methods have been reported which included modification of double disc method with cefepime, the use of chromogenic agar, three-dimensional methods and microdilution methods that use clavulanate plus a different β-lactam such as the cefepime (Pitout

& Laupland, 2008). These tests are technically demanding and also difficult to interpret, so these factors limited their widespread use (Vading, 2010). In addition they fail to detect ESBLs in bacteria such as *Enterobacter*, *Citrobacter* and *Serratia* species (Winstanley *et al*., 2005). This is because the inhibition of clavulanate by ESBLs is often masked by β-lactamase types like AmpC produced by these organisms (Livermore & Brown, 2001). According to HPA (2004) ceftazidime or cefpodoxime had to be included in the first line routine susceptibility of the isolate, because these two drugs are the best third generation cephalosporin substrates for TEM and SHV-derived ESBLs, whilst cefotaxime detected CTX-M enzymes. Following these recommendations the British Society for Antimicrobial Chemotherapy (BSAC) developed and approved the zone sizes in the routine disc diffusion tests and MIC breakpoints (Table 5) in the broth dilution method (Johnson, Woodford, & Livermore, 2004; Jonnathan, 2005), so a resistant breakpoint gives cause for suspicion of ESBL production. The breakpoints are as shown in the Table 5 below.

Table 5. Breakpoints recommended by BSAC for detecting ESBLs

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

(HPA, 2004)

The Clinical and Laboratory Standards Institute reviewed the BSAC methods and recommended the disc diffusion and the MIC methods for screening *Klebsiellae* and *E coli* including other coliforms for ESBL production, but suggested cephalosporin MIC of ≤8µg/ml as susceptible and MIC of >8µg/ml is considered resistant and a suspect for ESBL production (CLSI, 2005). The guidelines standardized the detection method for *Proteus* and recommended MIC of ≥2µg/ml for selection for confirmation. However, the Health Protection Agency (HPA) of United Kingdom maintained that MIC of ≥2µg/ml for both cefotaxime and cefpodoxime and MIC of ≥4µg/ml for ceftazidime should be used for screening all enterobacteria (HPA, 2004). An alternative suggestion from CLSI was that laboratories using disc diffusion methods for antimicrobial susceptibility testing must adopt the BSAC breakpoints for more than one indicator drug (cefpodoxime, ceftazidime, cefotaxime and ceftriaxone) as a screening test for ESBL detection (CLSI, 2005). Organisms producing zone sizes which give the suspicion for ESBL production are to be confirmed phenotypically (Paterson & Bonomo, 2005). Since the methods are many and confusing HPA in 2004 prepared the following guidelines in the choice of indicator cephalosporin for the screening of isolates for the ESBLs by suggesting the following general traits:

- TEM & SHV ESBL-producing enterobacteria are obviously resistant to ceftazidime, but the resuts are variable when tested to cefotaxime.
- CTX-M ESBLs producers are obviously resistant to cefotaxime; here too the results are variable to ceftazidime.
- All ESBLs type producers; however, have obvious resistance to cefpodoxime, so this drug may be the choice for the screening.

- Cefuroxime, cephalexin and cephradine are unreliable indicators. In any case the suspect organisms must be confirmed for ESBL production (HPA, 2004).

### *2.7.2. ESBL confirmatory test methods*

The confirmatory test methods are many; a few of them are as described below

#### *2.7.2.1. Double disc synergy test method*

This test employs test discs of third generation cephalosporins and augmentin (co-amoxiclav). The discs are placed 30 mm apart from centre to centre on inoculated Muller-Hinton agar. A clear extension of the zone of inhibition of the cephalosporin towards the augmentin disc or Keyhole formation is interpreted as positive for ESBL production (Jarlier *et al.*, 1988). A picture of the double disc synergy test method is presented in figure 5 below.

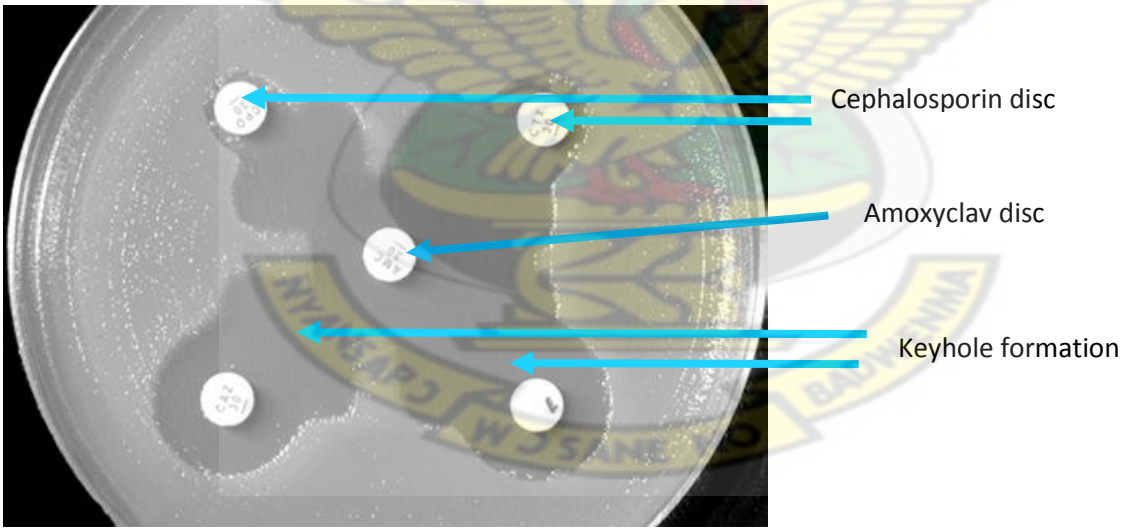


Figure 5. Double disc synergy method using four substrates for ESBL detection (Sturenburg *et al*., 2003). Single plate disc approximation method using four substrates for ESBL detection. Cefepime, ceftazidime, cefotaxime and cefpodoxime discs placed 2.5 cm away from a central co-amoxiclav disc. Exhibition of the keyhole phenomenon is indication for ESBL production (Sturenburg, *et al*., 2003)

*2.7.2.2 Combination disc method*

The combination disc test method uses an indicator cephalosporin (ceftazidime or cefotaxime) combined with an ESBL inhibitor, usually clavulanic acid (HPA, 2004). This is the method both the BSAC and Oxoid recommend for the detection of ESBLs among Enterobacteriaceae. A combination disc of cefpodoxime 10µg plus 1µg clavulanic acid is placed on Mueller-Hinton agar inoculated with the test organism. Another disc of the cefpodoxime (10 µg) alone is also placed on the agar. The set-up is incubated at 37 $^{o}$C overnight. The zones of inhibition of both discs are measured and compared. A zone difference between the two discs of >5mm is considered positive for ESBL production (Carter *et al*., 2000). Both BSAC and Oxoid validated this method and found it to accurately detect ESBL producing *Klebsiellae* with 100% sensitivity and specificity, but the test fails to detect organisms producing AmpC and KI enzymes (Grover, 2006). The set-up is as shown in the figure 6.

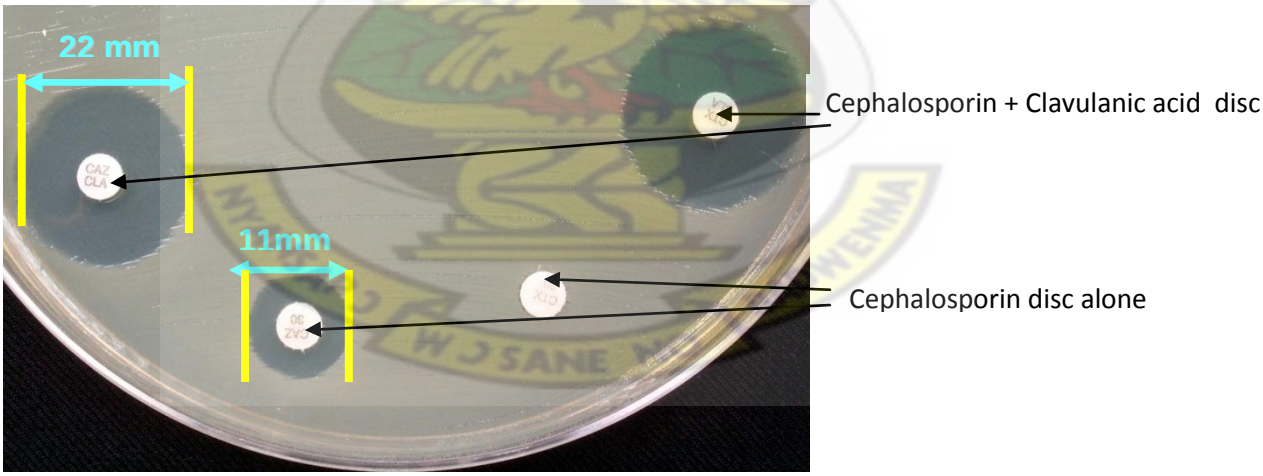


Figure 6. Combination disc method. Zone size of combined disc=22mm, that of cephalosporin alone is 11mm. Therefore 22mm-11mm > 5mm (or =5mm), so the isolate is an ESBL producer (Carter, et al., 2000)

2.7.2.3 Vitek ESBL test.

This is an automated method for the detection of ESBLs. The Vitek test system was produced by BioMeriex Vitek (bioMerieux Vitek, Inc. Hazelwood, Missouri) and uses cephalosporin and cephalosporin-inhibitor in wells on a card to detect ESBLs within 4-15 hours (Livermore & Brown, 2001).

Wells contained in cards are inoculated with test organism. A predetermined reduction in growth of a well containing cephalosporin plus clavulanic acid compared with growth in a well containing cephalosporin alone indicates presence of ESBL (Livermore and Brown 2001). Another automated system for ESBL detection was developed and known as Dade Behring MicroScan (Dade Behring Inc., West Sacramento, CA). It is capable of detecting ESBLs (Sturenburg *et al*., 2004). This one employs dehydrated serial dilutions of cephalosporins and clavulanate combinations in panels. Becton Dikinson Biosciences also produced the Automated Microbiology Systems which within 6 hours is able to measure growth responses to cephalosporins with or without clavulanate (Sanguinetti *et al.*, 2003). The reliability of automated antimicrobial susceptibility test systems for the detection of ESBL among *Enterobacteriaceae* other than *E. coli* and *Klebsiella* species remains a concern (Turng *et al*., 2002). These methods are available but apart from being limited in use are expensive and require training or expertise for their operation and maintenance.

*2.7.2.4 The three dimensional test*

To perform this test two types of inocula are prepared; the first one is usually the inoculum of a fully susceptible strain such as *E. coli* (ATCC 25922) for disc diffusion test with optical density

of 0.5 McFarland standards (Shahid *et al.*, 2004) . This is used to seed the agar plate. The second one is of denser concentration usually in the range of $10^9$ and $10^{10}$ CFU/ml of cells of the test organism. A slit is cut into the inoculated medium and the slit is filled with the test organism. Extended-spectrum cephalosporin disc is placed 3mm from the slit (Shahid *et al.*, 2004). The set-up is incubated at 37$^o$C overnight. A discontinuity in the circular zone of inhibition or the appearance of discrete colonies in the vicinity of the inoculated slit is considered positive for ESBL production. This test has been proven by (Menon *et al*., 2006) to be more sensitive than the double disc diffusion test, but has been found to be more technically challenging and more labour intensive.

2.7.2.5 *Inhibitor potentiated disc diffusion test*.

In this method, two plates of Mueller-Hinton Agar (MHA) are prepared. One plate is incorporated with clavulanic acid and the other plate is without it. The two plates are inoculated with the test organism. Cephalosporin discs are then applied and incubated overnight 37 $^o$C. More than 10 mm increase in the zone of inhibition on the clavulanate containing MHA plate indicates ESBL production (Queenan *et al*., 2004).

2.7.2.6 The E-test strip method

The E-test ESBL strip carries two gradients. On one end is usually ceftazidime and on the opposite end is ceftazidime plus clavulanic acid. A decrease in the MIC of ceftazidime of more than three dilutions in the presence of clavulanate is interpreted as a positive test for ESBL production, or when the MIC ratio of cephalosporin alone to cephalosporin plus clavulanic acid gave MIC ≥8 (Bradford *et al*., 2004; Queenan *et al*., 2004; Sridhar *et al*., 2008)).

The commonly used E-test ESBL strips contain Cefotaxime (CT) or Ceftazidime (TZ) on one end and on the other end has the corresponding antibiotic and clavulanate (AB BIODISK, Dalvägen 10; 169 56 Solna, Sweden). CT codes for the cefotaxime (0.25-16 μg/mL) gradient and CTL the cefotaxime (0.016-1 μg/mL) plus 4 μg/mL clavulanic acid. TZ codes for the ceftazidime (0.5-32 μg/mL) gradient and TZL the ceftazidime (0.064-4 μg/mL) plus 4 μg/mL clavulanic acid **(**AB BIODISK, Dalvägen 10; 169 56 Solna, Sweden). The E-test ESBL CT/CTL and TZ/TZL strips are made of a thin, inert and non-porous plastic carrier of dimensions 5 x 60 mm. On one side of the strip are calibrated MIC reading scales in μg or mL while the reverse side carries the two predefined exponential gradients of the antibiotic. The test needs to be set up according to standard E-test procedures for Gram-negative aerobes with at least both CT/CTL and TZ/TZL strips (Bradford, 1994; Cormican *et al*., 1996; Livermore & Brown, 2001). The presence of ESBL is confirmed by the appearance of a phantom zone or deformation of the CT or TZ ellipse or when either the MIC of CT or TZ is reduced by ≥ 3 $\log_2$ dilutions equivalent of MIC ≥8 in the presence of clavulanic acid (Bradford 1999). The E-test strips for cefotaxime/cefotaxime plus clavulanate (CT/CTL) and that of ceftazidime/ ceftazidime plus clavulanate (TZ/TZL) are shown in the figure 7.


Figure 7. The E-test strip method of ESBL testing. The E-test strips for cefotaxime/cefotaxime plus clavulanate (CT/CTL) and that of ceftazidime/ceftazidime plus clavulanate (TZ/TZL) **(**AB BIODISK, Dalvägen 10; 169 56 Solna, Sweden).

The results of the E-test can sometimes be difficult to interpret when clear-cut picture is not produced. Possible pictures an ESBL test can show with E-tests are presented in figures 8--a, -b, -c, and -d **(**AB BIODISK, Dalvägen 10; 169 56 Solna, Sweden). Interpretations of test results are also indicated according to the growth pattern shown along the test strip.


Figure 8a.Interpretation of the E-test strip method: Clear cut ESBL positive: MIC CT/CTL =1.5/0.047 = 32 **(**AB BIODISK, Dalvägen 10; 169 56 Solna, Sweden).


Figure 8b.The E-test strip method: A "rounded" phantom inhibition zone below CT is indicative of ESBL production **(**AB BIODISK, Dalvägen 10; 169 56 Solna, Sweden).

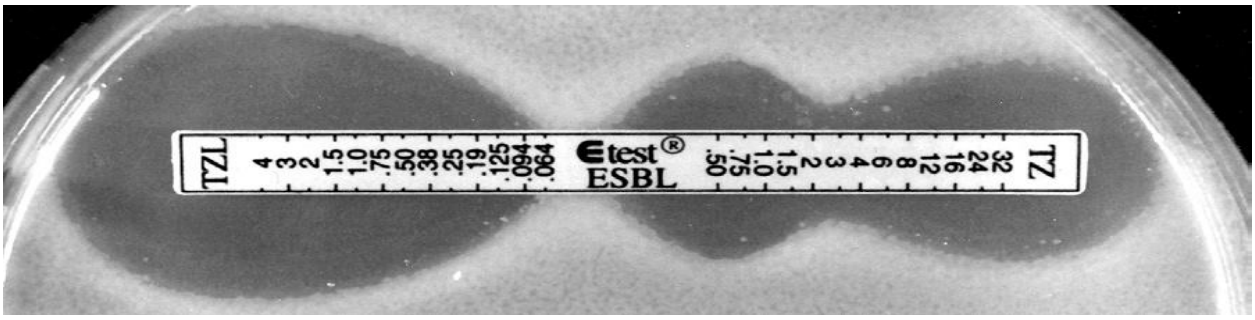


Figure 8c.The E-test strip method: Deformation of the TZ inhibition ellipse is indicative of ESBL**(**AB BIODISK, Dalvägen 10; 169 56 Solna, Sweden).




Figure 8d.The E-test strip method: When MIC values are above the test ranges, result is Non-Determinable (ND) **(**AB BIODISK, Dalvägen 10; 169 56 Solna, Sweden).

E-test strips containing cefotaxime/clavulanate gradients are definitive for the detection of CTX-M type enzymes (Bradford, 2004), but it is difficult to interpret with weak enzyme producers, as subtle zone formations are difficult to identify and interpret. The E-test is also too expensive for routine laboratory testing (Perez *et al.*, 2007). Other limitations of the ESBL E-test are that:

1. Inhibitor resistant TEM (IRT) enzymes cannot be detected by E-test ESBL strips.

2. An ESBL negative result with elevated MICs to CT/TL and TZ/TZL may be due to an IRT, AmpC or an ESBL masked by the concurrent presence of these enzymes and/or other resistance mechanisms.

3. Strains showing non-determinable (ND) results should be further investigated by genotyping.

4. Performance of E-test ESBL is based on the use of at least both TZ/TZL and CT/CTL strips simultaneously **(**AB BIODISK, Dalvägen 10; 169 56 Solna, Sweden).

Use of only one E-test ESBL strip to confirm the presence of ESBL is not valid **(**AB BIODISK, Dalvägen 10; 169 56 Solna, Sweden) because the two enzymes have different substrate affinities.



**2.8 Genotypic detection of ESBL**

In order to detect the genotype of ESBL (*Bla*$_{TEM}$, *bla*$_{SHV}$ or the *bla*$_{CTX-M}$) molecular or genetic methods have to be employed. Common molecular techniques for ESBL genotyping are mostly PCR based such as:

1) PCR with oligonucleotide primers

2) Specific DNA probes

3) oligotyping;

4) PCR followed by restriction fragment length polymorphism analysis

5) Ligase chain reaction and

6) Nucleotide sequencing (a variant of pyrosequencing) (Naas *et al*., 2007; Woodford *et al*., 2006).

Among all these PCR amplification with oligonucletide primers to determine genes of specific β-lactamase families is the most common method employed by many researchers (Bradford, 1999). This procedure does not distinguish between parent β-lactamases and derivatives of TEM, SHV, OXA and other ESBLs (Bradford, 1999). PCR followed by sequencing has made it possible to discriminate between non-ESBL parent enzyme and different variants of ESBL genes, and therefore has become the method of choice (Fluit & Schmitz, 2001). These genetic methods are not done routinely for clinical diagnostic purposes, because they are laborious and challenging to perform, so they are mainly restricted to reference laboratories and molecular surveillance studies in many countries (Woodford, *et al*., 2006; Woodford *et al*.,2007). Some attempts and progress have been made to develop simpler molecular techniques such as rapid-cycle sequencing and microarrays for genotyping ESBLs and these techniques have been tipped to be more user friendly, to become readily available and cost effective for diagnostic laboratories (Pitout & Laupland, 2008), but this hope has yet to be realized. Several other studies have described various molecular approaches for quick genotyping of ESBLs such as the multiplex PCR (Woodford *et al*., 2006) and pyrosequencing (Naas *et al*., 2007), but they too are not yet readily available for clinical diagnostic purposes.

All these methods share common advantages and disadvantages, except the nucleotide sequencing, a variant of pyrosequencing, which has the ability to detect all specific ESBL genes present in a bacterial strain (Bradford, 1994). The presence of multiple ESBL genes in a single bacterial strain of clinical isolate may not make this procedure straightforward and cost effective (Bradford, 1999). Nevertheless, nucleotide sequencing remains the gold standard for ESBL gene detection (Fluit & Schmitz, 2001).

These improvements in genetic technologies also resulted in improvement in ESBL testing methods (Bradford, 1999). It also resulted in achieving uniform standards of ESBL detection and confirmatory testing (Bradford, 1999). As a result accurate identification of ESBL-producing bacteria have been achieved, with easier to perform tests which have become common and more accessible to clinical laboratories (Fluit & Schmitz, 2001).

**2.9 PCR as genetic tools employed in Epidemiology**



PCR is a rapid and versatile *in vitro* method for amplifying defined target DNA sequences present within a DNA template (King, 2010). The PCR method permits selective amplification of a specific target DNA sequence(s) within a heterogeneous collection of DNA sequences (King, 2010). That is it targets a small unit of a specific DNA within the total genomic DNA of an organism (or a complex cDNA population). To achieve selective amplification, certain prior DNA sequence information is required from section of the targeted DNA. This information is used to design two oligonucleotide primers (amplimers) which are 15–25 nucleotides long and specific for the target sequence. When the primers are added to the denatured template DNA, they (primers) bind specifically to the target site of the denatured complementary DNA sequences (King, 2010). In the presence of a suitably heat-stable DNA polymerase (usually the Taq polymerase) and DNA precursors dNTPs which are the four deoxynucleoside triphosphates, designated dATP, dCTP, dGTP and dTTP), they initiate the synthesis of new DNA strands which are complementary to the individual DNA strands of the target DNA segment (King, 2010). The PCR is a chain reaction because newly synthesized DNA strands will act as templates for further DNA synthesis in subsequent cycles. After about 25 cycles of DNA synthesis, the products of the PCR will reach about $10^5$ copies of the specific target DNA sequence. This amount is easily visualized as a discrete band of a specific size when agarose gel electrophoresis

is performed on it (McPherson, 2000). A heat-stable DNA polymerase is used because the reaction involves sequential cycles composed of three steps:

i. Denaturation, which occurs typically at about 93–95°C.
ii. Reannealing at temperatures usually from about 50°C to 70°C depending on the melting temperature ($T_m$ ) of the expected duplex which is mostly $5^oC$ below the calculated melting temperature($T_m$).
iii. DNA synthesis, typically at about 70–75°C (King, 2010)

Heat-stable DNA polymerase enzymes (e.g. Taq polymerase) have been obtained from a microorganism, *Thermus aquaticus* whose natural habitat is hot springs. These enzymes are thermostable up to 94°C, but have an optimum working temperature of 80°C (King, 2010; Mullis, 1990).

## 2.10 Primer design and specificity of amplification

The specificity of primers depends on their ability to recognize and bind to the desired target sequences other than the unintended target DNA sequences (McPherson, 2000). In the genome of organisms with many chromosomes this effect of binding to unintended sites is overcome by using the following techniques:



1. **Nested Primers:** The products of an initial amplification reaction are diluted. The diluted product is then used as the target DNA source for a second reaction in which a different set of primers used, corresponding to sequences located close, but internal, to those used in the first reaction (McPherson, 2000)
2. **Hot-start PCR**. Initially mixing of all PCR reagents prior to the first heat denaturation step allows more opportunity for nonspecific binding of primer sequences to occur. To reduce this possibility, one or more components of the PCR are physically separated until the first denaturation step is passed. A popular approach is to use a specially formulated wax bead designed to fit snugly within a PCR reaction tube. The reaction components without the enzyme and reaction buffer are added to the tube followed by the wax bead in the molten state. The molten wax beads floats on top and then solidifies on cooling. The thermostable polymerase is then added with buffer. At the initial denaturation step the wax melts again and rises to the surface allowing all the reaction components to come into contact with each other, therefore bypassing the initial denaturation step with all reaction components in place (McPherson, 2000).
3. **Touch-down PCR**. Most thermal cyclers can be programmed to perform runs in which the annealing temperature is lowered incrementally during the PCR cycling from an

initial value above the expected $T_m$ to a value below the $T_m$. By keeping the stringency of hybridization initially very high, the formation of spurious products is discouraged, allowing the expected sequence to predominate (King, 2010; McPherson, 2000).

## 2.11 Applications of PCR

Although PCR was first developed only a decade and a half ago, the simplicity and the versatility of the technique have ensured that it is among the most ubiquitous of molecular genetic methodologies (McPherson, 2000), with a wide range of general applications as described below:

1. PCR enables rapid amplification of template DNA for screening of uncharacterized mutations

PCR is suitable for providing numerous DNA templates for mutation screening (Butler, 2005). Partial DNA sequences, from a gene associated with disease, or some other phenotype of interest, enable gene-specific PCR reactions to be designed. Amplification of the appropriate gene segment then enables rapid testing for the presence of the associated mutations (King, 2010).

2. PCR application for rapid genotyping for polymorphic markers

Polymorphisms can be typed using Southern blot hybridization to detect target DNA and RNA fragments that have been size-fractionated by gel electrophoresis (Brown & Razonable, 2010). A DNA probe representing the locus is hybridized against genomic DNA samples that have been digested with the appropriate restriction enzyme and size-fractionated by agarose gel electrophoresis. The resulting products which have two alleles corresponding to the presence or absence of the restriction site are targeted by the appropriate primers using PCR and then cut by

appropriate restriction enzyme and separating the fragments by agarose gel electrophoresis (Brown & Razonable, 2010).

A wide variety of PCR-based methods can be used to assay for known mutations. These include:

1. Allelic discrimination by size or susceptibility to restriction enzyme

   Here the small insertions or deletions (such as the three nucleotide deletion in the common cystic fibrosis can be detected by designing primers from regions closely flanking the mutation site and distinguishing the normal and mutant alleles by size on polyacrylamide or agarose gels (Meletis, *et al*., 2010).

2. Allelic discrimination by susceptibility to an artificially introduced restriction site

   Even if the mutation does not result in a restriction site difference, it may be possible to exploit the difference between normal and mutant alleles by amplification-created restriction site PCR (Ohnishi, 2008) . This is a form of mismatched primer mutagenesis which a primer is deliberately designed from sequence immediately adjacent to, but not encompassing, the restriction site (Ohnishi, 2008). The primer is deliberately designed to have a mismatched nucleotide which together with the sequence of the mutant site creates a restriction site not present in normal allele (Ohnishi *et al*., 2008).

3. Allele-specific PCR

In allele-specific PCR oligonucleotide primers are designed to discriminate between target DNA sequences that differ by a single nucleotide in the region of interest (Wangkumhang *et al*., 2007)

**2.12 Multiple loci variable repeat analysis**

Short tandem repeat polymorphism (STRP) is also called a microsatellite marker and consist of short sequences, typically from one to four nucleotides long (Shiu-Yun, 2007). These STRP can be tandemly repeated several times, and often characterized by many alleles (Butler, 2005). For example, $(CA)_n$ /$(TG)_n$ repeats are often polymorphic when *n* exceeds 12, and have been widely used as polymorphic markers in the human genome (Shiu-Yun Liang, 2007). In each case the STRPs can be typed conveniently by PCR (Butler, 2005). Primers are designed from sequences known to flank a specific STRP locus, permitting PCR amplification of alleles whose sizes differ by integral repeat units (Butler, 2005). An example would be "A-T-T-C-G-A-T-T-C-G-A-T-T-C-G" in which the sequence A-T-T-C-G is repeated three times. When between 10 and 60 nucleotides are repeated, it is called a minisatellite. Those with fewer are known as microsatellites or short tandem repeats (Butler, 2005).

When exactly two nucleotides are repeated, it is called a "dinucleotide repeat"; when three are repeated, it is called a "trinucleotide repeat". When the number is not known, variable, or irrelevant, it is often called a variable number tandem repeat (VNTR) (Butler, 2005). Tandem repeat analysis helps determine an individual's inherited traits, therefore enables the tracing or determining parentage in genealogical DNA tests. DNA is examined from *microsatellites* within the chromosomal DNA. PCR is performed on the minisatellite areas of each organism being tested. The amplified material is then run through electrophoresis. By checking the percentage of bands that match, parentage is determined and this is applicable in determining the clonal relationships in bacteria and other organisms (Butler, 2005). Standardized protocols have been established for multiple-locus variable number tandem repeat analysis (MLVA) to enable data comparisons from different laboratories (Katharine, 2008 ). This is to ensure reproducibility and high quality of data and to test the robustness MLVA in a multi-laboratory validation process

(Shiu-Yun *et al*, 2007). The ‘‘variable-number tandem repeat’’ (VNTR) regions have been identified in many pro- and eukaryotic species and have been successfully used for subtyping purposes (Shiu-Yun *et al*., 2007). In the simplest form, VNTR analysis may only include a single locus, and when multiple loci are targeted, the technology is often referred to as multiple-locus VNTR analysis (MLVA) (Miljković-Selimović, 2009). A typical MLVA protocol consists of multiplex PCR amplification of VNTR targets followed by fragment sizing using high-resolution capillary electrophoresis (Hyytia-Trees *et al*., 2006). Allele types for each VNTR locus are assigned either manually using arbitrary numbers as new types are discovered (Hyytia-Trees, *et al*., 2006) or automatically using specialized software to assign the actual copy

This protocol was intended to be used as a complimentary technique to pulsed-field gel electrophoresis (PFGE) which the PulseNet system uses as the ‘‘gold standard’’ method for subtyping foodborne bacterial pathogens (Gerner-Smidt & Scheutz, 2006). The MLVA assay shows great promise as a molecular epidemiologic tool particularly when used to further discriminate PFGE clusters of isolates with commonly seen restriction patterns (Hyytia-Trees *et al*., 2006). The discrimination ability and epidemiological concordance of MLVA were compared with those of PFGE and phage typing (Shiu-Yun *et al*, 2007). MLVA provided greater discrimination among non-epidemiologically linked isolates than did PFGE or phage typing (Shiu-Yun, 2007).

**2.13 Unresolved problems in ESBL testing**

Since ESBLs have been found to be responsible for multiple antimicrobial resistances, several attempts have been made to facilitate their detection in the laboratories. Although some successes have been achieved there are still some unresolved issues such as the non-specificity of the substrates against the enzymes they produce, inoculum effects and the expressing of different enzyme types by the bacteria species (Queenan, 2004).

### 2.13.1 ESBL enzymes and substrates specificity

In routine laboratory sensitivity testing the ceftazidime has been used extensively as a marker in the detection of ESBL producers (Bhavnani *et al*, 2006). Since many ESBL types are resistant generally to the ceftazidime, they are being replaced by the cefepime in many hospitals so ceftazidime is no longer tested in the first line routine testing (Bhavnani *et al*., 2006). Also ESBLs vary in their substrate specificity, for that matter not all ESBL producers are universally resistant to the indicator cephalosporin. Many CTX-M ESBL types are evolving without the ability to hydrolyze ceftazidime (Bonnet, 2004) making it more difficult in using one indicator drug in routine work for ESBL detection as some ESBL types may not even express resistance to their own substrate (Bhavnani *et al*., 2006).

### 2.13.2 Inoculums effects

The use of standard inoculum is strongly advocated in various antimicrobial sensitivity testing protocols including the disc diffusion method where 0.5Macfarland standard is used for appropriate interpretation of the test results (Nordmann, 2009). The inoculums effect is a complicating factor when determining susceptibility of enterobacteria for ESBL production because a $10^2$ increase or decrease in inoculums size results in a 4-fold increase or decrease in MIC (Queenan *et al*., 2004). The standard inoculum size for ESBL detection by disc diffusion is

$10^7$ cfu/ml and for broth dilution is $10^5$ cfu/ml (Andrews, 2001). Even with careful testing of ESBL with the standard protocols false results occur when lower or higher inocula are used in testing β-lactam antimicrobials; this is because a slight change in the observed MIC is sufficient to report an isolate as susceptible or resistant (Queenan & Bush, 2007; Queenan *et al*., 2004). The clinical relevance of inoculum effect is evident in serious infections with high load of infecting organisms, so as the bacteria are being killed by the antibiotic, the antibiotic is being degraded at that same time (Queenan and Bush, 2007). The active enzymes released by the dead bacteria decreases the effectual concentration of antibiotic in the surrounding to render the antibiotic less effective (Queenan *et al*., 2004).



### 2.13.3 Type of bacterial and EBL expression

The type of bacterial species may influence the type of ESBL being produced, but there is no specificity for the ESBL type, for an ESBL producing strain may harbor other enzyme types such as the AmpC which may change its ESBL phenotype (Queenan *et al*., 2004). The AmpC enzyme is broad spectrum conferring resistance to oxyimino-cephalosporins such as ceftazidime and cefotaxime and monobactams, but are resistant to inhibition by clavulanate so are grouped in the Ambler group 1 β–lactamases (Nordmann, 2009). The enterobacteria that are typically known to harbor AmpC chromosomal genes that may induce hyper-production of the enzyme are *Morganella morganii, Citrobacter freundii, Enterobacter cloacae, Enterobacter aerogenes, Serratia marcescens and Citrobacter koserii* (Bush, 2001) Though these organisms test positive in the CLSI screening tests, high production of chromosomal AmpC ESBL masks the synergy between the clavulanate and the corresponding cephalosporin in the confirmatory test thereby causing false negative results (Carter *et al*., 2000; Fife, 2009.). For this reason (Bhavnani *et al*., 2006; Phillippon, 2002) proposed that cefepime should be included in the test regime required

for patient treatment, because cefepime is more resistant to hydrolysis by AmpC enzymes during ESBL detection in such bacterial strains. Some strains of *Escherichia coli, Klebseiellae, Salmonellae* and *Proteus species* produce AmpC enzymes at low levels and these enzymes may be responsible for treatment failures with β-lactam-inhibitor combinations in such strains (Phillippon, 2002).

It has also been found that *Klebsiella oxytoca* produces chromosomally mediated β-lactamase (K1). K1 is placed in group 2be of the Bush-Jacoby-Medeiros classification (Bush, 2001). K1 production in the *K. oxytoca* strains arise as a result of mutations to hydrolyze penicillins and third generation cephalosporins and aztreonam but ceftazidime and cefotaxime retain susceptibility against these strains (Gheorghiu *et al*., 1997). It is therefore prudent to test-screen *K. oxytoca* for ESBL production with either only cefotaxime or ceftazidime regardless of the test procedure (Gheorghiu *et al*., 1997).

### 2.14 Determination of *E. coli* phylogenetic group

*Escherichia coli* cause a variety of intestinal and extraintestinal diseases, but these bacteria are known also to be normal inhabitants of the intestines of humans and animals causing both extraintestinal and intestinal diseases such as diarrhoea, septiceamia, neonatal meningitis, urinary tract and wound infections (Kappeli *et al*., 2011). *Escherichia coli* are composed of an enormous population of bacteria that exhibit high genetic and phenotypic diversity (Higgins *et al*., 2007). Differences among *E. coli* strains can be determined only by molecular techniques (Higgins, *et al*., 2007). For example, a strain of the normal flora may gain pathogenicity capacity and cause disease. The different strains may then develop the capacity to survive in particular body site or niche and also acquires the ability to resist antimicrobial agents (Higgins *et al*., 2007). Phylogenetic analyses have shown that *E. coli* strains fall into four main phylogenetic groups,

(A, B1, B2 and D) (Picard *et al*., 1999). The most virulent extraintestinal *E. coli* strains belong to the ECOR phylogenetic group B2 and to a lesser extent, to group D, whereas the commensal strains belong to group A (Picard, et al., 1999). The degree of pathogenicity is related to virulence determinant genes coding for adhesins, toxins and iron acquisition systems (Higgins, *et al*., 2007), therefore the more virulent determinant genes a strain possesses the more pathogenic it is.



**2.15 Treatment, Prevention and Control of ESBL infection.**

Treatment and clinical outcomes data indicate that ESBLs are clinically significant and, when detected, indicate the need for the use of appropriate antibacterial agents (Rupp and Fey, 2003). Unfortunately, the laboratory detection of ESBLs can be complex and at times misleading (Rupp & Fey, 2003).

Antibacterial choice is often complicated by multi-resistance as many ESBL-producing organisms also contain in them plasmids mediating aminoglycoside resistance (Nordmann, *et al.,* 2009; Rupp & Fey, 2003). In addition, there is an increasing association between ESBL production and fluoroquinolone resistance (Rupp & Fey, 2003). Although in *in-vitro* tests ESBLs are inhibited by beta-lactamase inhibitors such as clavulanic acid, the activity of beta-lactam/beta-lactamase inhibitor combination agents is influenced by the bacterial inoculum, dose administration regimen and specific type of ESBL present (Pitout & Laupland, 2008; Rupp & Fey, 2003). The carbapenems are the most stable against these factors (Pitout & Laupland, 2008). Currently, carbapenems are regarded as the drugs of choice for treatment of infections caused by ESBL-producing organisms (Jacoby & Munoz-Price, 2005; Pitout & Laupland, 2008; Song *et al*., 2006). Meropenem (MIC = 0.03-0.12mg/mL) is the most active carbapenem with

MIC generally lower than those of imipenem (MIC= 0.06-0.5mg/mL)(Pitout & Laupland, 2008). Unfortunately, use of carbapenems has been associated with the emergence of carbapenem-resistant bacteria species such as *Stenotrophomonas sp*. or *Pseudomonas sp* (Nordmann *et al*., 2009) and *Klebsiella pneumoniae* (Queenan & Bush, 2007).

In spite of this, carbapenems are still the most effective and reliable β-lactam antibiotics available as they are highly resistant to the hydrolytic activity of all ESBL enzymes due to the stability of the trans-6 hydroxy ethyl group (Rodriguez-Villalobos *et al*., 2010). ESBL producing organisms generally vary in their susceptibility to the different oxyimino-β-lactams, and despite resistance to some, they may appear sensitive to others. Organisms producing TEM and SHV type ESBLs are often sensitive to cefepime, and to piperacillin-tazobactam, but it must be noted that MICs of these agents rise dramatically as the inoculum is increased from $10^5$ to $10^7$ organisms/ml (Jacoby & Munoz-Price, 2005; Samaha-Kfoury & Araj, 2003). Strains producing CTX-M-type and OXA-type ESBLs are resistant to cefepime on testing, despite in-vitro use of standard inoculums (Jacoby & Munoz-Price, 2005). AmpC-producing strains are typically resistant to oxyimino-β-lactams and to cephamycins, but are susceptible to carbapenems, however diminished porin expression can make such a strain carbapenem resistant too (Ahmed *et al*., 2009; Jacoby & Munoz-Price, 2005).

Successful control of ESBL infections and outbreaks hinge on monitoring and institution of appropriate infection control measures, as well as good antibiotic management interventions. These assertions are supported by claims (Canton *et al*., 2008; Pfaller & Segreti, 2006; Qin, *et al*., 2008; Ramazanzadeh, 2010a; Sarma & Ahmed, 2010) that by careful monitoring of local prevalence data and instituting control programmes ESBL infections and outbreaks in particular can be controlled.

## 2.16 Possible solutions to antimicrobial resistance problems

One way of combating the development of antibiotics resistance is to control the usage with antibiotics policies (Kader *et al*, 2006). In order to establish antibiotics policy one of the determinants is the surveillance of the antibiotics susceptibility patterns of the organisms prevalent in the individual hospitals and communities (Pfaller, 2006). The continual surveillance of the prevalent bacterial strains and their resistance patterns enables hospitals to update their recommended antibiotic policy. These in turn help improve management of bacterial infections. However, data on prevalence of bacterial resistance in most countries are fragmentary and under-analyzed (Pfaller *et al*., 2006).

The successes achieved with the introduction of new antibiotics onto the market lasted for a while, because resistance develops soon after each new antibiotic became available. The current crisis is that several organisms are involved and no new antimicrobial agents are available, or will soon be available to treat these resistant microorganisms (Chaudhary and Aggarwal 2004).

Infections with ESBL- producing bacteria are not only encountered in hospital settings as nosocomial outbreaks (Revathi *et al*., 1998), they have been found also from the community (Shah *et al*., 2004; Pitout *et al*., 2005). The first approach to ESBL problems is to avoid its spread by preventing these organisms (CDC, 2010). These can be achieved by admitting patients with ESBL to a single room or cohorting colonised or such infected patients. Efforts must be made to avoid placing known ESBL positive patients with surgical patients or patients with central lines (CDC, 2010). Other approaches include strict hand hygiene, including meticulous line management, careful cleaning of all communal equipment including stethoscopes, X-ray and ultrasound machines. These implements must be wiped over with soap and water or followed by

alcohol towelettes. Hospital waste and ‘soiled’ linen should be handled carefully and correctly (CDC, 2010).

# CHAPTER THREE

## 3.0 Materials and Methods

### 3.1 The Study Setting.

A total of 405 consecutive (non-repeat) isolates of *E. coli*, *Klebsiella pneumoniae* and *Klebsiella oxytoca* were obtained from various clinical specimens at the Bacteriology Laboratory in the Clinical Microbiology Department of the Komfo Anokye Teaching Hospital (KATH), and also at the Microbiology laboratory, School of Medical Sciences (SMS), Kwame Nkrumah University of Science and Technology, all in Kumasi, Ghana. The study sample collection and preliminary analyses started from February to April 2008, when Mr Lord Ayisi worked on 300 isolates for ESBL phenotype studies at KATH (Ayisi, 2009). Later 105 isolates were added from March to July 2009 for the present study. The Komfo Anokye Teaching Hospital is a 1,200 bed capacity hospital situated in the middle section of Ghana. The Hospital has many wards and units designated on Blocks as A, B, C and D. Block A has many sections but generally is the Obstetrics and Gynaecology block. This block also contains labour ward, post caesarian lie-in ward. Block B has many sections as well. Here there are the children surgical wards, also known as the paediatric surgery ward and renal and cardiac units for children. Block C harbours the orthopaedic, general surgery, and general medical wards. Block D is the general medical ward that harbour tuberculosis and HIV cases, Eye, Ear, Nose and Throat (EENT) cases, neurosurgical cases and burns units. There is also the accidents and the emergency unit, which receives such cases temporarily and then transfer them to the wards on Blocks A, B, C and C. Due to KATH's position as Teaching Hospital, it is serviced by many specialist laboratories namely Biochemistry, Haematology and Blood Banking, and Microbiology. The Microbiology services are divided and performed in different rooms called units where specialist tests are performed.

These units under Microbiology are parasitology, serology and bacteriology. Tests performed at the bacteriology unit include diagnostic tests involving culture and sensitivity of various clinical samples submitted to the laboratory from the wards and out-patient department. The laboratories also receive clinical samples from other hospitals in the city and elsewhere for analyses. This study was partly performed (isolation and sensitivity testing) at the bacteriology unit where samples were received and cultured as routine diagnostic service to patients, after which ESBL phenotypes were determined. The bacteriology unit receives and analyzes more than 5000 blood cultures, 3000 urine samples and 2000 wound samples, including many other body fluids, for analyses annually. Many and various kinds of bacteria are isolated from these samples.

KATH is the second largest hospital in Ghana, serving the middle belt mostly the Ashanti and Brong-Ahafo regions, but also receives referral cases from the three Northern Regions (namely the Northern, the Upper West and the Upper East Regions). It receives patients from parts of the Western and Eastern Regions as well.

Kumasi is the capital of Ashanti region of Ghana and has a population of approximately 2,000,000. This city is in the tropical rain forest zone of Ghana. The inhabitants are farmers, merchants and petty traders. There are also artisans, government and company workers. The city has good water supply and drainage systems, but there are environmental sanitation problems from poor solid and liquid waste disposal.

### 3.2 Collection of isolates.

#### 3.2.1 Inclusion criterion

Only clinical isolates of enterobacteria, isolated from patients found to be the cause of an infection and identified to the species level, were included in the study.

### 3.2.2 Exclusion criterion

Multiple isolates of one type of organism obtained from a patient were excluded from the study to avoid duplications.

### 3.2.3 Ethical issues

Sample collection begun only after ethical clearance was granted by the joint Committee on Human Research Publications and Ethics of the School of Medical Sciences and the Komfo Anokye Teaching Hospital in February, 2008. The specimens collected and isolates obtained from them were given numbers, and all identities on samples were removed, which made the patients anonymous, so apart from the investigator the isolates could not be traced to the patient. To accomplish this task the investigator kept a record book for data storage which was different and separate from that used by the laboratory.

## 3.4 Samples

Clinical isolates of *Klebsiella pneumoniae* and *E. coli* obtained during routine diagnosis of patients at the Komfo Anokye Teaching Hospital were used in the study. For each isolate the corresponding patient data obtained include, personal data (age, sex), in/out patient, tentative diagnosis, and type of specimen submitted for analysis. This study was conducted between the periods of February to April 2008 and again from March to July 2009 at the Komfo Anokye Teaching Hospital, Kumasi. This involved the collection and storage of *Escherichia coli* and *Klebsiella* sp isolated from routine testing of clinical samples at the Clinical Microbiology Laboratory. The isolates were obtained by convenience sampling.

## 3.5 Data collection

Activities leading to the generation of data for the study were divided into five stages:

Stage 1-isolation, identification and storage of enterobacteria from clinical samples.

Stage 2- determination of antimicrobial susceptibility patterns of the isolates.

Stage 3- detection of ESBL phenotype among the isolates obtained.

Stage 4- determination of the ESBL genotype amongst the ESBL-positive phenotypes.

Stage 5-determination of the *E. coli* genotypes and clones circulating in Kumasi.



**3.6 Isolation, identification and storage of enterobacteria from clinical specimens.**

This stage involved the isolation, identification and maintenance of the enterobacteria isolates and sensitivity to antimicrobial agents testing. These activities were performed in the Bacteriology Laboratory of the Komfo Anokye Teaching Hospital, Kumasi.

*Blood.* To isolate enterobacteria from blood, blood samples were taken from the patients and inoculated into brain heart infusion broth and were received at the laboratory and incubated overnight. After overnight incubation the blood culture broths were sub-cultured onto blood agar and MacConkey agar and incubated at 37$^{o}$C. After overnight incubation the plates were read and the growths that occurred were identified using Gram stain, biochemical and sugar fermentation tests. The blood bottles from which no growths occurred were re-incubated and inspected daily for any signs of growth such as turbidity, haemolysis or surface pellicle formation until the 7$^{th}$ day when the last sub-culture was performed.

*Urine.* Urine samples received in sterile universal bottles were inoculated onto cystine lactose electrolyte deficient (CLED) agar or MacConkey agar and incubated at 37$^{o}$C overnight. The plates were then read and the growths that occurred were identified using the Gram stain, biochemical and sugar fermentation tests.

*Sputum*. Sputum samples were inoculated onto MacConkey agar, Chocolate agar and Blood agar.

The plates were incubated overnight at 37$^{o}$C and inspected for growth. The growths that occurred were identified using the Gram stain, biochemical and sugar fermentation tests.

*Miscellaneous samples.* Wound swabs, pus and body fluid aspirates were inoculated onto MacConkey agar and Blood agar. The plates were incubated overnight at 37$^{o}$C and inspected for growth. The bacterial growths that occurred on the agar plates were identified using Gram stain, biochemical and sugar fermentation tests.



Bacterial identification was done on single colonies from MacConkey agar plates which were pre-screened for typical *E. coli* characteristics (i.e. Gram stain negative rods, indole positive, methyl red positive, citrate negative, and Voges-Proskauer test negative), while *Klebsiellae* characteristics include Gram negative rods, indole negative, methyl red positive, citrate test positive and Voges-Proskauer test positive. Lysine decarboxylase, urease production and gluscose fermentation separated *Klebsiella pneumoniae* from K. oxytoca. Isolates with typical *E. coli* phenotypes were then classified using the API 20E identification system (bioMerieux SA, Marcy l'Etoile, France). Upon obtaining the tentative identification of the isolates, they were then fully identified using the API 20E rapid test strips (bioMerieux SA, Marcy l'Etoile, France).

### 3.6.1 Collection and maintenance of the isolates

After identifying the organisms using the API 20E rapid test strips (bioMerieux SA, Marcy l'Etoile, France), isolates that were identified to be *E. coli* and *Klebsiella* sp. were selected for storage.

### 3.6.2 Storage of Bacterial isolates (in Ghana)

To store the isolates 2% trypticase soy broth was prepared and sterilized by autoclaving. Glycerol was then added to form 10% glycerol-trypticase soy broth. Five mls of this broth was dispensed aseptically into Eppendorf tubes. An isolated colony of the isolate from a nutrient agar plate was cleanly picked with an inoculating wire and inoculated into the broth. The broth was then vortexed to mix. This set-up was then kept in a deep freezer at temperatures between -18 to -20$^{o}$C until they were processed further. Further processing involving antimicrobial susceptibility testing and ESBL phenotyping done in Ghana were done using materials taken from these broths. These broths were also transported on dry ice to the University of Michigan, United States of America (USA) for further processing by United Postal Services (UPS).

### 3.6.3 Transfer and storage of isolates (in the USA)

Material Transfer Agreement (MTA) was prepared, signed and then transported to Ghana. The MTA was used to transport the isolates through United Postal Services (UPS) to the School of Public Health, University of Michigan. Upon receipt, the isolates were subcultured onto Luria-Bertani (LB) agar and then transferred into 2% glycerol in LB broth and then stored at -80$^{o}$C. Further testing of the isolates was done using bacteria taken from these broths.

### 3.6.4 Antimicrobial susceptibility testing of the isolates.

The antimicrobial sensitivity of each of the isolates was determined using the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI, 2007). The isolates were removed from storage and allowed to thaw and brought to room temperature. They were then sub-cultured onto nutrient agar and incubated at 37$^{o}$C overnight. Isolated colonies from the overnight culture were suspended in peptone broth to obtain inoculums density equivalent to 0.5 MacFarland standard which gives counts equivalent to $10^{7-8}$ colony forming units (cfu/ml). This

inoculum density gave semi-confluent growth upon overnight incubation. The suspension was used to inoculate Muller-Hinton agar using cotton tipped swabs. The swab was dipped into the test suspension; excess fluid was removed by pressing and rotating the swab against the inside of the test tube above the fluid. It was then applied evenly onto the surface of the Muller-Hinton agar (Oxoid, UK) and left to stand for about 15minutes for the surface moisture to dry. Antimicrobial discs were applied to the agar surface using a pair of sterile forceps. The plates were incubated at 37$^{o}$C for 18-24 hours. The following antibiotics on discs were applied to the agar surface: ampicillin 10µg, co-trimoxazole 25µg, gentamicin 10 µg, ciprofloxacin 5µg, cefuroxime 30µg, cefotaxime 30µg, ceftriaxone 30µg and cefpodoxime (10µg). For every batch of tests performed a susceptible reference strain of *E. coli* (NCTC 19418) was included as control to check the performance of the antibiotic discs. After overnight incubation the diameter of the zone of inhibition around each antibiotic disc was measured using a ruler and recorded. The zone sizes obtained were compared with the zone diameter chart supplied by the CLSI (M100-S17, 2007) and interpreted accordingly.

Table 6 Breakpoints adopted for antimicrobial susceptibility testing

| | Antibiotic | Concentration (µg) | Susceptible breakpoint (mm) |
|---|---|---|---|
| 1 | Cefotaxime | 30 | ≥30 |
| 2 | Ceftriazone | 30 | ≥26 |
| 3 | Cefuroxime | 30 | ≥26 |
| 4 | Cefpodoxime | 10 | ≥21 |
| 5 | Ampicillin | 10 | ≥22 |
| 6 | Co-amoxiclav | 20/10 | ≥20 |
| 7 | Imipenem | 10 | ≥29 |
| 8 | Gentamicin | 10 | ≥23 |

| | | | |
|---|---|---|---|
| 9 | Nalidixic acid | 30 | ≥25 |
| 10 | Ciprofloxacin | 35 | ≥35 |
| 11 | Co-trimoxazole | 1.25/23.75 | ≥16 |
| 12 | Chloamphenicol | 30 | ≥29 |

(CLSI, 2007)

### 3.7 ESBL Phenotype detection

ESBL production by the isolates was determined by inoculating Muller-Hinton agar with overnight culture with a turbidity of 0.5 Macfarland standard (Dade Microscan turbidity meter, CA, USA) using cefotaxime (30µg) and cefpodoxime (10µl). A zone of inhibition shown by an isolate to either cefotaxime or cefpodoxime to be less than 29 mm and 25 mm respectively was indicative of resistance of the isolate to the drug (HPA, 2004) and a possible ESBL producer. Thus an isolate obviously resistant to cefpodoxime (zone diameter ≤25mm) and/or to cefotaxime (zone diameter ≤29mm) was reported as positive in the screening test for ESBL production. Isolates that were positive for ESBL production in the screening tests to any one of the screening agents were selected for confirmation of ESBL production.

### 3.7.1 Confirmation of Microbial ESBL Production Using the Combination Disc Method.

Isolates that tested positive in the screening tests were selected for confirmation of ESBL production by using the combination disc methods.

#### 3.7.1.1: Double disc synergy

In the double synergy method for testing ESBL production, an inoculums density of 0.5 McFarland density of the test organism was seeded on a Muller-Hinton agar. A 3$^{rd}$ generation disc (cefpodoxime) was placed on the seeded agar plate and another disc of amoxicillin-clavulanic acid was placed about 15mm from a third generation cephalosporin disc (e.g.

cefotaxime 30µg, ceftriaxone30µg or cefpodoxime 10µg). After overnight incubation the plates were examined for zones of inhibition measured and compared with a chart. Isolates that showed frank resistance and also showed key-hole patterns formed by the zones of inhibition between the co-amoxiclave (10µg/10µg) and a cephalosporin disc give a suspicion of ESBL production. In this study keyhole formation between the 3rd generation cephalosporin antibiotic disc and the co-amoxiclav disc confirms ESBL production CLSI.



**3.7.1.1: Combination disc method**

This method was used to further confirm ESBL production among the isolates which did not show clear keyhole formation in the double disc synergy test. In this method cefpodoxime discs 10µg alone and a combined disc of cefpodoxime (10µg) and clavulanic acid (10µg) were placed on the inoculated Muller-Hinton agar plates. This was done after the plates were seeded with 0.5 McFarland density of the test organism. After overnight incubation at 37$^{o}$C the diameters of the zones of inhibition were measured (using a ruler and compass) and recorded. Isolates that produced a

zone of inhibition of ≥5mm with the combined disc as compared with the value produced by cephalosporin disc alone was regarded as an ESBL producer.

Quality control was performed on each batch of the antibiotic discs using the following positive ESBL control strains: *Escherichia coli* (TEM3) NCTC 13351, *Escherichia coli* (CTX-M15) NCTC13353 and *Klebsiella pneumoniae* (SHV-3) NCTC 165032. *Escherichia coli* NCTC 10418 was used as ESBL negative control organism.



### 3.8 Detection of ESBL genes by PCR

Isolates that were positive in the screening and confirmative tests were further tested for ESBL genes. The genes tested for were *Bla*$_{SHV}$, *Bla*$_{CTX-M}$ and *Bla*$_{TEM}$. Total DNA was extracted from the clinical isolates and used as templates in PCR amplification for the detection of the ESBL genes following the protocol described by Monstein (2007).

#### 3.8.1 DNA extraction.

The isolates were inoculated into Luria-Britani broth (LB Broth) and incubated overnight at 37$^{o}$C. Aseptically 1ml of this overnight broth culture was pippetted into a 1.5ml micro-tube and heated for 10minutes to break the cells to release the DNA. The tubes containing the extracted DNA were stored in a freezer for the PCR

#### 3.8.2 DNA amplification using PCR: Setting up multiplex (duplex PCR) for *Bla*$_{CTX-M}$ and *Bla*$_{TEM}$

Using illustra PuReTaq Ready-To-Go™ PCR beads (GE Health Biosciences, Pittsburg, PA, USA) a 25µl volume of PCR mixture was prepared. The bead (in an eppendorf tube) already contained 10x buffer, dNTPs, $MgCl_2$, and pure Taq enzyme. To the bead were added 20µl of

double distilled (or PCR grade) water, 1µl of each primer pair of *Bla*$_{CTX-M}$ and *Bla*$_{TEM}$ (diluted 1/10) and 1µl of DNA template, making a 25µl PCR reaction volume. The primers were supplied by Integrated DNA Technologies, 1710 Commercial Park, Coralville, Iowa 52241, USA). The PCR reaction mixture was placed in a thermocycler (Eppendorf$^{TM}$, USA) for PCR. The cycling conditions were as follows: denaturation at 94$^{o}$C for 10 min, 30 cycles of denaturation at 94 $^{o}$C for 1 min, followed by annealing at 60 $^{o}$C for 1 min, extension at 72 $^{o}$C for 1 min and a final extension step at 72$^{o}$C for 7 min. A previously identified *K. pneumoniae* ESBL-positive isolate was used as a positive control, and a negative control (nuclease-free water) was included in each run. The primer pairs sequences, sizes and melting temperatures are as provided in Table 7.

Table 7 *Bla*$_{CTX-M}$ and *Bla*$_{TEM}$ primers used to determine ESBL genotypes of the isolates

| Primer Name | Primer sequence | Size | $T_M$ $^{o}$C |
|---|---|---|---|
| Bla-CTX-M forward | 5'ATGCGTTATATTCGCCTGTG-3' | 454 | 45 |
| Bla-CTX-M reverse | 5'-ATGCGTTATATTCGCCTGTG-3' | 454 | 45 |
| Bla-TEM forward | 5'-ATGTGCAGYACCAGTAARGTKATGGC-3' | 593 | 54 |
| Bla-TEM reverse | 5'-TGGGTRAARTARGTSACCAGAAYCAGCG-3' | 593 | 58 |

$T_M$ =Melting Temperature. Source: (Monstein & LE, 2007) Key for standard Mixed base symbols: R-A,G; Y-C,T; M-A,C; K-G,T; S-C,G; W-A,T; H-A,C,T; B-C,G,T; V-A,C,G; D-A,G,T(Integrated DNA Technologies, Inc , USA)

### 3.8.3 Setting up single PCR for *Bla*$_{SHV}$

The *Bla*$_{SHV}$ was determined by running single PCR on the isolates. The total reaction volume used which was 25µl contained 19µl of double distilled water (PCR grade), 2µl each of the *Bla*$_{SHV}$ primers (forward and reverse) and 2µl of DNA template added to the beads, which were illustra PuReTaq Ready-To-Go™ PCR beads (GE Health Biosciences, Pittsburg, PA, USA) already contained in a 25µl volume PCR (Eppendorf) mixture tube. The primers were supplied by Integrated DNA Technologies (1710 Commercial Park, Coralville, Iowa 52241, USA). The PCR reaction mixture was placed in a thermocycler (Eppendorf$^{TM}$, USA) for PCR. The cycling conditions were as follows: denaturation at 94 $^{o}$C for 10 min, 30 cycles of denaturation at 94 $^{o}$C for 1 min, followed by annealing at 59 $^{o}$C for 1 min, extension at 72 $^{o}$C for 1 min and a final extension step at 72 $^{o}$C for 7 min. The primer pairs sequences, sizes and melting temperatures are as provided in Table 8.

Table 8. *Bla*$_{SHV}$ primer used to determine ESBL genotype of the isolates

| Primer Name | Primer sequence | Size | $T_M$ $^{o}$C |
|---|---|---|---|
| Bla-SHV forward | 5'ATGCGTTATATTCGCCTGTG-3' | 747 | 45 |
| Bla-SHV reverse | 5'-ATGCGTTATATTCGCCTGTG-3' | 747 | 45 |

$T_M$=Melting temperature Source (Monstein & LE, 2007)

#### 3.8.3.1 PCR amplification and visualization

After amplification 10µl of reaction mixture for each sample was taken and examined by gel electrophoresis.

### 3.8.3.2. Gel preparation for electrophoresis

1X TAE buffer was first prepared by adding 20ml of the 50X TAE buffer to 1000ml of deionized water to form a working stock buffer. This stock buffer was used for the preparation of 2% agarose gel for electrophoresis.

The 2% agarose gel for electrophoresis was prepared by adding 2 gm of agarose powder (Bio-Rad Laboratories Inc., USA) to 100 ml of 1X TAE buffer in a conical flask. This was placed in a microwave oven and heated, and inspected every minute, till the agar was completely dissolved to form a clear solution. This was allowed to cool to about 55$^{o}$C still in the molten state. To the molten gel 4µl of ethidium bromide was added and swirled gently to mix avoid formation of bubbles. The molten gel was then poured into a gel casting system with a comb inserted at one end. The gel was allowed to cool to set and the comb was removed.

### 9.8.3.3 Loading of amplicons

Using a micropipette, 6µl of PCR product was placed on a parafilm sheet and thoroughly mixed with 4µl of loading dye. This was pipetted 2-3times to mix and 10 µl of the mixture was loaded into the agarose wells. The first lane and the last lanes were loaded with 6µl of 1Kb Plus DNA Ladder. The second lane was loaded with a known organism positive for the $Bla_{gene}$ (SHV, CTX-M or TEM) under test. The third lane was loaded with deionized water free of DNA. The remaining lanes, except the last one, were loaded with samples under test and electrophoresis was run in 1XTAE buffer at 70v for 40 minutes.

### 3.8.3.4 Visualization of amplicons

After the electrophoresis the resulting gel was visualized under ultraviolet illumination using image analysis system (UVIsave Gel Documentation system, Tokyo). All images were photographed with a digital camera (Kodak, Japan) and saved on a computer. The difference in

amplicon sizes enabled the separation of the bands of the PCR product to determine the ESBL genes, where the sizes were 747bp for *Bla*$_{SHV}$, 593bp for *Bla*$_{CTX-M}$ and 454bp for *Bla*$_{TEM}$ as shown in the figure 9 below.

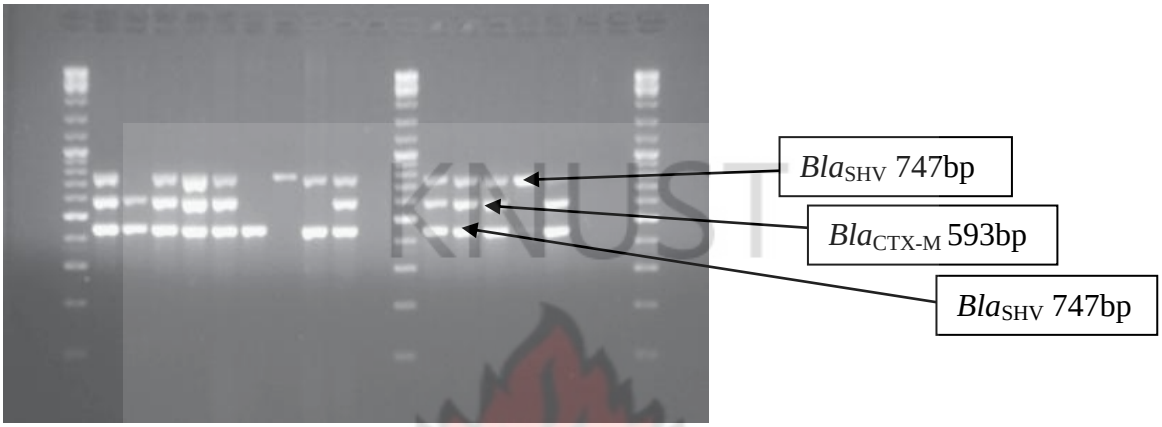


**Figure 9** Triplex PCR Gel pattern of *Bla*$_{SHV}$, *Bla*$_{CTX-M}$ and *Bla*$_{TEM}$. Adapted from Ehlers *et al.*, 2000.

### 3.9 Determination of the genotypes of ESBL-producing *E.coli*.

#### 3.9.1 DNA extraction

The *E. coli* isolates which were ESBL-producers were inoculated into Luria-Britani broth (LB Broth) and incubated overnight at 37$^{o}$C. 1in 10 dilution of this overnight culture was prepared. 1ml of the diluted broth culture was pippetted aseptically into a 1.5 ml micro-tube and heated for 10minutes on a heat-block to break the cells to release the DNA. The tubes containing the extracted DNA were stored in at -20 $^{o}$C for PCR.

#### 3.9.2 Multiplex PCR for the determination *E.coli* genotypes.

Multiplex (triplex) PCR was performed to determine the genotypes of the ESBL positive *E.coli*. using a 25µl mixture containing 2.5µl of 10X buffer, 2.5µl of deoxynucleoside triphosphate (dNTPs) at a concentration of 2mM, 2.0µl of $MgCl_2$, 0.5µl of chuA, 1.0µl each of yjaA and 1.5µl of TSPE4.C2 and 0.3µl of gold Tag(Quiagen, USA) and 2.0µl of crude extract of E.coli DNA. The PCR was performed with Eppendorf thermal cycler with MicroAm tubes under the following conditions: denaturation for 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and a final extension step of 7 min at 72°C. The sequences of the primer pairs were

ChuA.forward (5'-GACGAACCAACGGTCAGGAT-3')
ChuA reverse (5'-TGCCGCCAGTACCAAAGACA-3'),

YjaA forward (5'-TGAAGTGTCAGGAGACGCTG-3')
YjaA reverse (5'-ATGGAGAATGCGTTCCTCAAC-3'),

and
TspE4C2 forward (5'-GAGTAATGTCGGGGCATTCA-3')
TspE4C2 reverse (5'-GCGCCAACAAAGTATTACG-3').
Amplification of the above primers pairs generated 279bp, 211bp, and 152bp fragments, respectively for the *E. coli* genotype determination..

**3.9.2.1 PCR amplification and detection**

After amplification 10µl of reaction mixture for each sample was taken and run by electrophoresis.

**3.9.2.2 Gel preparation for electrophoresis**

1X TAE buffer was first prepared and used to prepare 1.5% agarose gel for electrophoresis.

The 1.5% agarose gel for electrophoresis was prepared by adding 1.5 gm of agarose powder (Bio-Rad Laboratories Inc., USA) to 100 ml of 1X TAE buffer in a conical flask and stained

with ethidium bromide. The gel was poured into a casting tank with a comb and allowed to cool to set.

#### 3.9.2.3 Loading of amplicons

Loading of PCR products was done as previously described for ESBL gene detection except for a few modifications as described below. Using a micropipette, 6µl of PCR product was placed on a parafilm sheet and thoroughly mixed with 4µl of loading dye added. This was pipette 2-3times to mix and 10 µl of the mixture was loaded into the agarose wells. The first lane and the last lanes were loaded with 6µl of 1Kb Plus DNA Ladder. The second lane was loaded with an organism previously known to be positive for the three genes (chuA, yjaA and TSPE4.C2). The third lane was loaded with deionized water free of DNA. The remaining lanes, except the last one, were loaded with samples under test and electrophoresis was run in 1XTAE buffer at 70v for 40minutes.

#### 3.9.2.4 Visualization of amplicons

After the electrophoresis the resulting gel was visualized under ultraviolet illumination using image analysis system (UVIsave Gel Documentation system, Tokyo). All images were photographed with a digital camera (Kodak, Japan) and saved on a computer. The difference in amplicon sizes enabled the separation of the bands of the PCR product to determine the *E.coli* genes with amplicon sizes 279bp, 211bp, and 152bp fragments for chuA, yjaA and TSPE4.C2 repectively.

### 3.10 *E.coli* genotyping

By the application of PCR using the *ChuA*, *yjaA* and TspE4.*C2* gene primers the genotypes of the ESBL positive *E. coli* were characterized. The phylogenetic group of the isolates was

determined using the dichotomous decision tree (Clermont *et al*., 2000). In this protocol strains possessing the *chuA* gene were assigned ECOR groups B2 and D, and all strains lacking it were assigned to ECOR groups A and B1. This allows separation of ECOR groups B2 and D from ECOR groups A and B1. Similarly strains positive for yjaA gene belong to B2 and separates them from D which lacks the yjaA gene. Finally strains possessing the clone TSPE4.C2 all belong to group B1 and thus separates group B1 from group A. Subjecting the triplex PCR results to the dichotomous decision tree (Figure 10) enabled the phylogenetic groups of the *E. coli* isolates to be determined.

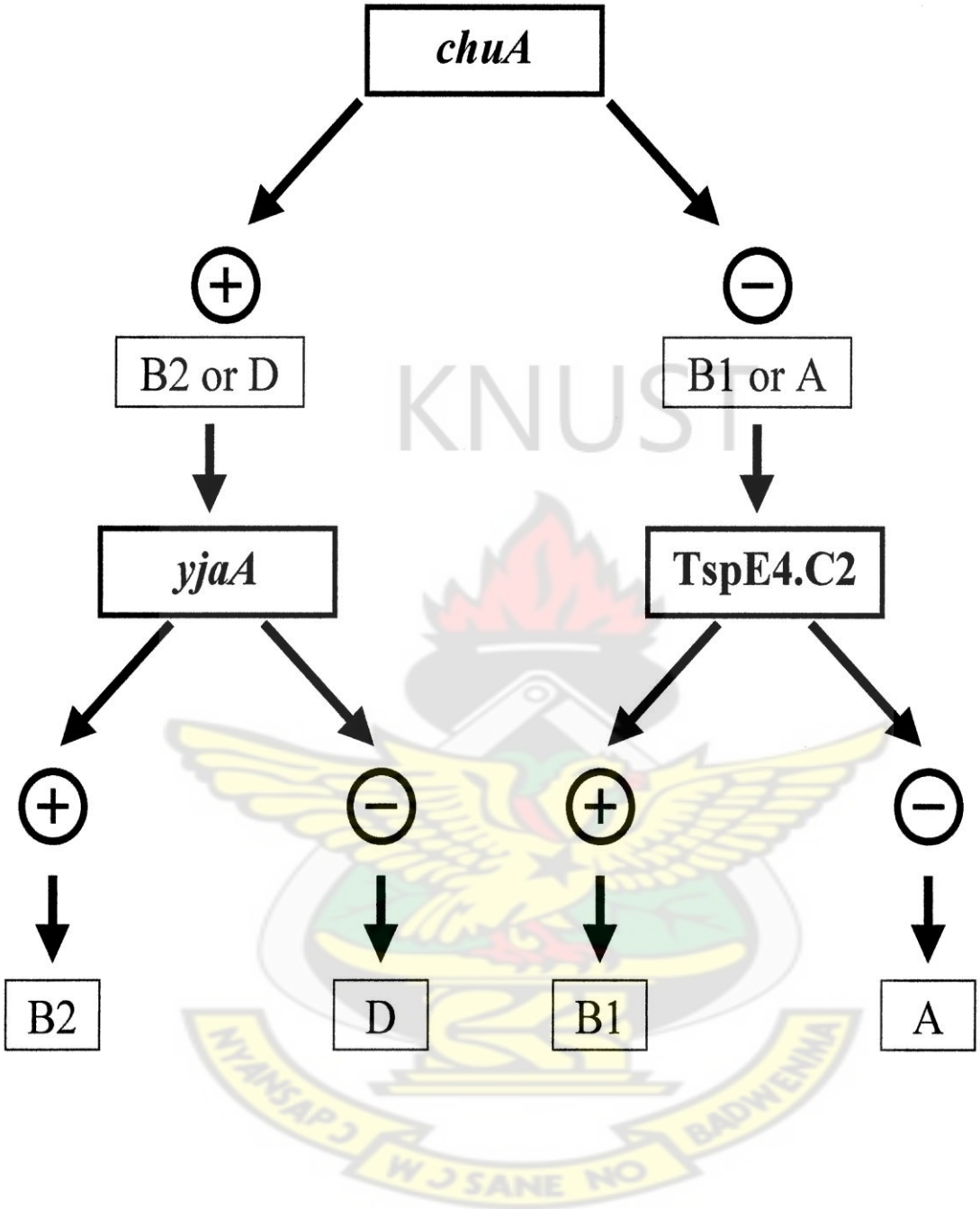


Figure 10 Dichotomous decision tree to determine the ECOR phylogenetic group of *E. coli* strains using results of PCR amplification of chuA, yjaA genes and DNA fragment TSPE4.C2 (Clermont *et al*., 2000).

### 3.11 Virulence determination of ESBL-producing *E. coli*

The multiplex PCR and decision algorithm of Tobias and Vitukuru (2011) was used to determine whether a subset of *E. coli* isolates belonged to a particular diarrhogenic pathotype, namely Enteroaggregasive *E. coli* (EAEC), Enterohaemorrhagic *E. coli* (EHEC), Enteropathogenic *E. coli* (EPEC) and Entero toxigenic *E. coli* (ETEC).

Pathotype identification was based on the presence/absence of 7 loci, corresponding to pCVD432, Eae, LT, STp, STh, VTcom, and Bfp.

The presence of 30 genes encoding for uropathogenic *E. coli* (UPEC) virulence factors or traits that increase general fitness in the urinary tract were assayed based on multiplex PCRs as described by Vigil *et al*, (2011) using the following genes:

1. Genes encoding adherence factors (*fimA*, *papA*),

2. Iron acquisition factors (*chuA*, *hma*, *iutA*, *iroN*, *fyuA*, *iha*, *ireA*), and

3. Toxigenic factors (*hlyA*, *cnf1*, *tosA*, *sat*, *pic*, *tsh*)

Fimbrial genes (*focH*, c1936, c2395, *ppdD*, *yadN*, *draC*, *yehA*, *aufA*, *ygiL*, *yfcV*, *sfaS*, *pixC*, *papG1*, *papG2*, and *papG3*) were identified as described by Spurbeck *et al* ( 2011) using three primer mixes were as follows:

mix 1 (*focH*, *c1936*, *c2395*, *ppdD*, and *yadN*),

mix 2 (*draC*, *yehA*, *aufA*, *ygiL*, *yfcV*, and *sfaS*), and

mix 3 (*pixC*, *papG1*, *papG2*, and *papG3*).

The following purified genomic DNAs were used as controls:

1. CFT073 (DNA positive for *focH, c1936, c2395, ppdD, yadN, yehA, aufA, ygiL, yfcV,* and *papG2*),

2.UTI89 (a cystitis isolate positive for *c1936, ppdD, yadN, yehA, aufA, ygiL, yfcV, sfaS,* and *papG3*),

3. 536 (a pyelonephritis isolate positive for *c2395, ppdD, yadN, yehA, aufA, yfcV, sfaS, pixC,* and *papG3*), and

4. BN406 (clone positive for *draC*) were included as positive-control templates.

5. A no-template control was also included.

This was done by Multiplex PCR using the Qiagen multiplex PCR kit. Thermocycler conditions were 95°C for 15 min, followed by 30 cycles of 30 s at 94°C, 1.5 min at 63°C, and 1.5 min at 72°C, and then final extension incubation for 10 min at 72°C. Characteristics of *E. coli* MLST house-keeping genes are listed in Table 8 and Primers sequences are listed in Table 9.

**Table 9 Characteristics of *E. coli* MLST house-keeping genes**

| Gene | Gene Product | Gene Size (bp) | Amplicon (bp) | Amplicon Range | Allele Sequence (bp) |
|---|---|---|---|---|---|
| *aspC* | aspartate aminotransferase | 1191 | 594 | 57..650 | 513 |
| *clpX* | ATP-dependent Clp protease | 1275 | 672 | 292..933 | 567 |
| *fadD* | cyl-CoA synthetase | 1686 | 580 | 768..1347 | 492 |
| *icdA* | isocitrate dehydrogenase | 1251 | 669 | 352..1020 | 567 |
| *lysP* | lysine-specific permease | 1470 | 628 | 36..663 | 477 |
| *mdh* | malate dehydrogenase | 939 | 650 | 130..779 | 549 |
| *uidA* | beta-D-glucuronidase | 1812 | 658 | 277..934 | 588 |

STEC Center (http://www.shigatox.net

**Table 10 Primers for multilocus sequence analysis of pathogenic *E. coli* (Seven loci for MLST)**

| Locus | Primer | Primer sequence | Position in gene | Size of amplicon |
|---|---|---|---|---|
| *aspC* | aspC-F4 | 5' - GTT TCG TGC CGA TGA ACG TC - 3' | 57..76 | 594 bp |
| | aspC-R7 | 5' - AAA CCC TGG TAA GCG AAG TC - 3' | 631..650 | |
| | | | | |
| *clpX* | clpX-F6 | 5' - CTG GCG GTC GCG GTA TAC AA - 3' | 262..281 | 672 bp |
| | clpX-R1 | 5' - GAC AAC CGG CAG ACG ACC AA - 3' | 914..933 | |
| | | | | |
| *fadD* | fadD-F6 | 5' - GCT GCC GCT GTA TCA CAT TT - 3' | 768..787 | 580 bp |
| | fadD-R3 | 5' - GCG CAG GAA TCC TTC TTC AT - 3' | 1328..1347 | |
| | | | | |
| *icdA* | icd-F2 | 5' - CTG CGC CAG GAA CTG GAT CT - 3' | 352..371 | 669 bp |
| | icd-R2 | 5' - ACC GTG GGT GGC TTC AAA CA - 3' | 1001..1020 | |
| | | | | |
| *lysP* | lysP-F1 | 5' - CTT ACG CCG TGA ATT AAA GG - 3' | 36..55 | 628 bp |
| | lysP-R8 | 5' - GGT TCC CTG GAA AGA GAA GC - 3' | 644..663 | |
| | | | | |
| *Mdh* | mdh-F3 | 5' - GTC GAT CTG AGC CAT ATC CCT AC - 3' | 130..152 | 650 bp |
| | mdh-R4 | 5' - TAC TGA CCG TCG CCT TCA AC - 3' | 760..779 | |
| | | | | |
| *uidA* | uidA-277F | 5' - CAT TAC GGC AAA GTG TGG GTC AAT - 3' | 277..300 | 658 bp |
| | uidA-934R | 5' - CCA TCA GCA CGT TAT CGA ATC CTT - 3' | 911..934 | |

STEC Center (http://www.shigatox.net)

### 3.12 Multilocus sequence typing (MLST)

Primers and sequencing methodology used for MLST are detailed at an online *E. coli* isolate, sequence, and metadata repository, known as the STEC Center (http://www.shigatox.net). Briefly, 2X coverage of the internal fragments of seven housekeeping genes (*aspC*, *clpX*, *fadD*, *icdA*, l*ysP*, *mdh* and *uidA*) were obtained by Sanger style sequencing for phylogenetic comparison at the University of Michigan DNA Sequencing Core. The SeqMan Pro™ program

(DNASTAR, Inc., Madison, WI) was used to edit and align raw sequencing reads before comparison using MEGA5 software (Tamura *et al*, 2011). A sequence type (ST) was identified using the analysis tool, EcMLST, on the STEC Center website. A neighbour-joining dendrogram (Kimura-2 parameter) representing the phylogenetic relationship between STs of the ECOR collection and the KATH ST88 clone was constructed from concatenated MLST loci using MEGA5 software.



**3.13 Data analysis**

Data generated in the various activities previously described are presented in summary tables and subjected to statistical analyses. Contingency (r x c) and summary tables were generated. The statistical analyses are performed using STATA (STATA$^{TM}$ 10, StataCorp., 4905 Lakeway Drive, College Station, Texas 77845 USA). These involved qualitative and quantitative analyses of information gathered to address the objectives of the study. The general characteristic of the study population was stratified by *Enterobacteria* species. Socio-demographic characteristic, sample type, place (in/out patient) and antibiotic resistance level of the isolates for study population is stratified by ESBL phenotype. The proportion of *Enterobacteria* isolates were also stratified by resistance to antibiotics, ESBL genotype and *E.coli* genotype. Furthermore the variables encountered in the analyses are categorical or continuous. Continuous data are presented as mean ± Sd and categorical data presented as proportions. Continuous data were compared to each other using unpaired t-test whilst categorical data were compared to each other using Chi-square analysis. Pearson chi-square test (p value <0.05) was used to assess the level of significance of an assumed hypothesis.

# CHAPTER FOUR



## 4.0 Results

### 4.1 General characteristics of the study population.

During the period February to April 2008 and March to July 2009 a total of 405 isolates of *Klebsiella* and *Escherichia coli* were obtained. The *Klebsiellae* isolates totaled 249 representing 61.5% of the isolates. Out of this total 234 (94.0%) were *Klebsiella pneumoniae* and 15(6.0%) were *K. oxytoca* isolates. The rest of the isolates 156 (38.0%) were *E. coli*.

### 4.2 Socio-demographic characteristics of the study population.

**4.2.1** *Age*

The mean (±SD) age of the patients was 30.8±27.2 years. The mean age of the patients infected with *E.coli* alone was 32.1±26.8 years whilst the mean age of patients infected with *Klebsiella pneumoniae* alone was 29.4±27.5 years and for *Klebsiella oxytoca* alone it was 47.9±18.8 years (Table 10). There was no significant difference in the mean age infected with *E.coli* and *Klebsiella pneumoniae* p=(0.3648), but *Klebsiella oxytoca* significantly affects older patients than *Klebsiella pneumoniae* (p=0.0224) and *E.coli* (P=0.0467).



### *4.2.2 Gender*

Gender distribution as shown in Table 10 indicated that the study population of 405 patients comprised 212 (52.3%) females and 193 (47.7%) were males. There were 156 patients infected with *E. coli.* Eighty three 83 (53.2%) of these were females while 73 (46.8) were males. There were 234 *Klebsiellae pneumoniae* isolates were obtained from 119 (50.9%) females. Out of the 15 patients infected with *K. oxytoca*, 10 (66.7%) were females. There were no significant sex differences between patients infected with *E. coli*, *K. pneumoniae*, and *K. oxytoca*).

### *4.2.3: Place (in/out-patient)*

Samples analyzed came from many places in the hospital with the majority 161(39.8%) coming from the Out-Patients Department (OPD). As shown in Table 10, there were no significant differences amongst isolate types and the places (MBU, PEU, OPD and others) they were isolated from.

### 4.2.4: Sample types

There was a significant difference between proportions blood isolates of *K. pneumoniae* and *K. oxytoca* (p=0.05). There were more *E. coli* isolates than *K. pneumoniae* isolates obtained from urine (p=0.01). So also the difference in proportions of isolates of *K. pneumoniae* from sputum as compared to proportions of *E. coli* was found to be significant. No significant differences

were observed amongst the isolates from wound when *E.coli* and *K. pneumoniae* were compared (p=0.4314), and also when *E.coli* and *K.oxytoca* were compared. So also there was no significant (p=0.1399) difference between *K. pneumoniae* and *K. oxytoca* when they were compared as shown in Table 10.

Table 10: General characteristic of the studied population stratified by enterobacteria isolate

| Variables | Total (n=405) | E coli (n=156) | K. pneumonia (n=234) | K. oxytoca (n=15) | P value |
|---|---|---|---|---|---|
| ***Socio-demographic data*** | | | | | |
| Age (yrs) | 30.8 ± 27.2 | 32.1 ± 26.8 | 29.4 ± 27.5 | 47.9 ± 18.8 | 0.3648 |
| Female | 212 (52.3%) | 83 (53.2%) | 119 (50.9%) | 10 (66.7%) | 0.6490 |
| ***Ward*** | | | | | |
| MBU | 47 (11.6%) | 15 (9.6%) | 32 (13.7%) | 0 (0.0%) | 0.2276 |
| PEU | 58 (14.3%) | 18 (11.5%) | 38 (16.2%) | 2 (13.3%) | 0.1947 |
| OPD | 161 (39.8%) | 65 (41.2%) | 85 (36.3%) | 11 (73.3%) | 0.2881 |
| Others | 139 (34.3%) | 58 (37.2%) | 79 (33.8%) | 2 (13.3%) | 0.4884 |
| ***Sample type*** | | | | | |
| Blood | 109 (26.9%) | 37 (23.7%) | 71 (30.3%) | 1 (6.7%) | 0.1521 |
| Urine | 155 (38.3%) | 75 (48.1%) | 72 (30.8%) | 8 (53.3%) | **0.0005** |
| Wound | 80 (19.8%) | 33 (21.2%) | 42 (17.9%) | 5 (33.3%) | 0.4314 |
| Sputum | 47 (11.6%) | 8 (5.1%) | 39 (16.2%) | 0 (0.0%) | **0.0006** |
| Others* | 14 (3.5%) | 3 (1.9%) | 10 (4.3%) | 1 (6.7%) | 0.2052 |

***Continuous data are presented as mean ± Sd and categorical data presented as proportion. Continuous data were compared to each other using unpaired t-test whilst categorical data compared to each other using Chi-square analysis. P value$^{a}$ = E coli vrs K. pneumoniae, P value$^{b}$ = E coli vrs K. oxytoca and P value$^{c}$ = K. pneumoniae vrs K. oxytoca, MBU = Mother-Baby Unit, PEU = Peadiatric-Emergency Unit and OPD = Out patient department, Others = Other wards, and Others* = other samples (ear, pus and aspirates).***

**4.3 Antibiotic susceptibility profiles of the isolates**.

A total of 405 enterobacteria were tested against 11 different antibiotics namely cefotaxime, ceftriaxone, cefuroxime, ampicillin, cefpodoxime, co-amoxiclav, imipenem, gentamicin, nalidixic acid, ciprofloxacin, co-trimoxazole and chloramphenicol as shown in Table 9. Higher proportions of isolates resistance to ampicillin 391 (91.7%) followed by cefpodoxime 299 (73.8%), cefuroxime 286 (70.6%), ceftriaxone 224 (55.3%) and then cefotaxime 195 (48.1%) amongst the β-lactam antibiotics. Amongst the non-β-lactams the proportions of isolates resistant to ciprofloxacilin was 77 (19.0%). The proportion of isolates resistant to other antibiotics tested ranged from 61% - 79%. The proportion of *E. coli* compared to *Klebsiella pneumoniae* resistant to β-lactam antibiotics was significant (p=0.01) except imipenem as shown in (Table 11). So also the proportions of resistant isolates between *K. oxytoca* and *E. coli* were significant. Again, differences in the resistance proportions between *K. oxytoca* and *E. coli* to cefpodoxime were significant for co-amoxiclav. There were no resistant isolates to imipenem, the only carbapenem antibiotic tested. *K. pneunoniae* isolates showed significantly higher proportions (P=0.0029) level of resistance more than *E. coli* to co-amoxiclav, so also there was significant (P=0.00285) resistance level between *E. coli* and *K. oxytoca*. Though the resistance level of *K. oxytoca* to co-amoxiclav was higher than *K. pneumoniae*, there was not significant (P=0.2214) as shown in Table 11.

Table 11: Proportion of *Enterobacteria* isolates stratified by resistance to antibiotics, ESBL genotype and *E. coli* genotype

| Variables | Total (n=405) | E. coli (n=156) | K. pneumoniae (n=234) | K. oxytoca (n=15) | P value[a] | P value[b] | P value[c] |
|---|---|---|---|---|---|---|---|
| ***β-lactam antibiotic resistance*** | | | | | | | |
| Cefotaxime | 195 (48.1%) | 61 (39.1%) | 125 (53.4%) | 9 (60.0%) | **0.0056** | 0.1159 | 0.6202 |
| Cefriaxone | 224 (55.3%) | 66 (42.3%) | 146 (62.4%) | 12 (80.0%) | < **0.0001** | **0.0051** | 0.1698 |
| Cefuroxime | 286 (70.6%) | 91 (58.3%) | 180 (76.9%) | 15 (100.0%) | < **0.0001** | **0.0015** | **0.0355** |
| Ampicillin | 391 (96.5%) | 143 (91.7%) | 233 (99.6%) | 15 (100.0%) | < **0.0001** | 0.2448 | 0.7997 |
| Cefpodoxime | 299 (73.8%) | 101 (64.7%) | 183 (78.2%) | 15 (100.0%) | **0.0034** | **0.0052** | **0.0426** |
| Co-amoxiclav | 90 (57.7%) | 272 (67.2%) | 169 (72.2%) | 13 (86.7%) | **0.0029** | **0.0285** | 0.2214 |
| Imipenem | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | 0 (0.0%) | NA | NA | NA |
| ***Non- β-lactam antibiotic resistance*** | | | | | | | |
| Gentamicin | 248 (61.2%) | 87 (55.8%) | 149 (63.7%) | 12 (80.0%) | 0.1176 | 0.0695 | 0.1998 |
| Nalidixic acid | 266 (65.7%) | 97 (62.2%) | 156 (66.7%) | 15 (100.0%) | 0.3631 | **0.0032** | **0.0070** |
| Ciprofloxacin | 77 (19.0%) | 29 (18.6%) | 43 (18.4%) | 7 (46.7%) | 0.9575 | **0.0108** | **0.0080** |
| Cotrimoxazole | 249 (61.5%) | 140 (89.7%) | 195 (83.3%) | 14 (93.3%) | 0.0748 | 0.6572 | 0.3066 |
| Chloramphenicol | 321 (79.3%) | 119 (76.3%) | 188 (80.3%) | 14 (93.3%) | 0.3372 | 0.1292 | 0.2126 |
| ***ESBL phenotype*** | | | | | | | |
| ESBL | 234 (57.8%) | 77 (49.4%) | 144 (61.5%) | 13 (86.7%) | **0.0174** | **0.0057** | 0.0506 |
| ***ESBL genotype*** | | | | | | | |
| SHV | 76 (32.5%) | 32 (41.6%) | 36 (25.0%) | 8 (61.5%) | **0.0110** | 0.1799 | **0.0050** |
| CTX-M | 221 (94.4%) | 72 (93.5%) | 136 (94.4%) | 13 (100.0%) | 0.7777 | 0.3444 | 0.3830 |
| TEM | 225 (96.2%) | 74 (96.1%) | 138 (95.8%) | 13 (100.0%) | 0.9228 | 0.4692 | 0.4530 |
| ***E. coli genotype*** | | | | | | | |
| B1 | | 57 (74.0%) | | | | | |
| B2 | | 20 (26.0%) | | | | | |

***Data were presented as proportions. P value[a] = E. coli vrs K. pneumoniae, P value[b] = E coli vrs K. oxytoca and P value[c] = K. pneumoniae vrs K. oxytoca, ESBL = Extended spectrum beta-lactamase.***

**4.4 *ESBL phenotypes***.

ESBL phenotypes were detected in high proportions in all the isolates tested. Generally ESBL production was detected at 234 (57.8%) for the isolates. ESBL production was found in 77(49.4%) *E. coli*, 144(61.5) *K. pneumoniae* and 13(86.7%) *K. oxytoca*. Similarly the proportion of *E. coli* producing ESBL were significantly lower compared to that of K. oxytoca (Table 11)

**4.5 ESBL Genotypes**:

The ESBL genotypes of *E. coli* and *Klebsiella pneumoniae* and *K. oxytoca* isolates were tested for $Bla_{SHV}$, $Bla_{CTX\text{-}M}$ and $Bla_{TEM}$ genes. Both $Bla_{CTX\text{-}M}$ and $Bla_{TEM}$ genotypes were more prevalent and were more commonly detected among isolates than the $Bla_{SHV}$. $Bla_{CTX\text{-}M}$ prevalence was 94.4% and for $Bla_{TEM}$ it was 96.2% amongst the isolates. $Bla_{SHV}$ genotypes prevalence among the isolates was 32.5%, which was much lower as compared to $Bla_{CTX\text{-}M}$ and $Bla_{TEM}$. Figures 10 and 11 show the separation of PCR amplicons on 2% agarose gel. Most of the isolates had the two genes of $Bla_{CTX\text{-}M}$ and $Bla_{TEM}$, except in a few cases where one band showed the presence of one gene.

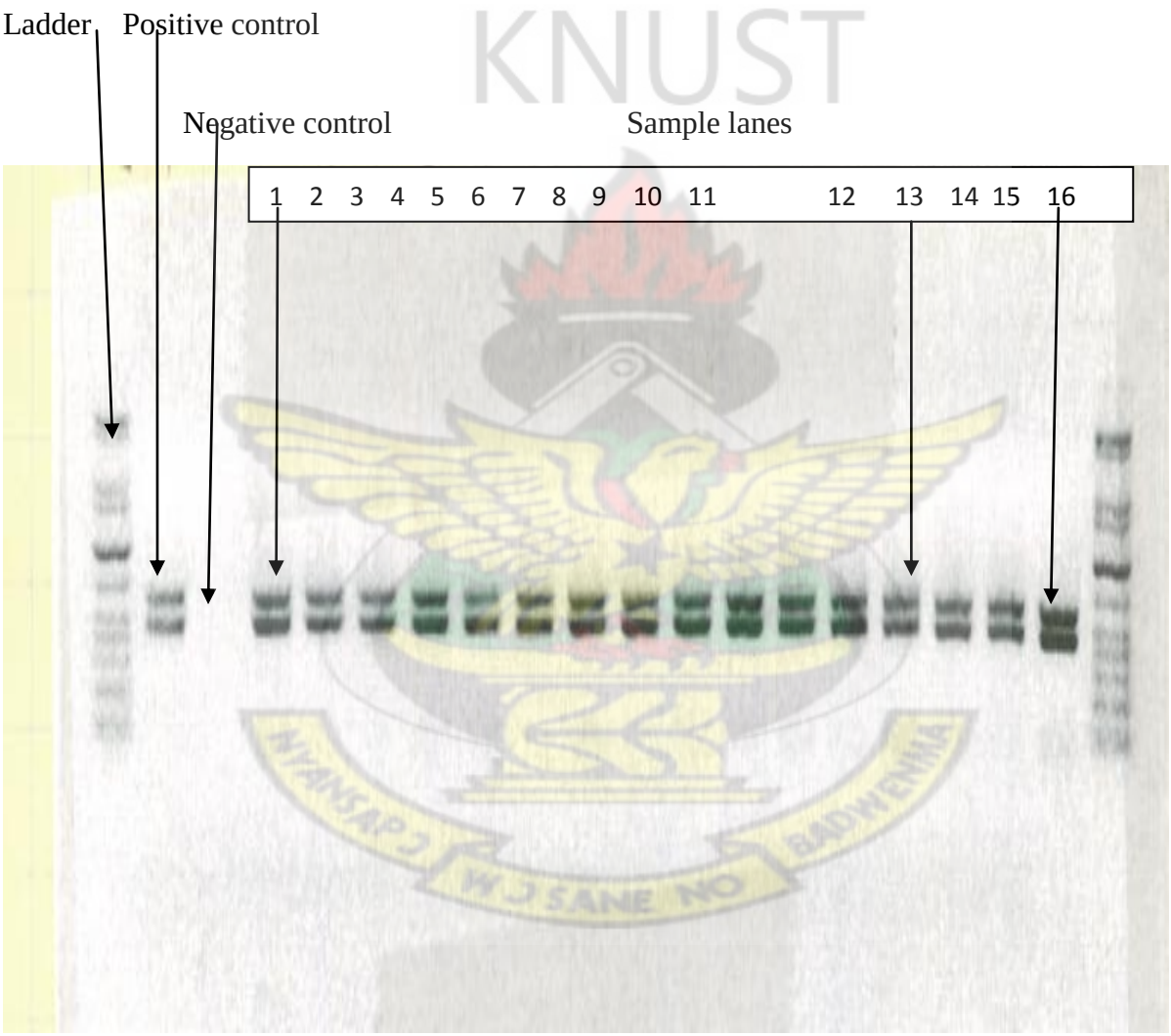


**Figure 11** Duplex PCR profiles for *Bla*$_{\text{CTX-M}}$ and Bla$_{\text{TEM}}$ of Entorobacteria isolates. Lane arrowed += positive control and lane marked -= negative control. All other lanes (bands) are positive for *Bla*$_{\text{CTX-M}}$ and *Bla*$_{\text{TEM.}}$

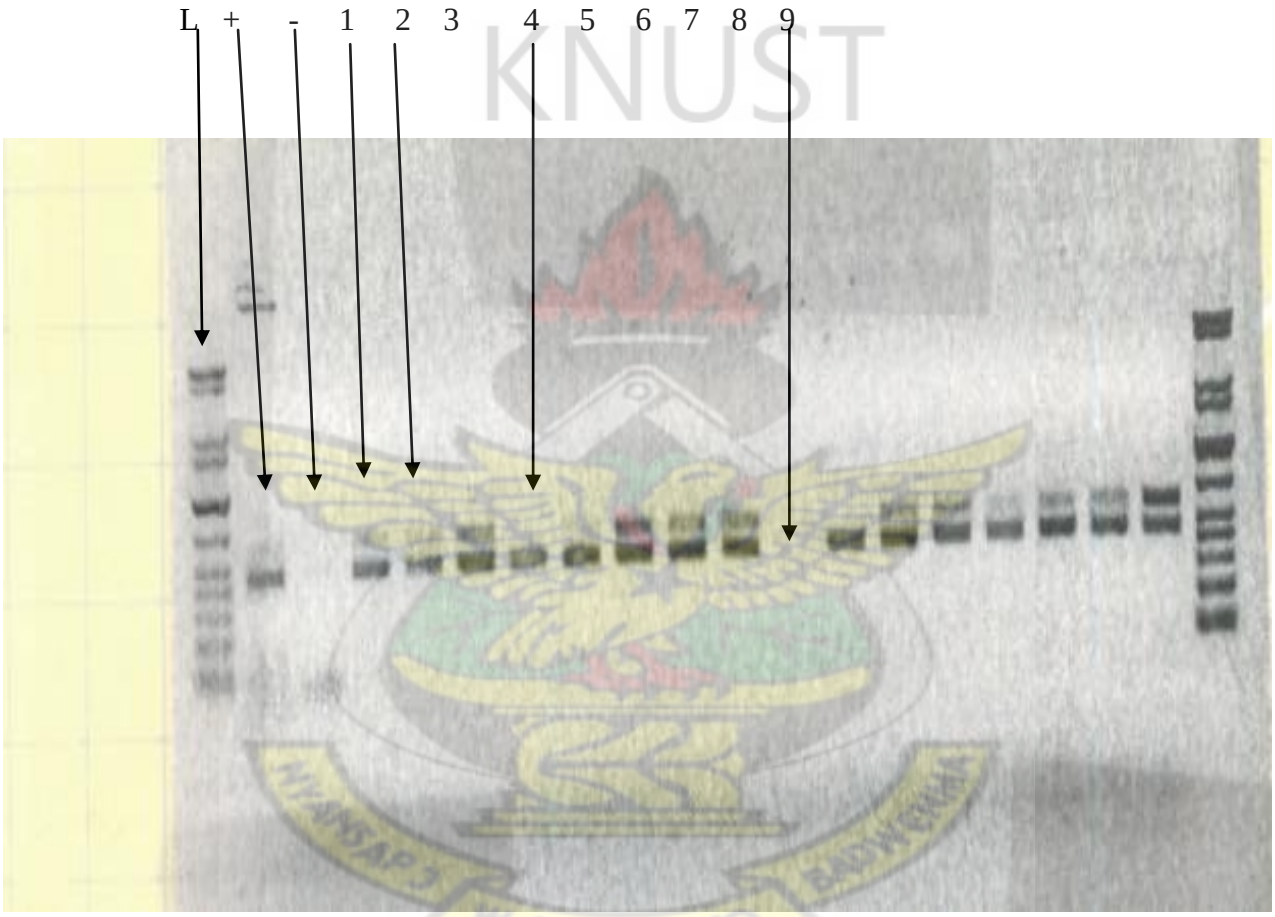


**Figure 12**. Duplex PCR gel pattern- Positive and negative lanes. Lane L = Ladder, + = positive control, - = negative control.

Lanes 1, 2, 3, 6, 7, 8 and other double bands are positive for *Bla*$_{\text{CTX-M}}$ and *Bla*$_{\text{TEM}}$ and lanes 4 and 5 and are positive for *Bla*$_{\text{TEM}}$ only and Lane 9 is negative for all three genes.

*Bla*$_{SHV}$ prevalence was significantly higher in *E. coli* 32 (41.6%) than in *K. pneumoniae* 36 (25.0%) where p=0.0050 (Table 12). *Bla*$_{SHV}$ prevalence again was significantly higher among *K. oxytoca* than among *K. pneumoniae* (p=0.0050). The prevalence of both *Bla*$_{CTX-M}$ and *Bla*$_{TEM}$ genes was high (above 90%) in all the isolates with the *Bla*$_{CTX-M}$ prevalence level in *E .coli* being 72 (93.5%), in *K. pneumoniae* 136 (94.4%) and *K. oxytoca* 13 (100.0%). The *Bla*$_{TEM}$ prevalence in *E. coli* was 74 (96.1%), in *K. pneumoniae* 138 (95.8%) and *K. oxytoca* 13 (100.0%).



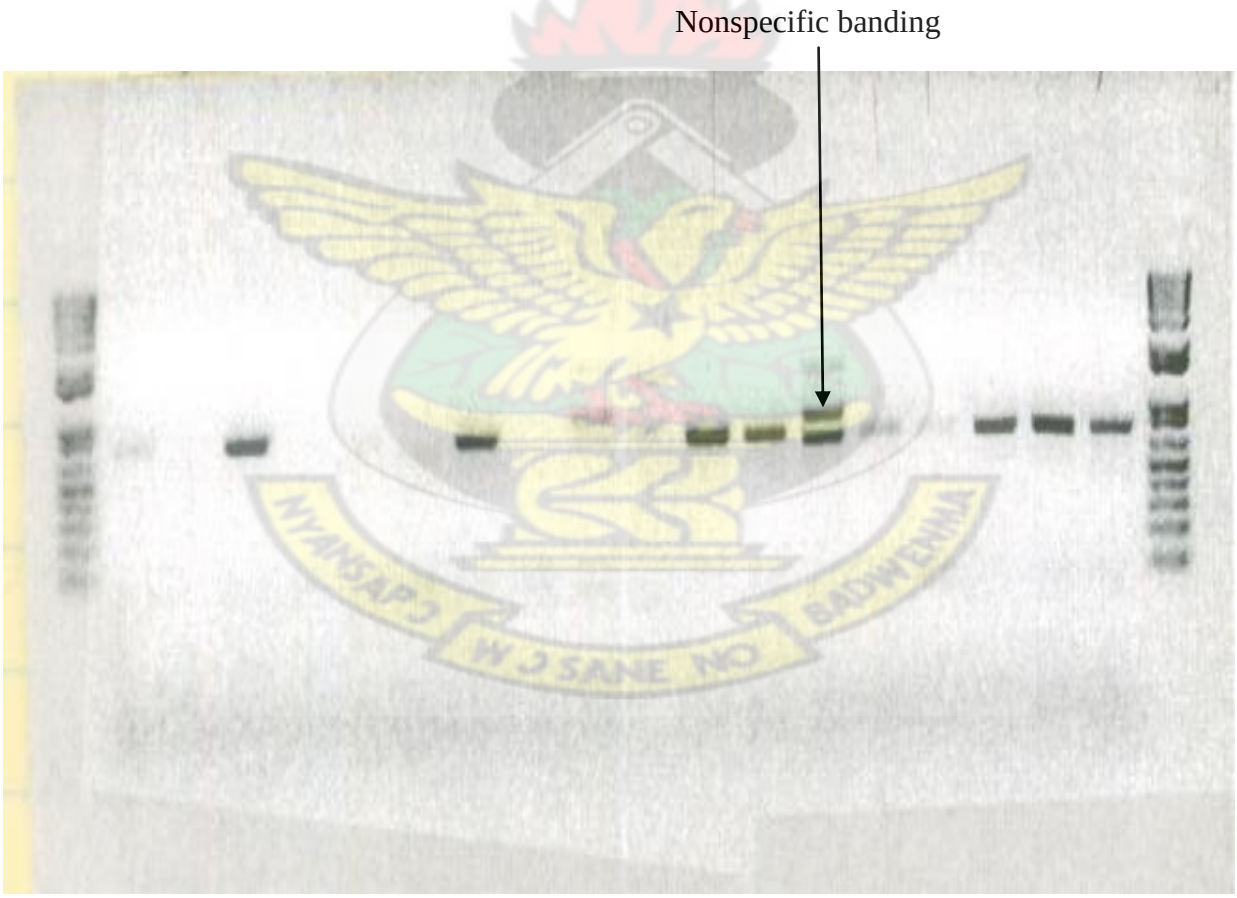


**Figure 13** Bands showing *Bla*$_{SHV}$ only. Areas with no bands are negative for *Bla*$_{SHV}$. Lane arrowed shows nonspecific band in addition to the *Bla*$_{SHV}$ band.

### 4.6 *E coli* genotypes

The *E. coli* isolates were found to belong to two main ECOR groups, namely B2 and B1. There were more isolates found to belong to group B1 with prevalence of 74.0% than those belonging to group B2 which had a prevalence of 26.0%). No isolates were found to belong to the groups A and D. The details of these results are shown in Table 12. The gel pattern from which the results were obtained are presented in figure 14.

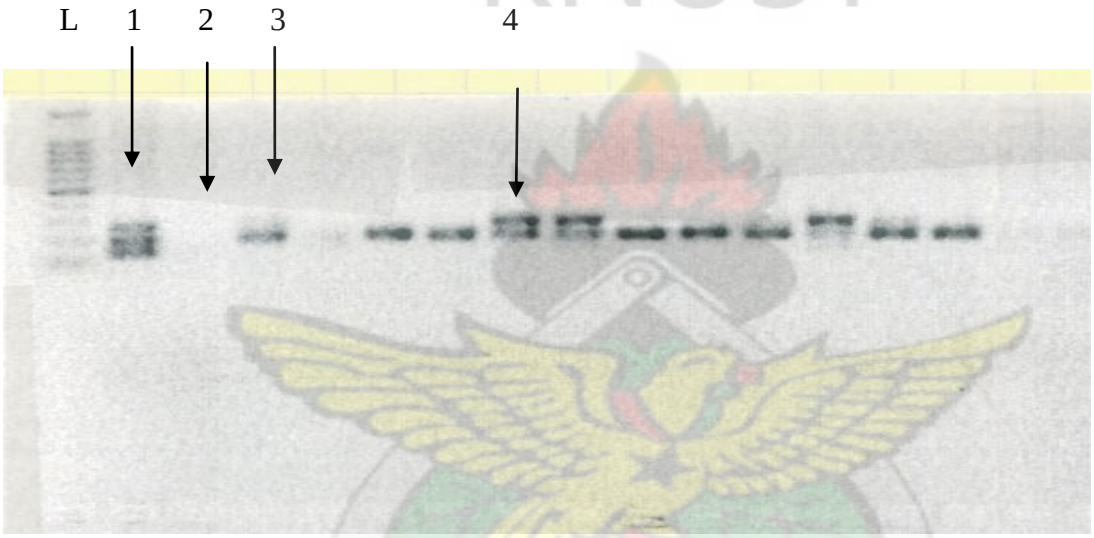


**Figure 14** Triplex PCR for *E. coli* genotype determination. Lane 1 = Positive control for chuA, yjaA and TSPE4.C2, lane 2=Negative control, lane 3=positive TspE4.C2 only (B1) and lane 4= positive for chuA and yjaA (B2).

### 4.7 Phylogenetic relatedness of ESBL-producing *E. coli* from KATH

All 29 ESBL isolates and ECOR-72 belong to the B1 *E. coli* phylogroup and sequence type (ST) 88 according to the STEC center database. All sequences generated in this study were identical to ECOR-72 (TW02070) and are publically available from the STEC Center website. Two ESBL ST88 isolates from KATH were screened for the presence of 37 virulence factor genes that are often carried by *E. coli* pathogens. Both isolates screened gave identical results and were positive

for c1936, *fimA*, *ppdD*, and *yehA* only. Phylogenetic relationships among isolates of the ECOR collection and the ESBL-producing ST88 clone from KATH (red arrow in figure 15) were constructed. Isolates were labeled according to ECOR numbers and STEC center identification numbers ('TW'). Colors were used to indicate *E. coli* phylogroup designations and only unique STs are shown (i.e. other ECOR representatives of the same ST are not shown). Inconsistencies between phylogroup designations between studies (Wirth *et al*, 2006) were labeled for reference with open triangles. A representative of the closest common ancestor to *E. coli* (Escherichia clade I) was used as an outgroup for the dendrogram (Walk *et al*, 2009) Figure 15.

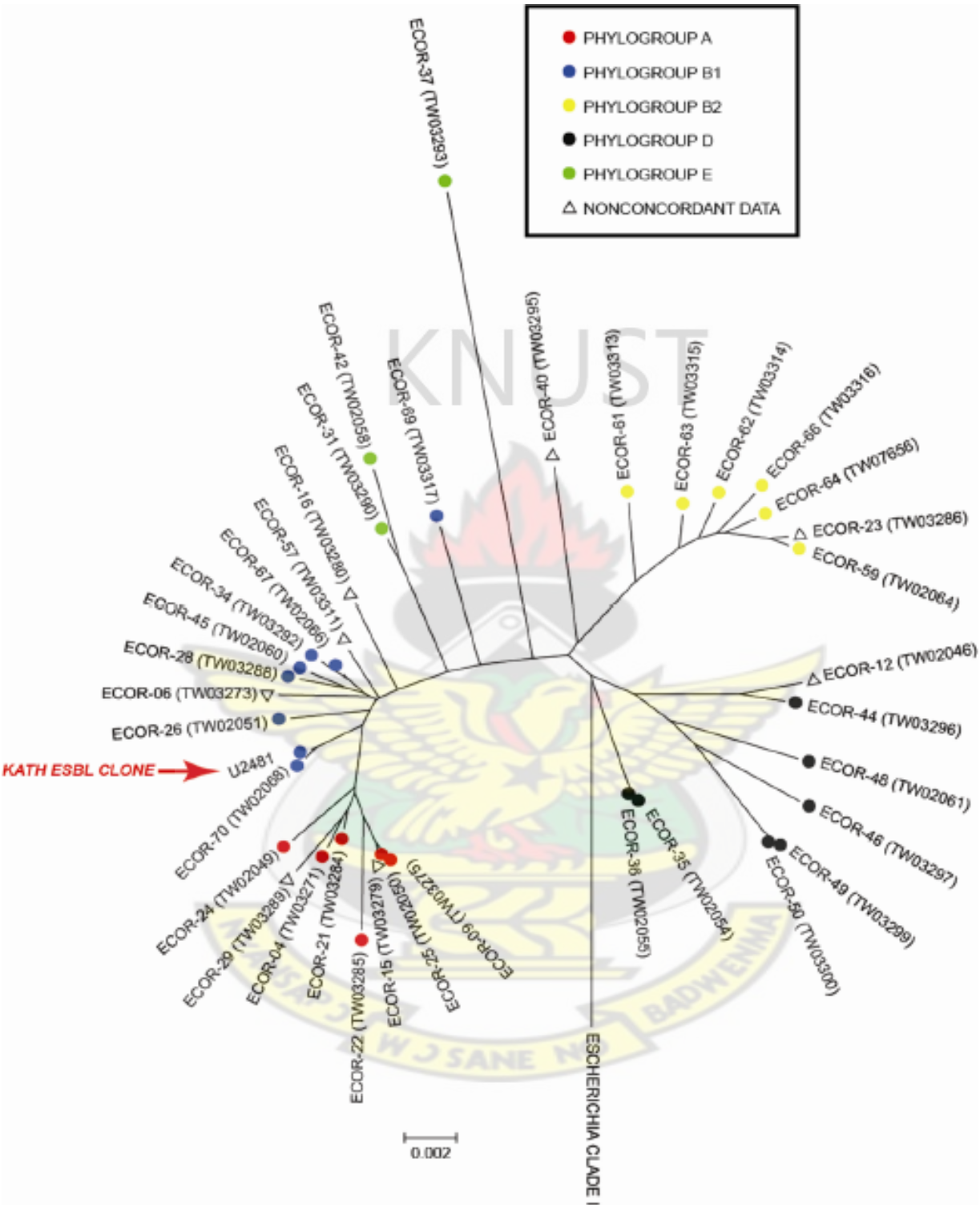


Figure 15 Phylogenetic relationships among isolates of the ECOR collection and the ESBL-producing ST88 clone from KATH (red arrow).

### 4.7 Socio-demographic characteristics of the study population in relation to ESBL isolates.

The socio-demographic characteristics of the patients from whom samples were taken and antibiotic resistance prevalence were stratified by ESBL phenotype (Table 13). The mean age of the patients infected with ESBL was 31.1±27.4 whilst the non-ESBL infected patients had mean age of 30.4±26.9. The proportion female patients infected with ESBL compared with that for male patients showed no significant difference.



Socio-demographic characteristic, sample type, ward and antibiotic resistance level of the study population stratified by ESBL phenotype.

Table 13: Socio-demographic characteristic, sample type, ward and antibiotic resistance level of the study population stratified by ESBL phenotype.

| Variable | ESBL (n=234) | Non-ESBL (n=171) | P value |
|---|---|---|---|
| ***Socio-demographic data*** | | | |
| Age (yrs) | 31.1 ± 27.4 | 30.4 ± 26.9 | 0.8023 |
| Female | 120 (51.3%) | 92 (53.8%) | 0.6161 |
| ***Ward*** | | | |
| MBU | 84 (35.9%) | 55 (32.2%) | 0.4344 |
| PEU | 29 (12.3%) | 18 (10.5%) | 0.5623 |
| OPD | 93 (39.7%) | 78 (45.6%) | 0.2374 |
| Others | 38 (16.2%) | 20 (11.7%) | 0.1973 |
| ***Sample type*** | | | |
| Blood | 64 (27.4%) | 45 (26.3%) | 0.8166 |
| Urine | 101 (43.2%) | 54 (31.6%) | **0.0178** |
| Wound | 41 (17.5%) | 39 (22.8%) | 0.1870 |
| Sputum | 24 (10.3%) | 23 (13.5%) | 0.3216 |
| Others* | 4 (1.7%) | 10 (5.8%) | **0.0243** |
| ***β-lactam antibiotic resistance*** | | | |
| Cefotaxime | 170 (72.6%) | 25 (14.6%) | **< 0.0001** |
| Ceftriaxone | 185 (79.1%) | 39 (22.8%) | **< 0.0001** |
| Cefuroxime | 212 (90.6%) | 74 (43.3%) | **< 0.0001** |
| Cefpodoxime | 214 (91.5%) | 85 (49.7%) | **< 0.0001** |
| Ampicillin | 231 (98.7%) | 160 (93.6%) | **0.0051** |
| Co-amoxiclav | 190 (81.2%) | 83 (48.5%) | **< 0.0001** |
| Imipenem | 0 (0.0%) | 0 (0.0%) | **NA** |
| ***Non-β-lactam antibiotic resistance*** | | | |
| Gentamicin | 176(75.2%) | 72 (42.1) | **< 0.0001** |
| Nalidixic acid | 183(78.2%) | 85 (49.7%) | **< 0.0001** |
| Ciprofloxacin | 69(29.5%) | 8 (4.7%) | **< 0.0001** |
| Co-trimoxazole | 218(93.2%) | 131 (76.6%) | **< 0.0001** |
| Chloramphenicol | 206(88.0%) | 116 (67.8%) | **< 0.0001** |

***Continuous data were presented as mean ± Sd and categorical data presented as proportion. Continuous data were compared to each other using unpaired t-test whilst categorical data compared to each other using Chi-square analysis. MBU = Mother-Baby Unit, PEU = Peadiatric-Emergency Unit and OPD = Out-patient department, Others = Other wards, and Others* = other samples (ear, pus and aspirates).***

### 4.8 ESBL distribution in wards.

ESBL prevalence levels among the wards were also determined. The Out-Patient Department (OPD) with prevalence 39.7% was the highest. The prevalence of ESBL phenotypes in all the wards ranged from 12.3% to 39.7%. The differences were all not significant when compared with prevalence of non-ESBL isolates (Table 13).



### 4.9 ESBL distribution in sample types

ESBL prevalence was determined for the sample types tested for the 234 ESBL phenotypes (Table 13). Urine isolates recorded a significant (p= 0.0178) prevalence of 101 (43.2%), compared to non-ESBL isolates (54 (31.6%). Another significant difference was detected for the non-ESBL isolates among the group of sample types labelled 'others' (pus, ear, aspirate) where the difference between the prevalent of non-ESBL isolates 10 (5.8%) and ESBL producers 4 (1.7%) was found to be significant (p=0.0243). Prevalence of isolates with ESBL from blood 64 (27.4%), wound 41 (17.5%) and sputum 24 (10.3%) were all not significant when the prevalence of non-ESBL compared respectively.

The distribution of B*la*$_{genes}$ was varied among the samples. There were 4 (1.7) isolates that were positive for ESBL phenotypes but none of the three genes (*Bla*$_{SHV}$, *Bla*$_{CTX-M}$ and *Bla*$_{TEM}$) was detected. Whilst 3(1.3%) were positive for *Bla*$_{TEM}$ only, 5 (2.1%) were positive for *Bla*$_{SHV}$ only. Some of the isolates had multiple genes. Both *Bla*$_{SHV}$ and *Bla*$_{TEM}$ were found in 1(0.4%) of the isolates and *Bla*$_{CTX-M}$ and *Bla*$_{TEM}$ were found in 151(64.5%) of the isolates, but there were no isolates with *Bla*$_{SHV}$ and *Bla*$_{CTX\_M}$. All the three genes (*Bla*$_{SHV}$, *Bla*$_{CTX-M}$ and *Bla*$_{TEM}$) were found in 70 (29.9%) of the isolates. The details are presented in Table 14.

Table 14 Prevalence of multiple ESBL genotypes and their distribution among the isolates.

| No(%) of isolates with multiple ESBL genes (n=234) | | | | | | | |
|---|---|---|---|---|---|---|---|
| None | Single gene | | | Two genes | | | Three genes |
| All genes absent | CTX-M only | SHV only | TEM only | SHV + CTX-M | SHV + TEM | CTX-M + TEM | SHV + CTX-M + TEM |
| 4 (1.7) | 0 | 5 (2.1) | 3 (1.3) | 0 | 1 (0.4) | 151 (64.5) | 70 (29.9) |



**4.10 ESBL production of the isolates in relation to their antimicrobial resistance**

Antimicrobial resistance for ESBL positive isolates and the non-ESBL phenotypes were compared to determine whether ESBL production influenced the antimicrobial resistance prevalence. With exception of imipenem, to which all isolates were sensitive, significantly higher proportions of ESBL-producers were resistant to the antibiotics that were tested. There were ESBL-producers found to be susceptible to the antibiotics including the β-lactam antibiotics tested. For the β-lactam antibiotics susceptible proportions registered by the ESBL producers were 64 (27.35%), 48 (20.51%), 40(17.02%), 22 (9.40%), 19 (8.12%) and 2 (0.08%), to cefotaxime, ceftriaxone, co-amoxiclav, cefuroxime, cefpodoxime and ampicillin respectively. The susceptible proportions of the ESBL producers for the non-β-lactam antibiotics were 165 (70.51%), 58 (24.79%), 50(21.37%), 27(11.54%) and 16(6.83%) to ciprofloxacin, gentamicin, nalidixic acid, chloramphenicol and cotrimoxazole respectively.

**Table 15**: Resistance to antibiotics as predictors of ESBL production

| Antibiotic | % ESBL Resistant | (%) ESBL producers susceptible | Odds ratio | P Value | Lower limit 95% | Upper limit 95% | Number (n) |
|---|---|---|---|---|---|---|---|
| **CTX** | **48.1** | **15.8** | **15.5125** | **0.0001** | **9.2525** | **25.8958** | **405** |
| **CTR** | **55.3** | **11.9** | **13.0449** | **0.0001** | **8.0889** | **21.0373** | **404** |
| **CXM** | **70.6** | **5.4** | **12.6315** | **0.0001** | **7.4116** | **21.5275** | **401** |
| **CPD** | **73.8** | **4.7** | **10.9981** | **0.0001** | **6.2934** | **19.2198** | **405** |
| **AMP** | **96.5** | **0.5** | **7.9906** | **0.0001** | **1.7366** | **36.3086** | **404** |
| GEN | 61.2 | 14.3 | 4.1724 | **0.0001** | 2.7291 | 6.3790 | 405 |
| NAL | 65.7 | 12.4 | 0.6415 | **0.0001** | 0.5550 | 0.7415 | 404 |
| CIP | 19.0 | 40.5 | 0.5139 | **0.0001** | 0.4041 | 0.6536 | 405 |
| COT | 61.5 | 3.9 | 4.1603 | **0.0001** | 2.2404 | 7.7254 | 405 |
| CHL | 79.3 | 6.7 | 0.6484 | **0.0001** | 0.5462 | 0.7697 | 404 |
| AUG | 57.7 | 10.1 | 0.5854 | **0.0001** | 0.5028 | 0.6816 | 399 |
| IMP | 0.0 | NA | NA | NA | NA | NA | 405 |

The results in Table 15 show ESBL phenotype against proportions of antibiotic resistance registered against the antibiotics tested. The levels of resistance as compared with those susceptible were highly significant (p=0.0000). The β-lactam antibiotics tested show large odds ratios indicating that there is close association between ESBL production and levels of antibiotics resistance by *E. coli* and *Klebsiella* isolates as compared to the low odds ratio for the non-β-lactam antimicrobials., where cefotaxime (OR=15.51), ceftriaxone (OR=13.05), cefuroxime (OR=12.63), cefpodoxime (OR=11.00) and ampicillin (OR=8.00) show close association with the corresponding level of antimicrobial resistance to the *E. coli* and *Klebsiellae*

isolates. Gentamicin and co-trimoxazole which were non-β-lactam antibiotics also show association with the odds ratio (OR) of 4.17 and 4.16; and confidence interval (CI) of 2.73 and 6.38 respectively.

# CHAPTER FIVE

## 5.0: Discussions

*Klebsiella pneumoniae, Klebsiella oxytoca* and *Escherichia coli* are bacteria commonly isolated from clinical sample types of patients, but these isolates have become multidrug resistant causing difficult-to-treat infections (Blomberg, *et al*., 2005). In this study these isolates were collected and characterized for their antimicrobial susceptibility and extended spectrum β-lactamase (ESBL) production. The isolates found to produce ESBL were further characterized for the ESBL genotype while the *E. coli* isolates were further analyzed for their phylogenetic diversity and relatedness.

This study has demonstrated the presence of ESBL and several studies (Jain, 2003; Knothe, 1983; Samaha-Kfoury, 2003) demonstrated that ESBL enzyme mediated antimicrobial resistance in *Klebsiellae* and *E. coli* isolates obtained from clinical samples at KATH. These isolates were obtained from patients with different and diverse socio-demographic characteristics. There were no significant age preferences for infection by *K. pneumoniae* and *E. coli*, but *K. oxytoca* tended to affect older patients as shown in Table 8. This observation may support another report, which suggests that *K. oxytoca* infections are more common in extremes of ages where it affects neonates and the aged (Nordmann *et al*., 2009). There were no significant differences in gender of patients infected with these isolates. This is probably because the organisms are opportunistic and do not have sex preferences. Both sexes are therefore infected when the chance is created (Nordmann *et al*., 2009). There are no significant differences in prevalence between the isolates from the community and the in-patients, where antimicrobial resistance levels are also very high.

With the exception of imipenem to which no resistant isolates were detected, high levels of antimicrobial resistance was detected among the isolates to all the other antimicrobials tested. The resistance levels ranged from 48.1% to cefotaxime to 91.7% to ampicillin. *Klebsiella* resistance to ampicillin was 100% where this proportion resistance is attributable to the intrinsic characteristics of the *Klebsiella (* Baron, 1996 *)*. It has been reported that *K. oxytoca* isolates are generally more resistant to antibiotics than *K. pneumoniae* (Feglo *et al*, 2010). In the present study it was found that *K. oxytoca* isolates were more resistant to the antibiotics tested than the other isolates. Also the *Klebsiellae* (*K. oxytoca* and *K. pneumoniae*) were more resistant than the *E. coli* to the antimicrobials tested. Apart from ciprofloxacin to which resistant proportion was about 19% all the isolates registered high resistance to all the isolates tested as has been previously reported in the Northern part of Ghana where proportion of enteric pathogens had between 81-90% resistance to the β-lactam antibiotics and chloramphenicol (Djie-Maletz *et al.*, 2008)). In the present study the proportion of resistance to the β-lactam antibiotics are considered very high among the cephalosporins, because cefotaxime, the best performing cephalosporin antibiotic registered resistance proportion of 48.1%. The other cephalosporin antibiotics recorded higher resistance proportions, so also resistance levels were high (>60%) against gentamicin, cotrimoxazole, nalidixic acid and chloramphenicol. These proportions of resistance are widespread among the isolates from the various sample types. These high proportions of antibiotics have been reported and attributed to indiscriminate sale and misuse of antibiotics in Ghana over the years by patients (Newman *et al.*, 2006) and also by farmers who employ the antibiotic in animal production (Tajick, 2006). Further compounding the problem is the manufacture and sale of substandard antibiotics in many developing countries, which was recognized long ago (Shakoor *et al*., 1977) but the practice still continues. The incorrect

concentrations of antibiotics make it difficult to prescribe the correct dosage of medicines by health professionals. In addition most of the counterfeit drugs are sold to patients outside the official health systems. In hospitals most of such drugs may not be identified as substandard, so the patient may not be given the correct dosage (Shakoor *et al*., 1977). Low dosages lead to treatment failures and hence repeated treatments. Such practices promote drug resistance among bacteria because the low dosage of antibiotics do not kill most bacteria but rather get the bacteria sensitized causing them to become resistant (Blomberg, 2008). The most abused drugs are the capsules and the tablets, because they are easy to administer so they are more abused than the injectables (Newman *et al*, 2006). The antimicrobial drugs such as the co-trimoxazole, ampicillin, and chloramphenicol can be administered easily. They are cheap and are easily accessible over the counter.

Another important but often overlooked cause of antimicrobial resistance is the discharge of antibiotic residues from industrial origin into the environment and especially water bodies potentially altering the microbial ecosystem (Baquero *et al*., 2008). Antibiotic-resistant organisms from human and animal sources enter into water environments and spread their genes to the indigenous microbes in the water environment. The bacteria in the water also contain resistance genes creating complex bacterial genomes that carry multiple resistance genes (Higgins *et al*., 2007). Humans and animals get infected with these strains resulting in difficult-to-treat infections. Failure of health authorities to control and enforce bye-laws on drug policy in Ghana (Djie-Maletz *et al.,* 2008) has resulted in the high proportions of microbes becoming resistant and the attendant treatment failures. Other important factors include poor prescribing practices (Newman *et al*., 2006) where physicians prescribe antibiotics without asking for microbiological tests, which are compounded by lack of competent personnel to perform

antimicrobial sensitivity testing. Even the antimicrobial sensitivity testing facilities are not available in many health facilities, except hospitals in large cities. Meanwhile these antibiotics are the major choice for the treatment of severe infections at KATH (Adu-Sarkodie, 2010). High proportions of resistance detected against second and third generation cephalosporins indicate that it may not be long when these antibiotics will no longer be effective in the treatment of infections caused by these enterobacteria. This is because of the resistance found among enterobacteria coupled with the production of ESBLs (Adu-Sarkodie, 2010) a *sine qua non* for the development of multidrug resistance. This present study sets to link these high antimicrobial resistant proportions to ESBL enzymes production and ESBL genotypes of the isolates obtained there.

Prevalence of ESBL production was detected among the isolates at a level of 57.8% which was found to be significant (p=0.0175) in this present study. Patients with infections due to ESBL enterobacteria tend to have less satisfactory treatment outcomes than those infected with pathogens that do not produce ESBLs (Paterson & Bonomo, 2005). This means that as more than half of the enterobacteria isolates tested produce ESBL treatment outcome for these patients was likely to be less satisfactory (Perez *et al*., 2007), and much less satisfactory with β-lactams treatment of ESBL infected patients (Perez, *et al*., 2007). A study adopting Clinical Laboratory Standards Institute (CLSI) breakpoint for cephalosporin treatment revealed that treatment failure may be as high as 80% and mortality more than 35% (Perez, *et al*., 2007). For example, *in-vitro* susceptible-cefepime treatment of ESBL producing *E. coli* and *Klebsiella pneumoniae* was associated with a failure rate of 23-83% (Projan, 2008). Aminoglycosides, sulphonamides and quinolones have been suggested as drugs which can be used when in-vitro results show that they are susceptible (Hawkey, 2008) but at KATH, there are high proportions of antimicrobial

susceptibility of *E. coli* and *Klebiellae* isolates to these non-β-lactam antibiotics (cotrimoxazole 61.5%, gentamicin 61.2%). Satisfactory clinical response can be achieved using quinolones (Projan, 2008). Unfortunately epidemiological studies reveal a strong link between fluoroquinolone resistance and ESBL production among enterobacteriaceae (Cremet *et al*., 2011). One of the determinants in achieving good treatment outcome of infected patient is choosing the appropriate empiric therapy within the first 24-48 hours of presentation (Perez, *et al*., 2007), but the issue of ESBLs have complicated the problem, because there are no available data to guide the clinician, because ESBL testing is not performed at KATH. There are also no reports on the treatment outcomes of ESBL infections at KATH. Results in this study found ESBL producers that were susceptible to antibiotics even to the β-lactam antibiotics, so laboratories may report ESBL producers as susceptible contrary to the CLSI recommendations (CLSI, 2009). Not testing enterobacteria isolates for ESBLs may have significant adverse impact on patient, especially those treated with cephalosporins (Perez *et al*., 2007). Since ESBL-producing enterobacteria are frequently susceptible *in vitro* to β-lactam/β-lactamases inhibitor combinations it is logical to assume that these combinations would be clinically effective (Perez, *et al*., 2007), but it is not always the case. The laboratory and the clinicians must be aware that chromosomal AmpC enzymes can be present in an isolate and the AmpC is normally resistant to inactivation by such drug combinations (Peterson, 2008). The dwindling spectrum from which to choose drugs for treatment compelled the CLSI to recommend reporting ESBL-producing strains of *Escherichia coli, Klebsiella* sp and *Proteus* sp as resistant to all penicillin, cephalosporin and monobactam antimicrobials (CLSI, 2009). These isolates should however be reported as susceptible to beta-lactam-beta-lactamase inhibitor combinations, when they test susceptible (CLSI, 2009). Current literature supports the action of piperacillin-tazobactam

against susceptible strains of ESBL-producing bacteria (Peterson, 2008) . This suggestion is based on the structure-activity relationship between inhibitors and the ESBLs, as well as on recent clinical outcome studies involving AmpC producing isolates (Peterson, 2008). Such AmpC enzymes are prevalent in *Enterobacter* sp, *Proteus mirabilis, Citrobacter freundii* and *Serratia marscescence* limiting β-lactam/β-lactam inhibitor combination therapy to only piperacillin-tazobactam (Jacoby & Munoz-Price, 2005). Therapy of infections caused by extended-spectrum beta-lactamase (ESBL)-producing bacteria with β-lactam antimicrobials only (without the inhibitor β-lactam inhibitor) to which they are susceptible results in treatment failure, higher cost and increased mortality (Peterson, 2008).

In this present study ESBL prevalence was detected in the following decreasing order: *K. oxytoca* (86.7%) followed by *K. pneumoniae* (61.5%) and then *E. coli* (49.4%) indicating differences in prevalence among strains (Table 9). The high prevalence in *K. oxytoca* is probably a reason why ESBLs were first detected among the *Klebsiellae* (Knothe, 1983). More importantly in this present study were 1.7% of the isolates that tested positive in the phenotype but had none of the SHV, CTX-M and the TEM enzymes. This observation confirms the assertion that an isolate could possess other enzyme types such as the AmpC. Meanwhile all the three $Bla_{\text{GENES}}$ were detected in the isolates confirming reports that these enzyme types are commonly found among the *E. coli* and *Klebsiella* (Jain *et al*, 2003). In this study all three genotypes $Bla_{\text{CTX-M}}$ and $Bla_{\text{TEM}}$ than $Bla_{\text{SHV}}$ were detected. TEM and SHV enzymes have a higher affinity for cefotaxime are said to be related to the broad spectrum β-lactamases, whilst the CTX-M group enzymes, which have a higher affinity for cefotaxime, had evolved from β-lactamases of *Kluyvera ascorbata* (Novais *et al*., 2008). It is unclear why $Bla_{\text{CTX-M}}$ type enzymes have spread much more in the community in Kumasi and the hospital than the $Bla_{\text{SHV}}$ enzyme.

The spread of an ESBL variant can be facilitated by a referral system, where the presence of a single ESBL variant in a different centre may be imported by a patient on referral to another centre (Nordmann *et al*., 2009. This situation can hold for KATH because it is a tertiary referral centre receiving patients from many parts of the country. ESBL gene-carrying plasmid within a hospital institution or country can easily be disseminated (Nordmann *et al*., 2009). For example, a single clone of $Bla_{\text{CTX-M}}$ type producing *Salmonella typhimurium* has been identified in Russia, Hungary and Greece (Tassios *et al*., 1999). The difference in $Bla_{\text{SHV}}$ prevalence between the *Klebsiella pneumoniae* and the *E. coli* were significant (p=0.0110), and also between *K. oxytoca* and *E. coli* was significant (p=0.0050) indicating that the $Bla_{\text{SHV}}$ are more prevalent among the *Klebsiellae* (Mbelle, 2010).

There are many ways by which an ESBL type can spread. An ESBL producer strain may appear in a hospital due to '*de novo'* selection, or a one that has been previously identified in another institution (convergent evolution). It may be imported by a patient from another centre, another city or even another country (Rodriguez *et al*., 2010). Once selected the ESBL variant may spread either by clonal dissemination of the producer strain or by horizontal transmission by plasmid carrying the gene (Soge *et al*., 2006) or by transposons or integrons (Rodriguez-Villalobos *et al.*, 2010) which facilitate such gene transfers. A single plasmid may carry resistance genes for multiple antibiotics such as was found in a study in Nigeria where 17 *Klebsiella pneumoniae* isolates were shown to have $Bla_{\text{CTX-M}}$ genes that were associated with a large plasmids Soge *et al*., 2006). The plasmid also carried tetracycline-resistance gene, and various aminoglycoside-resistance genes (Soge *et al*., 2006).

In this present study most of ESBL-positive isolates were isolated from urinary tract infections (43.2%) and blood stream infections (27.4%). Therefore the high rate of ESBL positive isolates obtained from these infections harbor these enzymes which are responsible for the antimicrobial resistance among these organisms (Ramazanzadeh, 2010a). Since the isolates were obtained from clinical samples they cannot be environmental saprophytes causing the pathological conditions among the patients.



In this study the ESBL producing isolates were more resistant to the antibiotics (both β-lactams and non β-lactams) tested than the non-ESBL producers and the differences were highly significant (p=0.0001). This suggests cross-resistance induced by the ESBL enzymes to the isolates against non-β-lactam antibiotics such as gentamicin, nalidixic acid, chloramphenicol, co-trimoxazole and the floroquinolones (Bonnet, 2004), because reports have it that the ESBL genes are located on transmissible or conjugative plasmids (Knothe, 1983; Lee *et al*., 2010; Levison, 2002). These transmissible plasmids carry along with $Bla_{GENES}$ other resistance genes for resistance to other antimicrobials including non-β-lactam antimicrobial agents (Pitout *et al*., 2005). The plasmids carrying the ESBL gene also carry multiple resistant genes which are transferred to another organism by conjugation (Sturenburg *et al*., 2004). The $Bla_{CTX-M}$ and $Bla_{TEM}$ are both prevalent at levels of 72 (93.5) and 74 (96.1) for *E. coli* respectively and $Bla_{SHV}$ prevalence of 32 (41.6%) in *E. coli* in this present study. Similar results were reported from Thailand where the prevalence of $Bla_{CTX-M}$ was high recording 99.6% for ESBL-producing *E. coli* and 99.2% for ESBL-producing *K. pneumoniae* (Kiratisin *et al.*, 2008), while the $Bla_{SHV}$ prevalence was 18.8%. These results are contrary to the much lower prevalence of 20.8% of $Bla_{CTX-M}$ reported from Iran (Ramazanzadeh, 2010a). CTX-M type ESBLs have become widely dispersed in many parts of the world and these enzymes confer higher levels of resistance to

cefotaxime than to ceftazidime (Bonnet, 2004). The widespread antibiotic resistance among the isolates may be explained by the spread of this *Bla*$_{CTX-M}$ enzyme among the isolates. This spread may be facilitated by cross infections that are likely to occur in the hospital and the community as a result of overcrowding, improper hand washing and improper application of disinfectants (Raymond *et al.,* 2007).

The detection of the ESBLs in all sample types demonstrates how widespread they are in both the community and hospital contrary to the notion that ESBLs are nosocomial associated (Rastogi, 2010). Though ESBLs have been reported from community setting (Rodriguez-Villalobos *et al.,* 2010) it is usually in low figures as opposed to the high level (39.7%) from KATH. The high spread of the ESBL types into the community is of no surprise because the patients who receive treatment from the hospitals upon discharge go back into the communities where they shed these organisms. This is facilitated by the unhygienic conditions that prevail in the communities. For example it is common to see large garbage situated in residential and market places unattended. Streams and rivulets in the city get choked with debris from human waste. Many insects and creatures breed at these sites. These insects carry and spread along with them bacteria to humans and animals in the communities. It is also common to see humans share the same compound with animals such as cattle, sheep and goats and fowls. Therefore microbes from humans and animals get into contact with each other and may exchange genetic material by conjugation (Leverstein-van Hall *et al.,* 2011).

The conjugative ability of the plasmids bearing the ESBL genes makes such spread quick to organisms of the same species and also of other species. Also the ability of the plasmid to spread makes it to attains outbreak status in wards (Rodriguez-Villalobos *et al.,* 2010), resulting in some isolates acquiring more than one gene type. In a study in Indian (Baby *et al.,* 2008), the majority

of isolates (82.6%) belonged to CTX-M type ESBLs, but they found that only one isolate was positive for both CTX-M and SHV gene, contrary to the results obtained in this present study where the CTX-M enzymes were found in close association with the TEM enzymes in more than 90% of the isolates. The detection of 70(29.9%) of the isolates to contain all the three $Bla_{\text{Genes}}$ and as many as 151 (64.5%) of the isolates possessing both $Bla_{\text{CTX-M}}$ and $Bla_{\text{TEM}}$, suggest that these two genes might have spread among the enterobacteria at a common point in time at KATH. The fact that most isolates simultaneously produced two or even three different β-lactamases indicates significant dissemination of ESBLs. Dissemination of SHV ESBLs may be largely mediated by plasmids or insertion elements (Messai *et al.*, 2008). Owing to insertion and transposable elements mediating the spread of ESBLs, the prevalence of *K. pneumoniae* and *E. coli* isolates with more than one β-lactamase has increased in recent years (Messai *et al.*, 2008). The possibility of multiple alleles in a single isolate had previously been identified and reported (Leinberger *et al.*, 2010). In this present study close to 30% of the isolates had all the three genes tested for namely $Bla_{\text{CTX-M}}$, $Bla_{\text{SHV}}$ and $Bla_{\text{TEM}}$. These results indicate that one isolate can carry all the three genes tested for; this is because the resistant genes are located on transmissible plasmid, which replicate and reasort independently, and are acquired by other close related organism by conjugation (Gonzalez-Sanz *et al.*, 2009). It is not surprising that isolates with multiple genes were found at KATH. Ciprofloxacin resistance was 29.5% in ESBL positive and 4.7% in ESBL-negative isolates and this difference was significantly (p=0.0001), an indication that ciprofloxacin resistance in the isolates was closely associated with ESBLs. This link between ESBL-production and resistance to ciprofloxacin is of grave concern since ESBL-producing isolates may carry plasmids that bear genes coding the resistance for other non-β-lactam antimicrobials including ciprofloxacin and β-lactam antibiotics (Phillippon, 2002).

Because the ESBL enzymes are plasmid mediated, the genes encoding these enzymes are easily transferable among different bacteria within the same species or to other genera (Phillippon, 2002). These plasmids not only have DNA-encoding ESBL enzymes but they also carry genes conferring resistance to several other non-β-Lactam antibiotics (Hawkey, 2008). As a result of this many isolates with ESBLs also develop resistance to many other classes of antibiotics other than the β-lactam antibiotics (Hawkey, 2008). Common co-resistance is found in ESBL-producing organisms are with the aminoglycosides, fluoroquinolones, tetracyclines, chloramphenicol, and sulfamethoxazole-trimethoprim. The emergence of epidemic clones harbouring several beta-lactamases simultaneously (*Bla*$_{\text{CTX-M}}$, *Bla*$_{\text{SHV}}$ and *Bla*$_{\text{TEM}}$) and perhaps of other mechanisms of resistance to fluoroquinolones and aminoglycosides warrants future surveillance studies at KATH. This is because treatment of these multi-drug-resistant organisms is a therapeutic challenge (Coque *et al.,* 2008).

*Bla*$_{\text{TEM}}$ being plasmid and transposon mediated whilst *Bla*$_{\text{CTX-M}}$ and *Bla*$_{\text{SHV}}$ are simply located on large transmissible plasmids facilitated their spread to other species of bacteria (Baby *et al.,* 2008). In addition local practices as regards selection pressure as a result of antibiotic use, prescribing practices in hospital and ease of access to antibiotics in the community kill the susceptible microbial strains and those carrying resistance strains survive. The killed ones will lyse and release their genome into the environment and the other survivors may get transformed by acquiring those chromosomes (Leverstein-van Hall *et al.,* 2011). Discharge of agricultural and factory effluence into the environment is another factor that sensitizes the microbes in the environment, but due to the dilute and low concentrations of the drugs the microbes are not killed, but are made to acquire resistant traits. As a result of these there are quite marked differences in geographic pattern of ESBL genes (Canton *et al.*,, 2008). ESBL prevalence in this

study was very high compared with Europe where prevalence of *Bla*$_{CTX-M}$ 62.5% and *Bla*$_{SHV}$ 34.4% have been reported and from Spain (Diestra *et al*., 2008) and 60% was reported in Portugal (Machado *et al.,* 2008) while 0-25% was reported from the USA (Canton *et al.*, 2008). This study determined whether the ESBL positive *E. coli* isolates under study were phylogenetically related or whether it was the same clones that were circulating among the patients. Out of the 77 ESBL positive isolates there were 74% that were found to belong to ECOR phylogenetic group B1 and the remaining 26% were in the group B2. Noting that the isolates came from the community and hospital patients, it is unclear why such a high percentage of isolates were found to belong to the ECOR group B1, which is said to be non-virulent (Picard *et al.,* 1999). The remaining 26% were found to belong to the well known virulent ECOR group B2. No isolates were found to belong in the ECOR groups A and D. The virulent B2 group is said to possess the haem transport genes which were acquired along the evolutionary path, rather than being present in a common ancestor (Picard *et al.,*1999). The haem genes are also present in the group D but it was completely lost in the groups A and B1. The yjaA genes possessare found only in the B2 group and are responsible for biofilm formation on inanimate objects (Sim *et al.,* 2010). Also yjaA is for cellular response to hydrogen peroxide and acidity, but their evolutionary origin is unknown (Nandi *et al.,* 2010; Serres *et al.,* 2001; Sim *et al.,* 2010). This B2 ECOR group is deeply divergent from the other strains and have been proven to be the cause of the majority of extraintestinal infections (Picard *et al.,* 1999), in contrast to ECOR group B1 found in this study. This group B1 strains were found in all sample types tested (ie blood, urine sputum wound). It is unclear whether antibiotic misuse and poor environmental sanitation can result in the selection of virulent strains or their independent evolution. It is however known that certain environmental microorganisms can cause severe human infections, even in the absence of

obvious pathogenicity traits a process called "accidental virulence" (Nandi *et al.,* 2010). It is also known that the pathogenicity of B2 could be related to host dependent factors such as susceptibility as linked to an underlying disease or site of infection by which the organism gains entry and initiate infection (Picard *et al.,* 1999). This is in agreement with reports that strains with no virulent traits can infect a host that has been made susceptible (Moreno *et al.,*2006).

General human practices in Kumasi, such as inappropriate antimicrobial prescription processes where antibiotics can be purchased off the counter to treat unknown infections perhaps due to viral causes can generate undue selective pressure (Baquero *et al.,* 2008). Also selective pressure can be induced as a result of agricultural use and discharge of antibiotic residues into the environment drive microbes to adapt new ways to survive (Baquero *et al.,* 2008). Also of importance is the inadequate diagnostics misleading clinicians to prescribe antibiotics needlessly, or rather treating unknown infections (Harris *et al.,* 2007). The bacteriological diagnostic tests often take a few days for the results to be obtained. So for the critically ill a physician has to make treatment decisions resulting in the prescription of broad-spectrum antibiotics, when a more specific treatment might be better. These are other factors that promote the emergence of antimicrobial resistance and the selection of virulent strains (Codruta-Romanita *et al.,* 2011). Hospital use and practices, where many sick people are brought together to one place (ward) and kept in close contact create a favourable environment for strains with self transferable plasmids carrying antimicrobial resistant genes to spread (Cremet *et al.,* 2011). At KATH crowding sometimes occurs in some of the wards resulting in the laying of patients on the floor whilst being attended to. There are also times when the municipal water supplies fail, so health workers depend on bowls of water for hand washing. Therefore health worker are unable to wash hands properly. This situation can lead to the dissemination of different bacterial clones in the hospital.

Generally patients seek antibiotic treatment when the infection may be of viral origin, so administering antibiotics in such situation may be of limited value. Efforts towards judicious use of antimicrobials are advocated. There is also a possibility that restricted use of antibiotics such as the third generation cephalosporins will lead to the reduction in ESBL production (Hunter *et al*., 2010). The withdrawal of antibiotics such as ampicillin, tetracycline and chloramphenicol to which resistance levels are high can lead to the withdrawal of selective pressure (Shiju *et al.*, 2010) and use of β-lactam and β-lactamase inhibitor combinations may exert reverse mutation on these resistant bacteria. Since resistance can be encoded on mobilizable plasmids, resistant ExPEC may emerge after selection of resistance-conferring plasmids in specific genetic backgrounds. For example, an ESBL-producing ExPEC clone, identified by MLST as ST131, recently emerged in countries around the world (Russo and Jonhson, 2003; Johnson *et al*, 2010; Nicolas-Chanoine *et al,* 2008) and the widespread dissemination is thought to have been facilitated by the acquisition of a plasmid-encoded CTX-M-15 β-lactamase by isolates with an *E. coli* phylogroup B2 chromosomal background {Johnson *et al*, 2010; Clermont *et al*, 2008).

An alarming proportion of ExPEC isolates from KATH were resistant to commonly used antibiotics and produced ESBLs. MLST results indicated that much of this resistance was due to a single clone, ST88. Interestingly, a historic representative of this lineage, ECOR-72, was originally isolated from a schoolgirl with pyelonephritis at the Children's Hospital in Goteborg, Sweden sometime between 1970 and 1971 (Ochman *et al*, 1984; Caugant *et al*, 1983), suggesting that this particular ExPEC lineage has been circulating in the human population for some time. When isolates of the ECOR collection were screened for resistance to 14 antibiotics against ECOR-72 in 2000 (Mazel *et al*, 2000), they were sensitive to each of these drugs, meaning that this lineage might have recently acquired resistance. It is also difficult to rule out

the possibility that *in vitro* passage of ECOR isolates selected for the loss of resistance determinants (esp. plasmids) or that ECOR-72 was not representative of other isolates in the lineage, especially as they were not isolated from KATH.

Results in this strongly suggest that ESBL-producing ST88 isolates have emerged among the KATH patient population. In particular, ESBL isolates appeared to be associated with in-patients undergoing treatment of extraintestinal infections. Finding that ST88 belongs to the B1 phylogroup is in agreement with the inability to detect virulence genes that are commonly associated with genomes of other ExPEC or diarrheagenic *E. coli* pathogens, most of which belong to either the B2 or D phylogroups. However, the fimbrial genes that were detected by PCR in ST88 isolates (c1936, *fimA*, *ppdD*, and *yehA*) have been shown to be associated with attachment and pathogenesis in the urinary tract (Vigil *et al*, 2011; Spurbeck *et al.*,2011). In particular, c1936 appears to be overrepresented among isolates that cause complicated UTI, cystitis, and pyelonephritis compared to commensal *E. coli* from human stool (Spurbeck *et al*, 2011).

A previous virulence factor screen of ECOR isolates by Johnson *et al (*2001) independently identified the presence of another gene of the type 1 fimbriae operon, *fimH*, in ECOR-72, suggesting that ST88 isolates carry genes for these fimbriae. Again, Johnson *et al (*2001) identified two other ExPEC virulence factors, *fyuA* (*Yersinia* siderophore receptor) and *ompT* (outer membrane protein T protease) in ECOR-72. However, a more recent screen by Vigil *et al* (2011) using a different primer pair was unable to identify *fyuA* in ECOR-72 (Vigil *et al.*, 2011), suggesting that this locus may not be present. To address these possible discrepancies, the primers used by Johnson *et al* (2001) were obtained and used to verify these claims. It was found

that both ECOR-72 and KATH ST88 isolates do not appear to carry either the *fyuA* or *ompT* loci. Therefore, the only difference between ECOR-72 and KATH ST88 isolates appears to be the presence of the usher protein gene for the Pix pilus, *pixC*. This gene was recently found in ECOR-72 (Spurbeck *et al*, 2011), but was not present in isolates from KATH.

There is growing awareness and concern over the emergence of non-ST131, fluoroquinolone resistance ($FQ^r$) ExPEC, particularly among virulent phylogroup B2 lineages (Platell *et al*, 2012). For example, approximately half of all $FQ^r$, non-ST131, B2 ExPEC isolates collected from humans and companion dogs were found to be clonally related (clonal complex 14) in a recent study (Platell *et al*, 2012), suggesting that the worldwide increase in the prevalence of $FQ^r$-B2-ExPEC may not have been solely the result of $FQ^r$-ST131 emergence, because flouroquinolone resistant ST88 have been recorded among the isolates from KATH. While more data are needed to determine the directionality of these evolutionary events, this study results support the suggestion by Platell *et al.* (2012) that $FQ^r$ ExPEC, should be a focus of strategic surveillance and control schemes, regardless of phylogroup.

### 5.1 Limitations of the study

The study depended on laboratory generated results, so relevant information missing on the laboratory report forms such as whether a urine sample was from a patient with an indwelling catheter could not be obtained. Also lacking was whether the patient had received any antibiotics prior to coming to hospital. Such data could not be obtained so could not be compared with the ESBL-colonizing isolates identified.

# Chapter Six

## 6.0: Conclusions and Recommendations

### 6.1: Conclusion

This study demonstrated that there are high antimicrobial resistance amongst *E. coli* and *Klebsiella* sp to the commonly prescribed antimicrobial drugs at KATH as a result of ESBL production by these isolates from the wards and the community. Ciprofloxacin and imipenem have excellent performance against the isolates tested and therefore are recommended for the treatment of infections caused by *E. coli* and *Klebsiella*. Isolates possessed all three ESBL genes (*Bla*$_{\text{CTX-M}}$, *Bla*$_{\text{TEM}}$ and *Bla*$_{\text{SHV}}$) tested for. Phylogenetic analysis of the ESBL producing *E. coli* strains classified the isolates into phylogenetic groups B1 and B2 only. A large proportion of ESBL-producing isolates at KATH therefore belong to a single clone ST88 that carries known ExPEC fimbriae and was prevalent among hospitalized patients with extraintestinal infections. The high levels of antimicrobial resistance and the widespread distribution of ESBL producing *E. coli* and *Klebsiellae* are reasons that emphasize the necessity to continuously do epidemiological monitoring and also adopt immediate intervention strategies to prevent severe nosocomial infections in the hospital.

### 6.2: Recommendation

1. It is recommended that clinical laboratories should be equipped and the staff trained on phenotypic testing of ESBLs of all isolates of enterobacteria. Also clinical staff and all prescribers should be trained on ESBL issues.

2. All isolates of enterobacteria must be tested for ESBL phenotype. This can be done together with the routine antimicrobial susceptibility testing by including co-amoxiclav disc 15mm away from a cefpodoxime disc.

3. Imipenem the only carbapenem tested could be introduced to treat severe infections. It recommended that Ghana National Health Insurance Authority consider introducing imipenem on the essential drug list for the hospital. But care must be taken in doing so to avoid the abuse of this drug, because carbapenemase producing *Klebsiella* have emerged in many parts of the world. Ciprofloxacin should be tested routinely. If it is found it sensitive should be chosen for the treatment of less severe infections including ESBL producers, because it was found to be highly sensitive to ESBL producing isolates.

4. Infection control measures need to be strengthened and implemented. Such measures should include the use of barriers so as to reduce other patients having contact with ESBL patients. Another control measure to consider should be isolation of patients with ESBL-producing isolates to reduce the ESBL spread in the hospital.

5. A more comprehensive study involving the molecular characterization for more ESBL gene types (e.g. AmpC, OXA, KPC and IMP and VIM) in more members of the *Enterobacteriaceae* and *Pseudomonas* is recommended. This is because some of these ESBL types, especially AmpC prevent susceptibility of β-lactam plus β-lactamase inhibitor combinations.

6. It is recommended that more data should be collected to determine more accurately the phylogenetic diversity of *E. coli* strain types circulating at KATH. Given the current impact of

the ESBL ST131 pandemic, it will be important to more accurately determine the incidence of ESBL ST88 disease at KATH and in the surrounding community and also to determine whether such isolates are geographically dispersed.

7. The isolates should be sequenced to determine their pathogenetic traits and the antimicrobial resistance genes.

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