Molecular characterization of multi-drug resistant *Escherichia coli* isolated from urine samples in Mater Hospital, Nairobi, Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This thesis is dedicated to my wife and my children who have tirelessly supported me all the way since the beginning of my studies. Their prayers and moral support have been the driving momentum that has enabled me to achieve the targeted goal in my studies.

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LIST OF ABBREVIATIONS

AMP	Ampicillin
API 20E	Analytical profile index 20 *Enterobacteriaceae*
ATCC	American type culture collection
ATM	Aztreonam
AUG	Augmentin
BD	Becton, Dickinson and Company
Bp	Base pair
BSAC	British society for antimicrobial chemotherapy
C	Chloramphenicol
CAZ	Ceftazidime
CGA	Clonal group A
CIP	Ciprofloxacin
CLED	Cysteine lactose electrolyte deficient medium
CLSI	Clinical Laboratory Standards Institute
CMR	Centre for Medical Microbiology Research
CN	Gentamycin
CRDR	Centre for Respiratory Disease Research
CRO	Ceftriaxone
CXM	Cefuroxime
DC	Direct current
DNAase	Deoxyribonuclease
E.COLI	*Escherichia coli*

EDTA	Ethylenediaminetetraacetic acid
ERB	Ethical Review Board
ESBL	Extended Spectrum Beta-Lactamase
F	Nitrofurantoin
F primer	Forward primer
GIT	Gastrointestinal tract
IDSA	Infectious Disease Society of America
LB	Luria bertani
MDR	Multi Drug Resistance
MH	Mueller-Hinton Agar
MIC	Minimum Inhibitory Concentration
NA	Nalidixic Acid
NON-ESBL	Non-extended spectrum beta-lactamase
PCR	Polymerase Chain Reaction
PFGE	Pulsed field gel electrophoresis
R primer	Reverse primer
RNase	Ribonucleic Acid
S	Streptomycin
SCC	Scientific Steering Committee
SDS	Sodium dodecyl sulphate
SHV	Sulphydryl variable
SMZ	Sulphamethoxazole
SPSS	Statistical Package for Social Sciences
SPP	Species

SXT	Cotrimoxazole
TE	Tetracycline
T-E	Tris-Ethylenediaminetetraacetic acid
TEM	Temoniera
THP	Tamm-Horsfall proteins
TMP	Trimethoprim
µg	Microgram
UPEC	Uropathogenic *E.coli*
UTI	Urinary tract infection
ZX	Cefepime
χ^2	Chi-Square

ABSTRACT

Multi-drug resistance among Gram negative bacteria is on the increase due to acquisition and expression of extended spectrum β-lactamases (ESBL). The presence of ESBL producing organisms have been reported to affect the course and the outcome of infections. Therefore, infections due to ESBL-positive strains pose a major challenge in the management of infections worldwide. The aim of this study was to determine the antimicrobial susceptibility patterns and the genetic basis of antibiotic resistance in uropathogenic *E.coli* isolates from Mater hospital, Kenya. These isolates were tested for antimicrobial susceptibility and analyzed for the presence of ESBL genes. Polymerase chain reaction (PCR) was conducted to confirm the presence of ESBL enzymes and pulsed field gel electrophoresis (PFGE) was performed to determine the genetic relatedness of the strains. Of the 384 isolates submitted for analysis, 56 (14.6%) tested positive for ESBL. Out of these 56 isolates 9 (16%) had both TEM and SHV and 12 (21%) had TEM alone. The difference in resistance levels between ESBL and non-ESBL producing isolates for ciprofloxacin, ceftriaxone, cefuroxime, ceftazidime, ampicillin, cotrimoxazole, augmetin and nalidixic acid ($p \leq 0.001$) was statistically significant. This study reports a prevalence of (14.6%) ESBL producers among uropathogenic E.coli isolates from Mater Hospital. There are two predominant genes TEM and SHV among uropathogenic strains circulating in this hospital, presumably, this could have been the cause of resistance among these pathogens. The PFGE confirmed that the E.coli pathogenic strains were genetically diverse since the majority of the isolates came from community-acquired infections where the patients were not demographically related.

CHAPTER ONE

1.0 INTRODUCTION

1.1 **Background information**

Resistance to broad spectrum cephalosporins and other antimicrobial agents among clinical isolates of Gram negative bacteria is on the increase worldwide (Iroha *et al.*, 2009). The development of resistance to commonly used antibiotics has been due to lack of compliance to treatment **(**Davies and Davies, 2010), availability of antibiotics over the counter and the supply of poor quality products (Iruka *et al*., 1999; Raúl and Claude, 2000). Other contributory factors include improper diagnoses, inadequate resources and facilities to conduct extended spectrum β-lactamase identification, extensive use of broad spectrum antibiotics and long hospitalization of patients (Ndugulile *et al.*, 2005).

Antimicrobial drugs that are resistance among the pathogenic agents of UTI, for example, uropathogenic *E.coli* (UPEC) continues to cause setbacks in developed and developing countries, contributing a great challenge in the management of UTI. The most prevalent mechanism of bacterial resistance among Gram negative bacteria is the production of ß-lactamase enzymes (Iroha *et al.*, 2009). Extended spectrum β-lactamase (ESBL) enzymes have been reported in a number of species in Gram-negative bacteria. The ESBL are usually plasmid mediated and are capable of hydrolyzing and inactivating a wide variety of β-lactam antibiotics, including third-generation cephalosporins, penicillins and aztreonam, but are susceptible to β-lactamase inhibitors such as clavulanic acid and tazobactam (Rawat and Nair, 2010).

Many ESBL producers also carry other genes that confer resistance to other antimicrobial agents such as aminoglycosides and fluoroquinolones. Very few studies have reported on antimicrobial resistance associated with ESBL in urinary tract pathogens particularly in Kenya. The ESBL reported in *Escherichia* species include TEM, SHV and CTX-M, although the later was not characterized in this study. Problems associated with ESBL have led to the limited use of commonly available antibiotics, leading to more expensive treatment options that have impacted negatively on clinical outcomes (Menashe *et al.,* 2001).

1.2 Research problem

Multi-drug resistant *Escherichia coli* from UTI is on the increase in Kenya. Concurrent resistance to antimicrobial of different classes has arisen in a multitude of species and may complicate the therapeutic management of infections, including those of the urinary tract (Perfecta and Gondek, 2002). The changing etiology of UTI and increasing resistance require periodic monitoring and possibly modification of empirical regimens (Griebling, 2005). Clinical failure of cephalosporin and monobactams therapy (due to ESBL) is a growing problem in hospitals as they go undetected by current isolation and susceptibility tests. Unfortunately ESBL producing organisms often possess resistance determinants to other important antibiotic groups, such as aminoglycosides and fluoroquinolones, leaving an extremely limited range of effective agents (Kariuki *et al.*, 2007). Therefore it is important to determine the contributing factors that cause multi-drug resistant to commonly available antibiotics in order to institute effective guidelines for management of UTI.

1.3 Justification

Escherichia coli are the most common cause of uncomplicated urinary tract infections and accounts for approximately 75 to 95 percent of all infections. Trimethoprim-sulfamethoxazole has been the standard therapy for urinary tract infection; however, *E.coli* is becoming increasingly resistant to this antibiotic combination. The emerging resistance among antimicrobial drugs has been associated with the production of ESBL (TEM-1 and SHV-1) in *E.coli* strains. In Kenya, few studies have been done in public hospitals but limited information is available on molecular characterization of multidrug resistance in private hospitals (Kariuki *et al*., 2006). This study aims to address resistance types and molecular basis for resistance in *E.coli* from patients suspected to have UTI at Mater hospital.

1.4 Research question

Is multidrug resistance in *E.coli* isolates as a result of ESBL spread through plasmid mediated genes TEM and SHV?

1.5 Hypothesis

1.5.1 Null hypothesis

Extended spectrum β-lactamases are not responsible for multidrug resistance among uropathogenic *E.coli* isolates.

1.5.2 Alternative hypothesis

Extended spectrum β-lactamases are responsible for multidrug resistance among uropathogenic *E.coli* isolates.

1.6 Objectives

1.6.1 Main objective

To determine the antimicrobial susceptibility patterns and the genetic basis of antibiotic resistance in uropathogenic *E.coli* isolated from patients suspected with urinary tract infection in Mater Hospital.

1.6.2 Specific objectives

1) To determine the prevalence of ESBL producing *E.coli* isolates among patients suspected to have urinary tract infection.
2) To establish the genetic basis of antimicrobial resistance in uropathogenic *E.coli* isolates from patients suspected to have UTI in Mater Hospital.
3) To determine the clonal relatedness of *E.coli* isolated from patients suspected to have UTI.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Species of the genus *Escherichia*

The genus *Escherichia* has several species mainly *E.albertii, E.blattae, E.fergusonii, E.hermanii, E.senegalensis, E.coli* and *E.vulneris* which are the major cause of infections in human. The characteristics of *Escherichia* species are distinguished from other enterobacteria by motility, and other biochemical analyses. Among these species *E.coli* is well recognized and specifically subdivided into biotypes and serotypes (Okerman and Devriese, 1985). *Escherichia coli* are Gram-negative non-sporing bacilli, facultative anaerobes, rod shaped bacteria with some having prominent polysaccharide capsule. This capsule encases the large appearance of the organism on the Gram stain, and provides resistance against many host defense mechanisms.

The genus *Escherichia* typically expresses three types of antigens on their cell surfaces. The first is a lipopolysaccharide (O antigen), the other capsule polysaccharide (k antigen) and the last one is the flagella polysaccharide (H antigen). The virulence of all serotypes appears to be similar. These three components contribute to the pathogenecity of the organism. The serotypes responsible for UTI are 01, 02, 04, 07, 09, 011, 018, 039, and 075 (Gruneburg and Bettelheim, 1969). As an alternative to serotyping, colicine typing may be employed in epidemiological studies (McGeachie and McCormick, 1967; Moulin-Schouleur *et al.,* 2007). The structural variability of these antigens forms the basis for classification into various serotypes.

2.1.1 Uropathogenic *Escherichia coli*

Uropathogenic *Escherichia coli* (UPEC) are Gram negative rods found in gastrointestinal tract (GIT) of humans and animals as normal flora. They grow well on standard bacterial culture media. Uropathogenic *Escherichia coli* are responsible for UTI seen in individuals with ordinary anatomy (Gilad *et al.,* 2000) and are the leading cause of UTI, causing up to 90% of UTI in adults. In ascending infections, fecal bacteria colonize the urethra and spread up to the urinary tract and finally to the urinary bladder. Since women have a shorter urethra compared with that of men, they are 14-times more likely to suffer from ascending infections (Edelita, 2008). Uropathogenic *Escherichia coli* utilize P-fimbriae (pyelonephritis-associated pili) to bind urinary tract epithelial cells and colonize the urinary bladder (Rendón *et al.,* 2007).

The frequency of the distribution of these host cell receptors plays a role in susceptibility to infection and this explains why certain individuals have repeated UTI caused by *E.coli* (Reid and Sobel, 1987). The uncomplicated UTI virtually never occurs in individuals lacking the receptors (Stapleton, 2005). Uropathogenic *Escherichia coli* produce alpha and beta-hemolysins, which cause lysis of urinary tract cells (Foxman, 2003). The intestinal pathogenic *Escherichia coli* strains colonize patients with uncomplicated cystitis then enter into the urinary bladder from the periurethral region during sexual intercourse (Nicolle, 2003). The morbidity and financial burden associated with UTI is substantial, almost half of all women experience at least one UTI in their lifetime (Tartof *et al.*, 2005). The overall expenditure for UTI treatment in women in countries such as the United States and Canada was approximately $2.47 billion in 2000, (Foxman, 2003; Nicolle, 2003; Griebling, 2005).

Many experts support using ciprofloxacin treatment for UTI as an alternative and, in some cases, as the preferred first-line agent (Bader *et al*., 2010). However, others caution that widespread use of ciprofloxacin will promote increased resistance (Mehnert-Kay, 2005). Antimicrobial resistance among UPEC continues to rise in the United States, contributing to greater difficulty in the management of UTI (Gupta *et al.,* 2001; Perfecta and Gondek, 2002). Studies carried out in France by Leflon-Guibout *et al.* (2000) and in the United States by Kaye *et al.* (2004) have shown a steady increase in resistance to most commonly used antimicrobial drugs for treatment of UTI caused by *E.coli.* The changing etiology of UTI and increasing antibiotic resistance require periodic monitoring and possibly modification of empirical regimens (Griebling, 2005).

In Africa, broad spectrum antimicrobial drugs such as fluoroquinolones, β-lactam inhibitors, and cephalosporin have lost their effectiveness over the years (Kariuki *et al.,* 2007). A study conducted in Tunisia revealed that the susceptibility of bacteria to the principal antibiotics used for the treatment of UTI was characterized by low sensitivity of the Gram negative rods to amoxicillin (41.2%) for *E.coli* and (22%) *Proteus spp* (Boukadida *et al.,* 2002). However, a similar study carried out in Kenya revealed a similar trend in antimicrobial resistance among UPEC (Kariuki *et al.,* 2007). Fluoroquinolones have become popular treatment for patients with uncomplicated UTI, but a study conducted by Kariuki *et al.* (2007), showed that UPEC are becoming resistant to fluoroquinolones among other antibiotics.

The alarming reports of community-acquired UTI caused by fluoroquinolone-resistant *E.coli* strains in some parts of the world suggest that this will results in an evolution of resistance to these agents just like sulfonamides, ampicillin, oral cephalosporin, and trimethoprim–sulfamethoxazole unless aggressive approach to the control of antimicrobial resistance is undertaken (Sandel *et al.,*2002). A study in Tanzania revealed that the resistance to third generation cephalosporin was due to acquisition and expression of ESBL enzymes among Gram-negative bacteria. This was attributed to the indiscriminate use of these antibiotics (Ndugulile *et al.,* 2005).

In Kenya, the upsurge of resistance is due to frequent misuse and prolonged use of certain drugs that expose these strains to adapt resistance (Mitema and Kikuvi, 2004). The strains may acquire resistance to several other antibiotics through acquisition of transmissible plasmids from resistant bacteria population (Smith and Halls**,** 1968; Boyd *et al*., 2004**)**. A strain with resistance that is dependent on mutated chromosomal genes or transmissible plasmids is commonly present in hospital patients receiving antibiotics. For example urological wards, where they commonly cause nosocomial infections (Childs, 1991; Oni *et al*., 2003). The bacteria responsible for UTI after urological or gynecological procedures sometimes are transmitted to the same patient or to other patients by cross-infection (Iwakiri *et al*., 2002).

2.1.2 Epidemiology of *Escherichia coli*

Escherichia coli are facultative anaerobes, normal flora; found in gastrointestinal (GTI) of humans and animals. Transmission of pathogenic *E.coli* often occurs via fecal contamination, dirty hands (Salyers *et al.*, 2004), and through sexual contact (Brown

and Foxman, 2000; Gupta *et al*., 2001). Since bacteria can enter the urinary tract through the urethra (an ascending infection), poor toilet habits can predispose to infection, but other factors such as pregnancy in women and prostate enlargement in men are also important (Lane and Mobly, 2007). However, in most cases the initiating cause is unclear. Among the elderly, UTI frequency occurs equally in women and men (Alraek *et al.*, 2002).

The use of urinary catheters in both women and men who are elderly, people experiencing nervous system disorders and people who are convalescing or unconscious for long periods may result in an increased risk of UTI for a variety of reasons (Molander, 2002). The urinary bladder wall is coated with various mannosylated proteins, such as Tamm-Horsfall proteins (THP), that block the binding of bacteria to the uroepithelium. As binding is an important factor in establishing pathogenicity for these organisms, its disruption results in reduced capacity for invasion of the tissues. Due to poor adhesion, the unbound bacteria are more easily removed from the bladder by voiding urine hence reducing the chance of infection.

The use of urinary catheters (or other physical trauma) may physically disturb this protective lining, thereby allowing bacteria to invade the exposed epithelium (Esposito *et al.*, 2008; Alexander *et al.*, 2009). Elderly individuals, both men and women, are more likely to harbor bacteria in their genitourinary system at any time (Alexander *et al.*, 2009). This colonization may be accompanied by symptoms of infection that may necessitate treatment. The presence of bacteria in the urinary tract of older adults, without symptoms or associated consequences, is also a well recognized phenomenon

which may not require antibiotics. This is usually referred to as asymptomatic bacteriuria. The overuse of antibiotics in the treatment of bacteriuria is of concern and a controversial issue (Rodhe *et al.,* 2009).

2.1.3 Pathogenesis of *Escherichia coli*

Uropathogenic strains of *E.coli* possess other determinants of virulence in addition to P fimbriae. Type 1 fimbriae provide a supplementary mechanism of adherence or play a role in aggregating the bacteria to a specific manosyl-glycoprotein that occurs in urine (Rendón *et al.,* 2007). Uropathogenic strains usually produce siderophores that play an essential role in iron acquisition for the bacteria during or after colonization. An alternative strategy for obtaining iron and other nutrients for bacterial growth may involve the lysis of host cells to release these substances. Infectious strains may therefore produce hemolysins which are cytotoxic due to formation of transmembranous pores in host cell membranes (Duguid *et al*., 1955). The activity of hemolysins is not limited to red cells since the alpha-hemolysins of *E.coli* also lyse lymphocytes, and the beta-hemolysins inhibit phagocytosis and chemotaxis of neutrophils (Stapleton, 2005).

Another factor thought to be involved in the pathogenicity of the UPEC is their resistance to the complement-dependent bactericidal effect of serum (Stapleton, 2005). The K antigens of *E.coli* are "capsular" antigens that may be composed of proteinaceous organelles associated with colonization, or made of polysaccharides. Regardless of their chemistry, these capsules promote bacterial virulence by decreasing the ability of antibodies and/or complement to bind to the bacterial surface, and the ability of phagocytes to recognize and engulf the bacteria (Rendón *et al.,* 2007). The best studied

K antigen, K-1, is composed of a polymer of N-acetyl neuraminic acid (sialic acid), which besides being antiphagocytic, has the additional property of being an antigenic disguise (Nowicki *et al.,* 1989).

2.1.4 Bacterial resistance to antimicrobial drugs

Escherichia coli isolates belonging to clonal group A (CGA), has been described as a disseminated cause of drug-resistant in urinary tract infections in humans (Boczek *et al.,* 2007). Human CGA isolates have typically exhibited a number of traits that set them apart from other uropathogenic or drug-resistant *E.coli* isolates. These characteristics include their virulence factor profile, several distinctive O antigens, the H18 flagellar antigen, and multidrug resistance, including resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline, and trimethoprim (Johnson and Russo, 2005).

Fluoroquinolones have become popular therapy for patients with uncomplicated UTI because of emerging resistance to other common medications. The reported prevalence of *E.coli* resistant to ciprofloxacin is still very low at less than 3% (Chun-Yu *et al*., 2008).

The Infectious Diseases Society of America (IDSA) guidelines recommend the use of fluoroquinolones (ciprofloxacin, fleroxacin, norfloxacin, and ofloxacin) as first-line treatment of UTI in communities with greater than 10 to 20% resistance rates to trimethoprim-sulphathoxazole (TMP-SMX). In contrast to a study conducted by Kariuki *et al.* (2007) that revealed that fluoroquinolone-resistant, UPEC are becoming increasingly prevalent in hospitals in Kenya, posing a major challenge in the treatment and management of UTI.

2.1.5 Bacterial mechanisms of antibiotic resistance

In addition to adding and deleting genes on the chromosome, bacteria can change phenotype through the gain or loss of plasmids (Leflon-Guibout *et al.*, 2000). Antibiotic resistance, for example, can be transferred via plasmid and has been demonstrated to be transmitted across bacterial species (Sahm *et al.,* 2001). An outbreak would follow the path of the plasmid (horizontal and vertical gene transfer) rather than clonal spread in the path of a particular bacterial clone. The identification of uropathogenic factors and their mode of transmission between pathogens would greatly assist our understanding of UTI epidemiology and pathogenesis and our ability to prevent disease via vaccination or other strategies (Weagant *et al.,* 1999). These types of studies require epidemiologic methods to collect appropriate sample isolates from well-defined populations and to make appropriate inferences about the findings based on laboratory analyses. The genotypic characterization of pathogens therefore has become an important objective in epidemiologic investigations of infectious agents.

Broad spectrum oxymino-cephalosporin (e.g. ceftazidime and cefotaxime) are widely used to treat infections caused by antibiotic-resistant organisms such as Gram positive and Gram negative bacteria (Tumbarello *et al.,* 2006). The union of the oxymino chain with the 2-amino-5-thiazolyl nucleus stabilizes these drugs against the effects of the common TEM-1 and SHV-1-β lactamases (Bradford, 2001). Resistance can develop in *E.coli* isolates that produce ESBL enzymes which hydrolyze all cephalosporins, penicillins and monobactams and which are inhibited by clavulanic acid, sulbactam, and tazobactam (Reid and Sobel, 1987). Although ESBL have been detected in most Gram negative species, *E.coli* is one of the most frequently reported producers of these

enzymes. *Escherichia coli* often carry multidrug resistant plasmids and under stress readily transfer those plasmids to other species.

2.1.6 Extended spectrum β-lactamase enzymes

Extended spectrum β-lactamase enzymes (ESBL) are modified β-lactamase enzymes mainly derived from TEM1/2, SHV-1 and CTX-M plasmid mediated enzymes, which hydrolyse expanded spectrum cephalosporin to varying degrees (Thomson *et al.,* 2007). The extended spectrum β-lactamase enzymes are widespread all over the world, but the prevalence and phenotypic characteristic among clinical isolates may vary between geographical areas (Sirot *et al.,* 1991; Jacoby *et al.,* 2006). The original TEM was first discovered in *E.coli* isolates in a patient named Temoniera in Greece, but it spread rapidly to other bacteria. The production of plasmid mediated ESBL have emerged as an important mechanism of resistance to β-lactam antibiotics among *E.coli* (Kaye *et al.,* 2004).

These conjugative plasmids are responsible for dissemination of resistance to other members of Gram negative bacteria in hospitals and in the community (Sirot *et al.,* 1991; Jacoby *et al*., 2006). Unfortunately ESBL producing organisms often possess resistance determinants to other important antibiotic groups, such as aminoglycosides and fluoroquinolones, leaving an extremely limited range of effective agents (Kariuki *et al*., 2007). The production of other types of ESBL, like CTX-M are more selective to cefotaxime than to other broad cephalosporins, have been increasingly detected previously in *E.coli* as shown by Leflon-Guibout *et al.,* (2000).

These ESBL enzymes are plasmid borne and have evolved from point mutations altering the configuration of the active site of the original and long known β lactamases designated TEM-1, TEM-2, and SHV-1 (Bradford, 2001). The activity of these enzymes is limited to ampicillin, penicillin, and carbenicillin. Initially these bacteria contained a single ESBL gene, but later multiple ESBL genes are commonly present in a single strain, further complicating the process of detecting them and identifying an appropriate treatment regimen (Boyd *et al*., 2004). To date, more than 90 TEM-type and more than 25 SHV-type β lactamases have been identified (Joumana and George, 2003). The AmpC β-lactamases hyper producing enterobacteriaceae have been reported worldwide, but few data are available about their prevalence in human clinical specimens (Philippon *et al*., 2002). These enzymes are numerous, and they mutate continuously in response to the heavy pressure of antibiotic use, leading to the development of ESBL (Joumana and George, 2003).

Lack of awareness, resources and facilities to conduct ESBL identification, contribute to the spread of multidrug resistance in most *Enterobacteriaceae* organisms (Urban *et al.*, 2008). It has been observed that while antibiotics revolutionized the treatment of infectious diseases in the 20th century, resistance threatens to render these drugs ineffective in the 21st century (Salyers *et al*., 2004). As soon as a new antimicrobial agent is discovered or synthesized, bacteria evolve mechanisms to overcome the effects of the new agent (Sean and Pitman, 2004).

2.1.7 Detection of extended spectrum β-lactamase

The increased prevalence of *Enterobacteriaceae* producing ESBL creates a great need for laboratory testing methods that accurately identify the presence of these enzymes in clinical isolates (Bradford, 2001). Although most ESBL confer resistance to one or more of the oxyimino-β-lactam antibiotics, the β-lactamase does not always increase the MICs to high levels to be called resistant by the Clinical Laboratory Standards Institute (CLSI) interpretive guidelines (Wayne, 2008). The sensitivity and specificity of a susceptibility test to detect ESBL vary with the cephalosporin tested. Several ESBL detection tests that have been proposed are based on the Kirby-Bauer disk diffusion test methodology (Bradford, 2001).

2.1.8 Double disk tests

A plate is inoculated as for a routine susceptibility test. Disks containing cefotaxime and ceftazidime 30 µg (or cefpodoxime 10 µg) are applied either side of one with co-amoxiclav (amoxicillin and clavulanic acid, respectively) 20+10 µg; and set 15 mm away from it (Jun-ichi *et al.*, 2004). Extended spectrum β-lactamase production is inferred when the zone of inhibition of cephalosporin is expanded by the clavulanate. The method is cheap, but the optimal disk separation varies with the strain and some producers may be missed. This method is no longer recommended for routine use.

2.1.9 Combination disk method

This compares the zones of cephalosporin disks to those of the same cephalosporin plus clavulanate. According to the supplier, either the difference in zone diameters, (Oxoid), or the ratio of diameters, is compared (Mast and BD) with zone diameter increases of

>5 mm or >50% in the presence of the clavulanate implying ESBL production. These tests are cheap and do not require critical disk spacing.

2.2.0 Etest for extended spectrum β-lactamase strips

These Etest ESBL strips (AB Biodisk, Solna, Bio-Stat, Stockport, UK) have a cephalosporin gradient on one end and a cephalosporin plus clavulanate gradient on the other. Users follow the manufacturer's instructions, including for a heavier inoculum than in BSAC disk tests. Extended spectrum β-lactamase production is inferred if the MIC ratio for cephalosporin alone: cephalosporin + clavulanate MIC is >8. Etest strips are accurate and precise, but more expensive than combination disks.

2.2.1 Fluoroquinolone resistance

The fluoroquinolone class of antimicrobial agents has had broad acceptance in hospitalized and community patients, and usage appears to be increasing (Hooper, 2001). Fluoroquinolones have become popular treatments for patients with uncomplicated UTI because of emerging resistance to other common medications (Chun-Yu *et al*., 2008). Mechanisms of bacterial resistance to fluoroquinolones fall into two principal categories, alterations in drug target enzymes and alterations that limit permeation of drug to the target, both resulting from chromosomal mutations (Hooper, 1999) and plasmid-mediated fluoroquinolone resistance via *qnrA, qnrB, qnrS* and *aac (6')-Ib-cr*.

Drug permeation is altered by mutations that increase expression of endogenous multidrug efflux pumps; alter outer membrane diffusion channels or both (Viveiros *et al*., 2007). Broad use of fluoroquinolones has been followed by emergence of

resistance, which has been due mainly to chromosomal mutations in genes encoding the subunits of the drugs' target enzymes, DNA gyrase and topoisomerase IV, and in genes that affect the expression of diffusion channels in the outer membrane and multidrug-resistance efflux systems (Hooper, 2001). The reported resistance rate of *E.coli* to ciprofloxacin is still very low at less than 3% (Chun-Yu *et al*., 2008). As we approach the halfway point of the second decade of fluoroquinolone use, resistance has already emerged in species of bacteria and clinical settings **(**Hooper, 2001).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study site

The study was carried out at the Mater Hospital, Nairobi, Kenya between January 2009 and August 2009. The hospital is situated two kilometres away from the city centre. This is a middle-class hospital and situated in Nairobi the capital city of Kenya and serves a wide range of patients across the country. It accommodates 100 in-patients and receives approximately 200 outpatients per day. The laboratory receives an average of 15 urine samples per day from patients suspected to have urinary tract infection.

3.2 Study design

A cross sectional study of *E.coli* isolates obtained from patients presenting with UTI at the Mater hospital.

3.3 Sample size determination

There is no data on the prevalence of ESBL-producing *E.coli* in Kenya neither is there data concerning isolation rates of pathogenic *E.coli* from patients suspected to suffer from UTI in Kenya. Determination of the number of *E.coli* strains used for screening ESBL producers was therefore done empirically based on a study by Urassa *et al*. (1997). In this study conducted in a neighboring country (Tanzania), a total of 232 urinary tract pathogens were isolated from hospitalized and non-hospitalized patients in a period of two months. Among the isolates, 200 (86.2%) were Gram negative bacilli, including *E.coli* 109 (54.5%).

Therefore the prevalence of 54.5% was used to calculate the minimum number of *E.coli* strains required to screen for ESBL producers. The antimicrobial susceptibility

profile for all the *E.coli* isolates was determined and the candidate strains were further screened for ESBL production.

Sample size determination was reached using the Fisher's (Thomas and Conlon, 1992) formula.

$$n = \frac{Z^2_{1-\alpha/2} P (1-P)}{d^2}$$

Where:

$Z_{1-\alpha/2}$= 1.96 (95% Confidence limit)

α = the level of significance

d= 0.05 (5% Absolute precision)

P= Prevalence of a previous study 54.5% (Urassa, *et al.*, 1997).

= 0.545 Probability of pathogen isolation.

$$n = \text{Minimum sample size } \frac{(1.96)^2\ 0.545(1-0.545)}{(0.05)^2} = 381$$

The minimum sample size of *Escherichia coli* isolates required to be screened for ESBL production was 381.

3.4 Inclusion criteria/ exclusion criteria

Only *E.coli* isolates from patients with suspected UTI were analyzed, while *E.coli* isolates from other specimens such as blood, stool and wound were excluded from the study.

3.5 Study assumptions

The following assumptions were made in this study:

- That the prevalence of isolation of *E.coli* from UTI patients in Kenya is not significantly different from Tanzania.

- That the patients sampled during the study period was a true representation of the patients in this hospital at any given time.
- That the proposed methods used were sensitive enough to identify ESBL producers among *E.coli*.

3.6 Laboratory procedures

3.6.1 Collection and identification of *Escherichia coli* isolates

Escherichia coli were isolated from patients diagnosed with UTI in Mater Hospital laboratory. The primary isolation was carried out on Cysteine lactose electrolyte deficient medium (CLED) and the organisms appeared yellow in colour. These isolates were stocked in the vials containing freezing media (tryptone soya broth and glycerol) then transported in a cool box to the KEMRI laboratory. On arrival at the KEMRI laboratory, isolates were sub-cultured on MacConkey agar media (Oxoid), and plates were incubated at 37°C for 24hrs. Pink colonies with a darker center appearing on the MacConkey agar plate, were re-identified using biochemical tests. The analytical profile Index 20 *Enterobacteriaceae* (API 20E bioMerieux ® S.A) was used for biochemical tests as shown in Figure 3.1.

The API 20E system consists of plastic strips of 20 individual, miniaturized test tubes (cupules) each containing a different reagent used to determine the metabolic capabilities, and ultimately, the genus and species of enteric bacteria in the family *Enterobacteriaceae.*

Figure 3. 1 The analytical profile index 20 *Enterobacteriaceae* strip

The identification of *Escherichia coli* was done using several biochemical tests. The metabolic capabilities are used to determine the genus and species of enteric bacteria in the family *Enterobacteriaceae* as shown in Table 3.1.

Table 3. 1 The interpretation of biochemical tests

Substrate	Results	Interpretation
ONPG-ortho-nitrophenyl-galactoside	Yellow	Positive
ADH-arginine	Yellow	Negative
LDC-lysine	Yellow	Negative
ODC-orthinine	orange/red	Positive
CIT-sodium citrate	Yellow	Negative
H_2S-hydrogen sulphide	green/yellow	Negative
URE-urea	Yellow	Negative
TDA-tryptophane	Yellow	Negative
IND-tryptophane	red ring	Positive
VP-sodium pyrute	pink/red	Positive
GEL-Kohn's galatin	no diffusion black pigment	Negative
GLU- glucose	Yellow	Positive
MAN-mannitol	Yellow	Positive
INO- inositol	blue/green	Negative
SOR- sorbitol	Yellow	Positive
RHA –rhamnose	Yellow	Positive
SAC-sucrose	blue/green	Negative
MEL –melibiose	Yellow	Positive
AMY–amygdalin	blue/green	Negative
Arabinose	Yellow	Positive

Individual reagents used to determine the metabolic capabilities of *Enterobacteriaceae*

3.6.2 Antimicrobial susceptibility testing

The antimicrobial susceptibility tests were performed using the Kirby Bauer disk diffusion technique (Furtado and Medeiros, 1980) with commercially available disks on Mueller Hinton agar plates. Antibiotics disks viability was quality controlled using *E.coli* ATCC® 25922.

This was done by emulsifying discrete colonies from an overnight culture of *E.coli* ATCC® 25922 in normal saline and comparing the turbidity with 0.5 McFarland equivalence standards. The ATCC® 25922 organisms were inoculated on Mueller-Hinton agar plates, disks placed and incubated at 37°C for 18-24 hours at the same time with the test organisms.

The diameter of the zone of inhibition for each antibiotic was measured in millimeters and interpreted as resistant, intermediate and sensitive according to Clinical Laboratory Standards Institute criteria (Wayne, 2008) as shown in Table 3.2.

Table 3. 2 Standard antimicrobial inhibition zones according to Clinical Laboratory Standards Institute

Antibiotics	Resistant	Intermediate	Sensitive
ciprofloxacin (5μg)	≤15	16-20	≥21
gentamicin (10μg)	≤12	13-14	≥15
Ampicillin (10μg)	≤13	14-16	≥17
Chloramphenicol (30ug)	≤12	13-17	≥18
Augmentin (30μg)	≤13	14-17	≥18
Cefuroxime (30μg)	≤14	15-17	≥18
Nitrofurantoin (10μg)	≤14	15-16	≥17
Tetracycline (30ug)	≤11	12-14	≥15
Nalidixic Acid (30μg)	≤13	14-18	≥19
Ceftazidime (30ug)	≤14	15-17	≥18
Cotrimoxazole (30μg)	≤10	11-15	≥16
Aztreonam (30μg)	≤14	16-21	≥18
Ceftriazone (30μg)	≤13	14-20	≥21
Cefepime (30μg)	≤14	15-17	≥18
Streptomycin (10μg)	≤10	12-14	≥15

Key- ≤ , ≥ , μg, antibiotics

3.6.3 Phenotypic confirmatory disk diffusion test for β-Lactamase

The test required a combination of ceftriazone (30µg), or cefepime (30µg), ceftazidime (30µg) and amoxicillin-clavulanic acid (30µg). The disk containing amoxicillin-clavulanate was placed at the centre of the plate, and disks containing one of the oxymino-β-lactam antibiotics are placed 15mm (centre to centre) from the amoxicillin-clavulanate disk. An increase in zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone or the formation of a phantom indicates production of ESBL enzymes as shown in Plate 3.1. *Escherichia coli* ATCC® 25922 and *Klebsiella* pneumonia ATCC ®700603 were used as negative and positive controls for ESBL production, respectively.

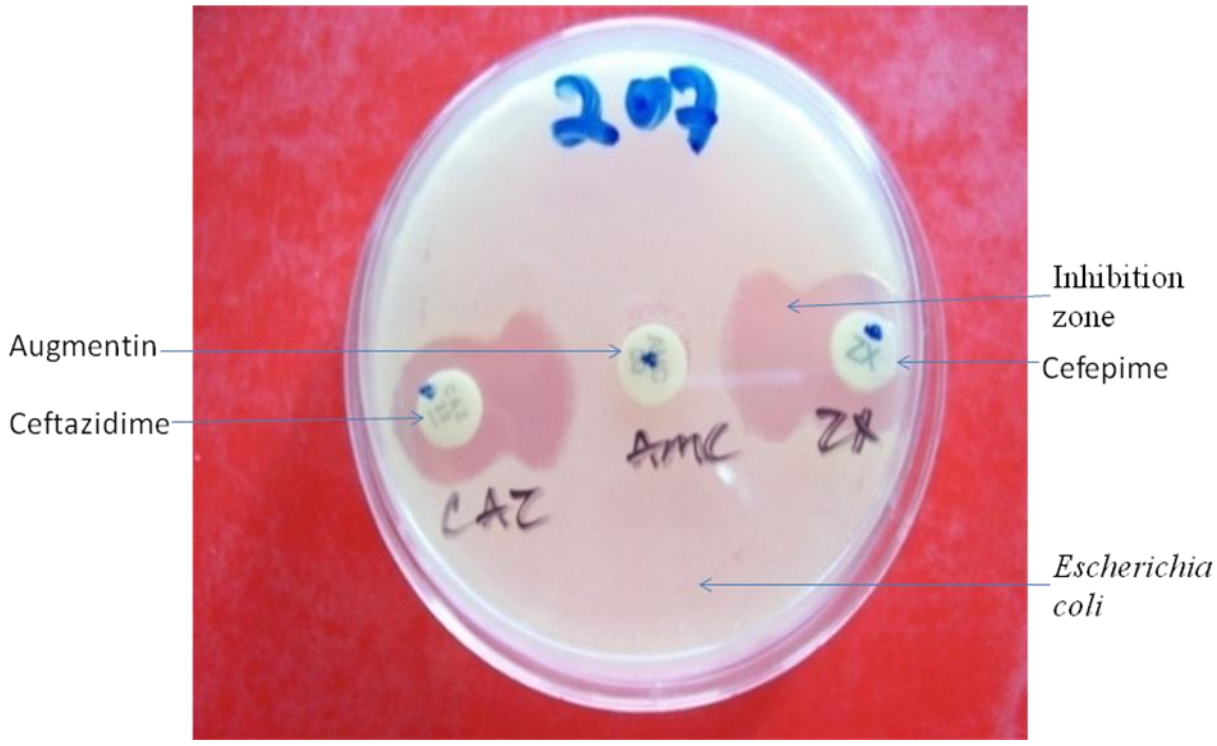

Plate 3. 1 *Escherichia coli* demonstrating zone of inhibition

The above plate demonstrates the production of extended spectrum β lactamase enzymes from *Escherichia coli* strain.

3.6.4 Detection of β-lactamase genes by Polymerase Chain Reaction

Bacterial DNA extract was obtained using boiling method as follows; DNA was prepared from overnight cultures grown on Mueller Hinton agar plates. The culture was suspended in1ml of sterile distilled water or in pure deionised sterile water and boiled at 100°C for 5 minutes. Upon boiling, the preparation was centrifuged at 14,000 rpm for 5 min. The supernatant was retained and stored at-20°C until further use while cell debris was discarded. The DNA extract was used as template in the specific PCR amplifications for detection of bla-$_{TEM}$ and bla-$_{SHV}$ genes. A total volume of 20µL master mix primer reaction was diluted as follows: (PCR mix, one tube) starting with distilled water- 1.6µl follwed by F primer (20pmol/µl) 0.2µl and R primer (20pmol/µl) 0.2µl then 2x Reddy mix (Thermo) 10.0µl and lastly the DNA 8.0µl was added. The PCR was performed with 5µl DNA from the sample and master mix comprising (*Taq* polymerase, dNNT, TEM and SHV (primers-Forward, Reverse) SHV F1-TCA GCG AAA AAC ACC TTG, SHV R1- TCC CGC AGA TAA ATC ACC, TEM F2-CTT CCT GTT TTT GCT CAC CCA, TEM R2-TAC GAT ACG GGA GGG CTT AC according to the manufacturer's instructions. The PCR conditions were as follows: 5 min at 95°C and 30 cycles of 1 min at 94°C for the denaturation of the template, 45 seconds at an annealing temperature of 58°C and 1 min 30 seconds at 72°C extension before a final extension at 72°C. The resulting PCR products were mixed with the loading dye and carefully loaded into the wells prepared in 1.5% agarose gel then analyzed by electrophoresis for 2 hours at 100 volts DC. The gel was stained with ethidium bromide. Photography of the gel was done by ultraviolet illumination using a transmitted illumination camera fitted with a Polaroid film (Brody and Kern, 2004).

3.6.5 Bacterial conjugation experiment

A half a litre brain heart infusion or Soya broth was prepared and dispensed into 3ml bijou bottles and labeled. Both donors (*E.coli*) and recipients' J53 (sodium Azide resistant F-) were grown in MacConkey or nutrient agar overnight. All the donors were resistant to Ampicillin (MIC= or ≥ 32µg/ml). Both donor and the recipient strains were sub-cultured into 3ml broth and incubated at 37°C for 3 hours in a rotating incubator or shaker. A fresh warm (37°C) broth was dispensed into 4.5ml sterile test tubes or universal containers. The donor and recipient cultures were diluted into 1:10 for example 0.5ml into 4.5ml fresh broth. The donor and recipient were mixed in equal proportions for example, 2ml and 2ml. Then one group of mixture was incubated at 37°C and the other at room temperature overnight. The cells were pelleted by centrifugation (13,000xg) for one minute in micro centrifuge (use 1.5ml tubes). The pellets were washed with sterile phosphate buffered saline by suspending cells using a vortex. The isolation of the conjugant was done using MacConkey media containing sodium azide and ampicillin (30µg/ml). The bacterial cells from each mixture were sub-cultured onto MacConkey agar containing 30µg/ml ampicillin and sodium azide to select transconjugant using sterile loop. Two control plates were used; MacConkey containing 30µg/ml ampicillin for *E.coli* and MacConkey agar containing sodium azide for J53 *E.coli*.

3.6.6 Plasmid extraction studies

The *E.coli* bacteria resistant to two or more antibiotics were selected for plasmid analysis using the protocol by Sambrook *et al*. (1989). Frozen bacteria at-70°C were revived by subculturing on MH agar and incubated at 37°C for 24 hours aerobically.

Plasmids from *E.coli*, V517 and R39 were used as controls. Briefly, a single bacteria colony was transferred into 2 ml of Luria Bertani (LB) medium containing ampicillin antibiotic in a loosely capped 15ml bottle to promote growth of R-plasmid containing isolates for analysis. The culture was incubated overnight at 37°C with vigorous shaking. Then culture was transferred into 1.5ml of microfuge tubes and centrifuged at 12,000g for 30 seconds at 4°C. The remainder of the culture was stored at 4°C. The medium was removed by aspiration leaving the bacterial pellet dry. Bacteria pellet obtained was resuspended in 100 µl of ice cold Solution I (as provided in the QIAprep®Spin MiniprepKit), vigorously vortexed followed by addition of 200 µl of freshly prepared solution II (as provided in the kit). Then 150µl of ice cold Solution III (as provided in the kit) was added and gently shaken. The solution was centrifuged at 12,000g for 5 minutes at 4°C and the supernatant was transferred to a fresh tube. The double stranded plasmid DNA was precipitated with 2 volumes of ethanol at room temperature, vortexed and allowed to stand for 2 minutes at room temperature. Centrifugation was then done at 12,000g for 5 minutes at 4°C and the supernatant was removed by gentle aspiration. The tube was placed in an inverted position to drain all the fluid. The pellet was rinsed with 1 ml of 70% ethanol at 4°C, the supernatant was removed and the pellet was air dried for 10 minutes. The nucleic acid was dissolved in 50µl of TE (pH 8.0) containing DNAse free pancreatic RNAase (20µg/ml). Brief vortexing was done and DNA stored at -20°C.

3.6.7 Gel electrophoresis

Plasmid DNA was analyzed by electrophoresis, the process included co-electrophoresis with plasmids of known sizes from *E.coli* strains V517 (NCTC 50193) and 39R861

(NCTC 50192) as controls and size markers. The Voltage was applied at 100 Volts DC. The image was digitized using a Polaroid film under ultraviolet light.

3.6.8 Preparation of agarose embedded deoxyribonucleic acid for pulsed field gel electrophoresis

The *E.coli* isolates were grown overnight on petri plates with the appropriate media to isolate a single colony. A suspension buffer 110µl was dispensed into an eppendorf tube and a single colony was emulsified. The bacterial cell suspension was mixed with agarose gel solution in equal proportion to give a final concentration 1% cell-gel mixture. A sterile pipette was used to transfer 100µl of the mixture into one well of the plug mold. Two wells per sample were filled and allowed the agarose to solidify for 30 to 45 minutes at room temperature. A sterile culture tube of 5ml was used for each sample and 1ml of Lysis Buffer and 40µl of Lysozyme was added to each tube. The mixture was gently mixed by inversion. The tape on the plug mold was removed and the solidified agarose plugs pushed into the 5ml sterile culture tubes containing the Lysis Buffer 1 and Lysozyme then incubated for 1 to 2 hours at 37°C without agitation. The Lysis Buffer 1 was aspirated and the plugs rinsed with 1.5ml wash Buffer. Wash Buffer x1 was removed and 1ml of proteinase K Buffer and 40µl of proteinase K solution were added into the tubes. The mixture was gently inverted and incubated for 16-20 hours (overnight) at 50°C with agitation. Proteinase K solution was aspirated and washed the plugs in 1x Wash Buffer for 30 to 60 minutes at room temperature with gentle agitation on a rocker. The 1x Wash Buffer was aspirated and 1.5ml of 1x Wash Buffer and 20µl 100mM PMSF were added into the tubes. The mixture was washed for 30-60minutes at room temperature with gentle agitation on rocker. The above was repeated. The solution

was aspirated and 1ml x Wash buffer added into the tubes. The plugs were stored at 4°C and they are stable for 3-6 months.

3.6.9 Deoxyribonucleic acid analysis by field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed as described by Gautom, (1997). The DNA of all isolates was digested using *Xba*I (New England BioLabs, Beverly, Mass, USA) at 37°C for 4 hours, according to supplier's instructions. The slices of the digested DNA were loaded into the wells of the poured gels. Electrophoresis agarose gel (Promega, Madison, USA) with a concentration of 1% was used for organisms in 0.5 × Tris-Borate-EDTA buffer using contour–clamped homogenous electric field apparatus (CHEF-DR III; Bio-Rad, Richmond, Calif., USA). A 48.5 kb lambda PFGE marker 50 µg/ml (New England BioLabs) was used as a marker. The conditions for electrophoresis were angle 120° gradient, 6 V/cm; temperature, 14C; pulse times ranging from 10 to 45 s and running time was 20 hours. The photography patterns were interpreted as described by Tenover *et al*. (1995).

3.7 Ethical consideration

The approval to carry out this study was given by the Kenya Medical Research Institute (KEMRI) Ethical Committee (Appendix 4) and Jomo Kenyatta University Agriculture and Technology. The permission to obtain isolates was sought from the Standard and Ethics Sub-Committee of Mater Hospital. The sample collection and primary isolation was done at Mater Hospital diagnostic laboratory**.** The collection of isolates started immediately the approval was given by KEMRI and the collection lasted for five months. The study isolates were delinked from patient information. The isolates were stored without any patient identifiers but recorded as *E.coli*. The investigator was not

involved in isolation of the organisms, but collected the isolates for further analysis at KEMRI.

3.8 Expected benefits of the research

The data generated from this study will help in the management of UTI caused by *E.coli*. This will reduce the cost of hospitalization by ensuring that only effective drugs are prescribed. The data from this work will provide a platform for advocacy for evidence-based prescription.

3.9 Data Management and Analysis

Laboratory procedures and results were recorded in the laboratory note book. All the data was transferred to Microsoft office excel 2007 linked software and saved in the hard disk drive and flash disks. The notebook, filed sheets were kept securely in a lockable cabinet. A password was used for the data in the computer hard disk drive. Data presentation was done by use of bar graphs and tables where graphs were not applicable. Chi-square was used for the test of association. Data analysis of the collected data was done by use of Statistical Package for Social Sciences (SPSS) software 12.

CHAPTER FOUR

4.0 RESULTS

4.1 Bacterial isolates and antimicrobial susceptibility profile

A total of three hundred and eighty four (384) *E.coli* isolates were isolated from patients suspected of having urinary tract infection. The antimicrobial susceptibility is as shown in Table 3. Susceptibility to cefepime was (86.7%), aztreonam was (82.6%), nitrofuranto in was (81.3%), ceftazidime was (80.0%), ceftriazone was (73.5%), gentamycin was (73.4%), ciprofloxacin was (63.0%), cefuroxime was (59.1%), chloramphenicol was (57.8%) and nalidixic acid (55.5%). The highest resistant rates of these Gram negative isolates were found against ampicillin (81.3%), cotrimoxazole (75.0%), tetracycline (68.8%), augmentin (52.3%) and streptomycin (56.5%) as shown in Figure 4.1.

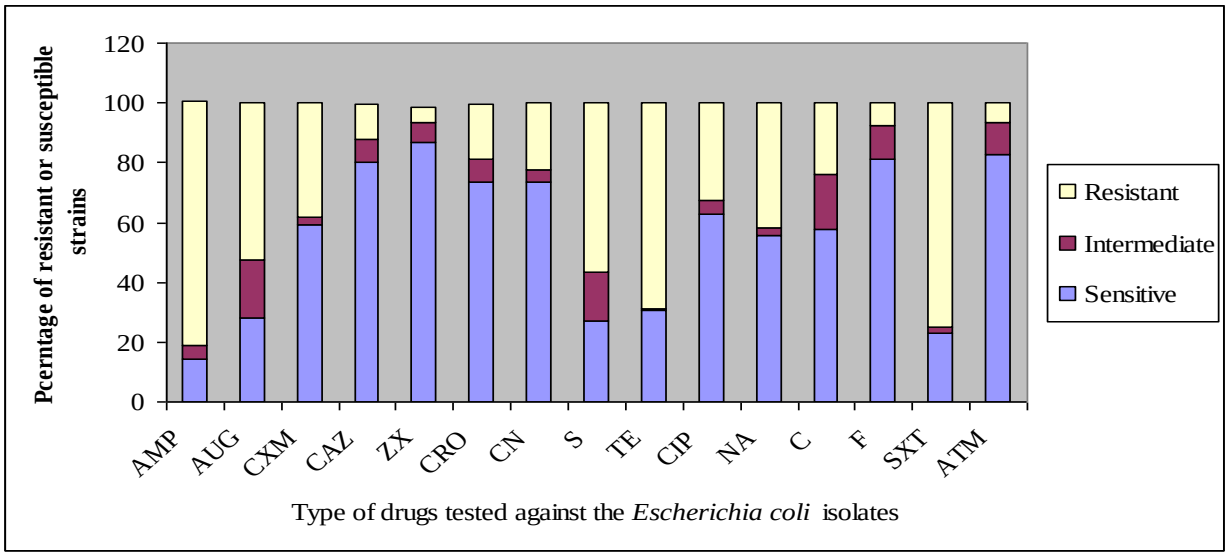

Figure 4. 1 Antimicrobial susceptibility profile for Escherichia coli isolates

AMP - ampicillin, AUG - augmetin, CXM - cefuroxime, CAZ - ceftazidime, ZX - cefepime, CRO - ceftriazone, CN - gentamycin, S - streptomycin, TE -tetracycline, CIP- ciprofloxacin, NA - Nalidixic acid, C - chloramphenicol, F - nitrofurantoin, SXT - cotrimoxazole and ATM aztreonam.

The *E.coli* isolates were sensitive to cephalosporins, nitrofurantoin, gentamycin and monobactams but resistant to β-lactams, streptomycin, tetracycline and cotrimoxazole Table (4.1).

Table 4. 1 Antimicrobial susceptibility profile for *Escherichia coli* isolates

Percentages, resistant, intermediate and the sensitivity of *E.coli* isolates from urine (=384).

Sensitive	(%)	Intermediate	(%)	Resistant	(%)	Antibiotics
54	14.1	18	5.0	312	81.3	Ampicillin (10µg)
107	27.9	76	19.8	201	52.3	Augmentin (20/10 µg)
227	59.1	11	2.9	146	38.0	Cefuroxime (30 µg)
307	80.0	29	7.6	48	12.0	Ceftazidime (30 µg)
333	86.7	30	6.6	21	5.5	Cefepime (30 µg)
284	73.5	30	7.8	70	18.2	Ceftriazone (30 µg)
282	73.4	16	4.2	86	22.4	Gentamycin (10µg)
105	27.3	62	16.1	217	56.5	Streptomycin (10µg)
117	30.5	3	0.8	264	68.8	Tétracycline (30µg)
242	63.0	16	4.2	126	32.8	Ciprofloxacin (5 µg)
213	55.5	10	2.6	161	41.9	Nalidixic Acid (30µg)
222	57.8	71	18.5	91	23.7	Chloramphenicol (30µg)
312	81.3	42	10.9	31	8.1	Nitrofurantoin (300 µg)
88	23.0	6	2.0	290	75.0	Cotrimoxazole(1.25/23.75
317	82.6	41	10.7	26	6.8	Aztreonam (30 µg)

Key- %, µg

Of the three hundred and eighty four (384) *E.coli* isolates, fifty six 56(14.6%) positively expressed ESBL enzymes. The antimicrobial susceptibility patterns of ESBL producing *E.coli* showed that the resistance was higher to the cephalosporins, ampicillin, nalidixic acid, ciprofloxacin and cotrimoxazole **(**Figure 4.2). Overall the prevalence of ESBL producing *E.coli* (14.6%) was of serious concern as compared to non-ESBL (85.4%) since the ESBL producing *E.coli* had a wide range of resistance to many antibiotics.

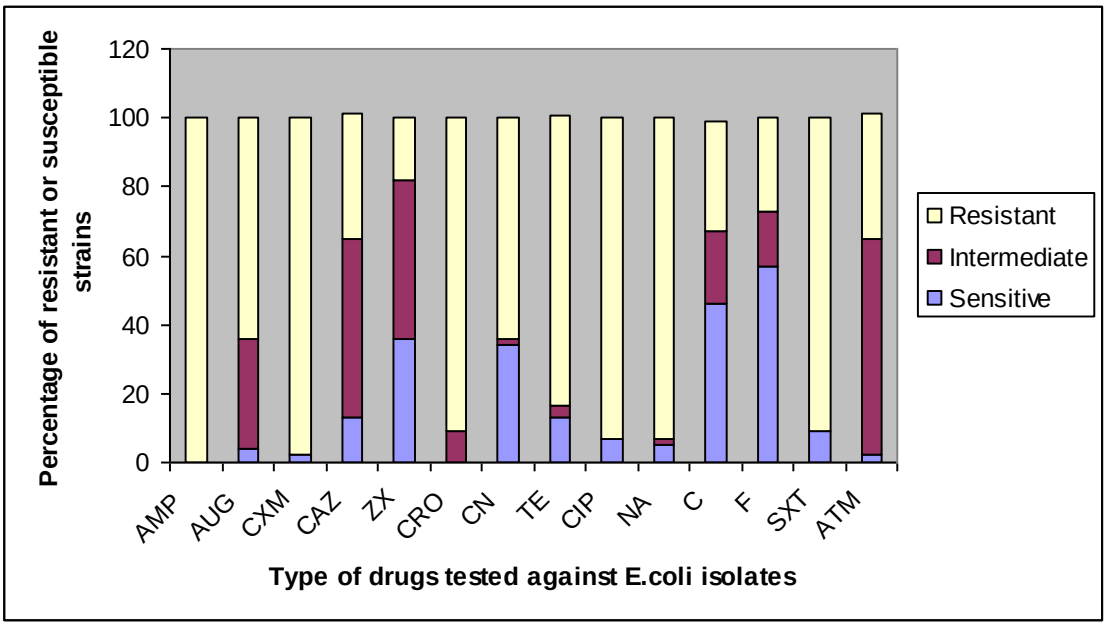

Figure 4. 2 Antimicrobial susceptibility profile for β-lactamase producers among *Escherichia coli* isolates

AMP - ampicillin, AUG - augmetin ,CXM - cefuroxime, CAZ - ceftazidime, ZX - cefepime, CRO - ceftriazone, CN - gentamycin, S - streptomycin, TE - tetracycline, CIP - ciprofloxacin, NA - nalidixic acid, C - chloramphenicol, F - nitrofurantoin, SXT - cotrimoxazole and ATM - aztreonam.

The extended spectrum β-lactamase producers among *Escherichia coli* isolates were resistant to cephalosporins, fluoroquinolones and β-lactams but susceptible to nitrofurantoin and chloramphenicol. The intermediate results were none on ampicillin, cotrimoxazole, ciprofloxacin and very low on nalidixic acid, gentamycin, tetracycline and ceftriazone respectively as shown in Table 4.2.

Table 4. 2 Susceptibility tests for β-lactamase producers among *Escherichia coli* isolates

Percentages for *Escherichia coli* isolates, ESBL producers (=56).

Sensitive	(%)	Intermediate	(%)	Resistant	(%)	Antibiotics
2	4	18	32	36	64	Augmentin (20/10 µg)
1	2	-	-	55	98	Cefuroxime (30 µg)
7	13	29	52	20	36	Ceftazidime (30 µg)
20	36	26	46	10	18	Cefepime (30 µg)
-	-	5	9	51	91	Ceftriazone (30 µg)
7	13	2	3.5	47	84	Tetracycline (30µg)
4	7	-	-	52	93	Ciprofloxacin (5 µg)
3	5	1	2	52	93	Nalidixic Acid (30µg)
19	34	1	2	36	64	Gentamycin (10µg)
26	46	12	21	18	32	Chloramphenicol (30µg)
32	57	9	16	15	27	Nitrofurantoin (300 µg)
5	9	-	-	51	91	Cotrimoxazole (1.25/23.75)
1	2	35	63	20	36	Aztreonam (30µg)
-	-	-	-	-	100	Ampicillin (30µg)

Key- %, µg, ESBL, *E.coli*

The suspected positive ESBL isolates were confirmed phenotypically by disk diffusion technique and identified by formation of phantom or a clear zone of inhibition (Plate 4.1).

Plate 4. 1 Disk confirmation test for extended spectrum β-lactamase enzymes

A double disk approximation or a double disk synergy test detection of ESBL producing bacteria. The clear zone (*phantom*) illustrates the presence of enzymes capable of hydrolyzing and inactivating a wide variety of β-lactam antibiotics, including third-generation cephalosporins, penicillins and aztreonam.

The ESBL and non-ESBL *Escherichia coli* isolates were analyzed using (Chi-square) χ^2 test. The difference in resistance levels between ESBL and non-ESBL producing isolates for all the antimicrobial drugs were statistically significant, $p<0.05$ as shown in Table 4.3.

Table 4. 3 Antimicrobial susceptibility patterns for β-lactamases and non-β-lactamases producing Escherichia coli isolates from urine

	ESBL N=56 (14.6%)	Non-ESBL N=328 (85.4%)		
Antimicrobial drugs	**% Resistant**	**% Resistant**	**χ^2**	**P-value**
Augmentin(20/10µg)	36(64)	165(50.3)	χ^2=4.55	**p<0.032**
Cefuroxime 30µg)	55(98)	91(28.0)	χ^2=114.51	**p<0.001**
Ceftazidime (30 µg)	20(36)	28(8.5)	χ^2=22.73	**p<0.001**
Ceftriazone (30µg)	51(91)	19(5.8)	χ^2=151.0	**p<0.001**
Tetracycline (30µg)	47(84)	217(66.0)	χ^2=14.21	**P<0.001**
Ciprofloxacin (5µg)	52(93)	74(22.5)	χ^2=102.62	**p<0.001**
Nalidixic acid (30µg)	52(93)	109(33.0)	χ^2=81.17	**p<0.001**
Cotrimoxazole (1.25/23.75µg)	51(91)	239(73.0)	χ^2=19.60	**p<0.001**
Ampicillin (30µg)	56(100)	256(78.0)	χ^2=24.60	**p<0.001**

Key - µg, %, χ^2

4.2 Antimicrobial susceptibility profile for *E.coli* transconjugants

The sensitivity tests for the fifty one (51) transconjugants were done for twelve (12) antibiotics. The majority of the isolates were sensitive to the antimicrobial drugs nitrofurantoin (98%), chloramphenicol (75%), augmentin (73%), ciprofloxacin (73%), nalidixic acid (67%), cotrimoxazole (59%), cefepime (53%) and ceftazidime (51%) that

are available in most hospitals. Nevertheless, these transconjugant isolates were highly resistant to ampicillin (98%), tetracycline (92%), cefuroxime (82%) and ceftriazone (78%) as shown in Table 4.4.

Table 4. 4 Antimicrobial susceptibility profile for transconjugant *Escherichia coli* isolates

Percentages of resistant, intermediate and sensitivity for Tranconjugant *E.coli* isolates.

Organisms	Sensitive	(%)	Intermediate	(%)	Resistant	(%)	Antibiotics
51	37	73	7	14	8	16	Augmentin (20/10 µg)
51	5	10	-	-	42	82	Cefuroxime (30 µg)
51	26	51	15	29	11	22	Ceftazidime (30 µg)
51	27	53	12	24	13	25	Cefepime (30 µg)
51	5	10	7	14	40	78	Ceftriazone (30 µg)
51	5	10	-	-	47	92	Tetracycline (30µg)
51	37	73	-	-	15	29	Ciprofloxacin (5 µg)
51	35	67	2	4	15	29	Nalidixic Acid (30µg)
51	38	75	2	4	12	24	Chloramphenicol (30µg)
51	50	98	1	2	1	2	Nitrofurantoin (300 µg)
51	30	59	-	-	22	43	Cotrimoxazole (1.25/23.75
51	2	4	-	-	50	98	Ampicillin (30 µg)

Susceptibility testing for the transconjugants

Key - No.organisms, %, µg, *E.coli*

The transconjugants and the donor *Escherichia coli* isolates were extracted for plasmid DNA analyses. Two controls *E.coli* R39 and *E.coli* V517 were ran concurrently with the test samples. The pattern showed that the majority of the donor strains had exchanged their genetic material to the recipients (Plate 4.2). The transconjugants had plasmid DNA of molecular weight of 43bp.

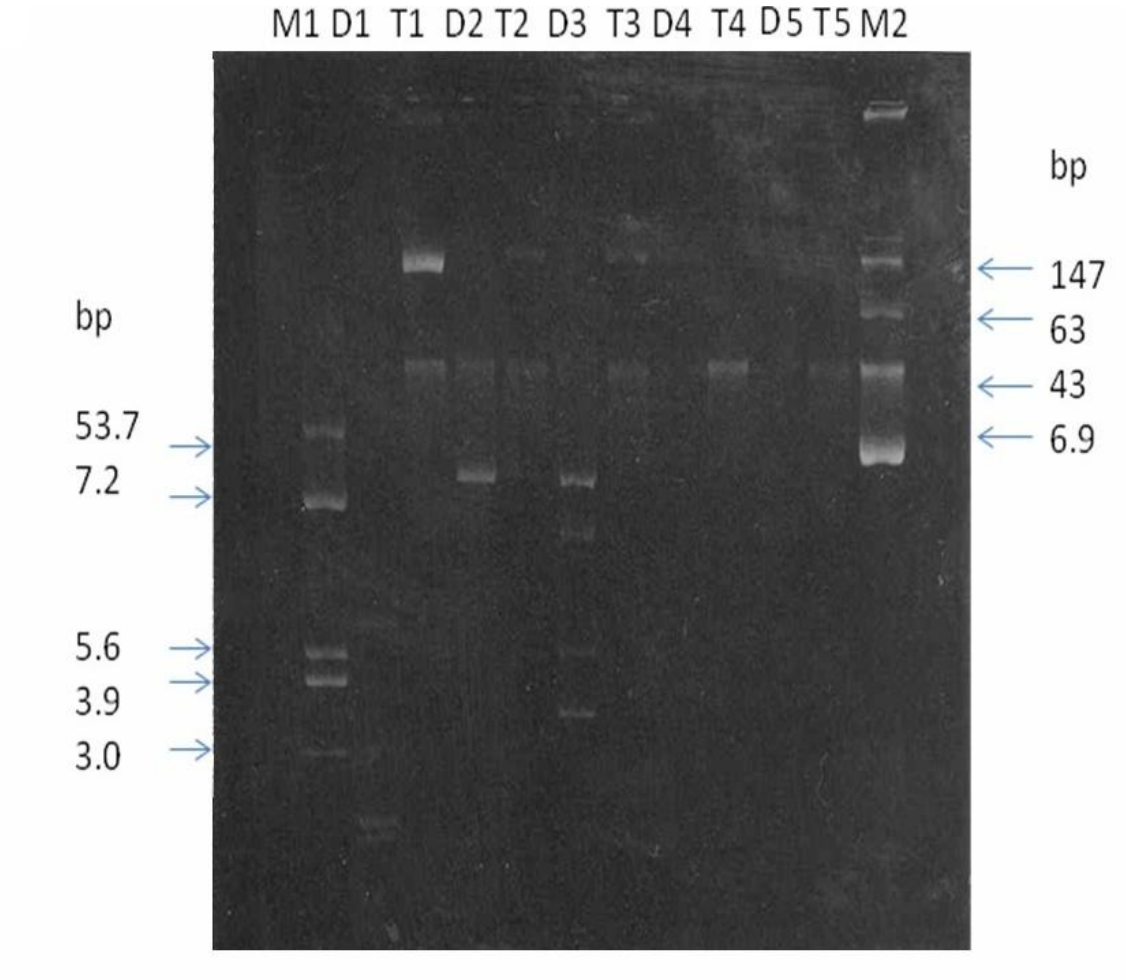

Plate 4. 2 Plasmid analysis for the resistant Escherichia coli isolates

Transconjugants and *Escherichia coli* donor strains; Lanes M1 and M2 control makers *E.coli* R39, *E.coli* V517 with standard molecular weights respectively; Lanes D1- D5 represents donor strains, plasmid 3.0 to 43bp; lanes T1-T5 represents transconjugants, plasmid-43bp.

Of the fifty one isolates, twenty two isolates were selected at random and analyzed for ampicillin resistance and thirteen had transferred their plasmids 43bp to J53 *E.coli* but nine did not transfer their genes (Table 4.5). Some isolates had one or two bands arranging from (3.0-43.0bp) as compared to the molecular markers (39R861) and (V517).

Table 4. 5 Donor and transconjugant plasmid profile for deoxyribonucleic acid

Org No.	Donor	positive	negative	Transconj.	positive	Negative	Control R39	Control V517
1	27	2	0	27	2	0		
2	113	1	0	113	1	0		
3	114	2	0	114	2	0	4	7
4	128	2	0	128	0	0		
5	131	1	0	131	0	0		
6	160	7	0	160	7	0		
7	223	0	0	223	0	0		
8	224	2	0	224	2	0		
9	236	5	0	236	6	0		
10	293	5	0	293	5	0	4	7
11	295	1	0	295	0	0		
12	301	2	0	301	3	0	3	5
13	303	2	0	303	2	0		
14	307	1	0	307	0	0		
15	309	0	0	309	0	0		
16	318	3	0	318	3	0		
17	319	1	0	319	0	0		

Key – No. organisms, R39, V517, donor, transconjugant

The ESBL-producing *E.coli* were identified by PCR that revealed the presence of TEM genes as demonstrated on (Plate 4.3). All the lanes with bright bands represent the targeted TEM genes. The banding patterns across had similar molecular weight of 50 - 100bp. Out of 56 isolates that exhibited ESBL production, nine (16.1%) had both TEM and SHV and twelve (21.4%) had TEM alone.

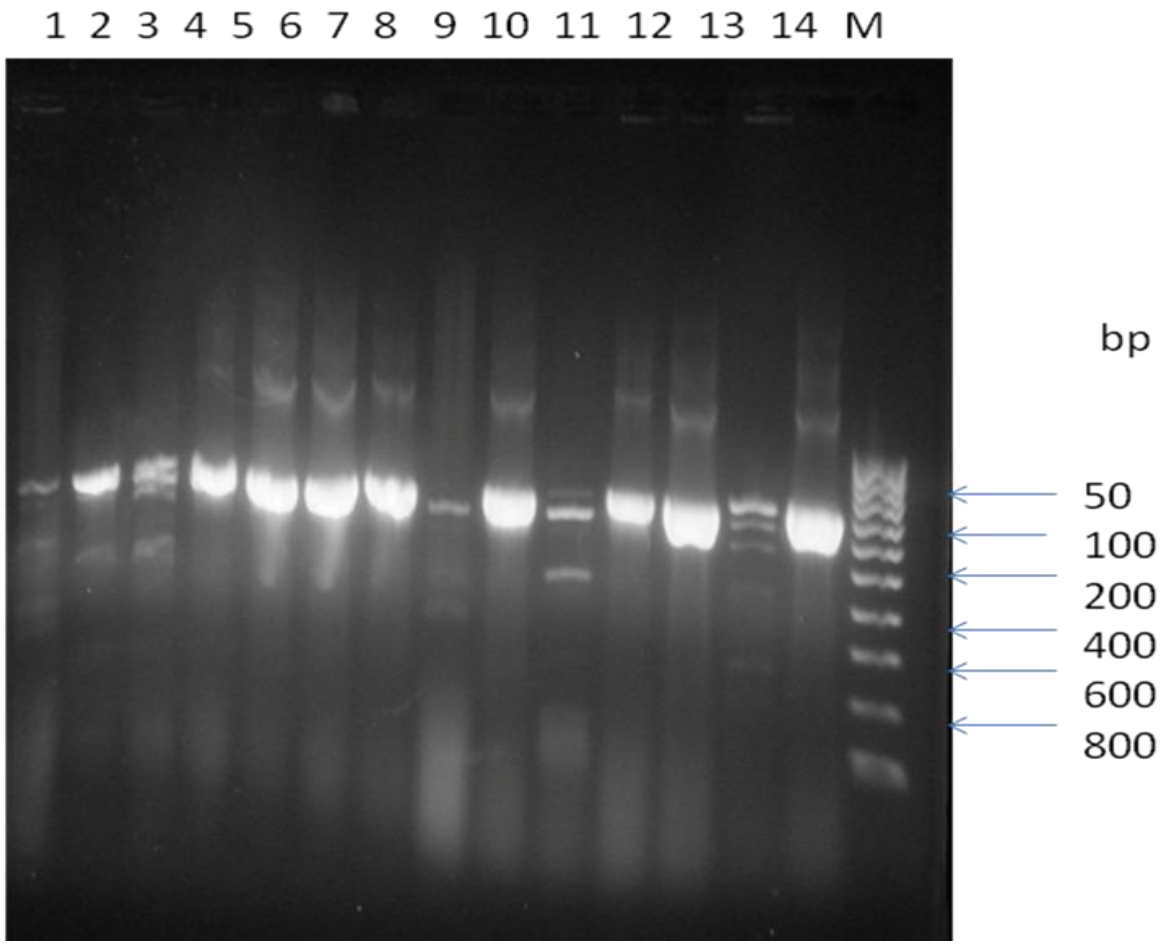

Plate 4. 3 Identification of genotypic resistant Escherichia coli isolates

Identification of antibiotic resistant *E.coli* strains that produce extended spectrum beta lactamases using PCR; Lane M, 100bp DNA ladda; lane 1-14, represent ESBL producing *Escherichia coli*; TEM 50-100bp.

PFGE was performed for thirteen non-ESBL *Escherichia coli* isolates using X-ba1. The PFGE bandings had no similar pattern thus suggested the isolates came from different sources. The banding patterns were indistinguishable and consisted of 10 to 19 bands ranging from 97.0 to 873.0bp of molecular weights. These *E.coli* strains were uniquely unrelated and therefore genetically different from each other (Plate4.4).

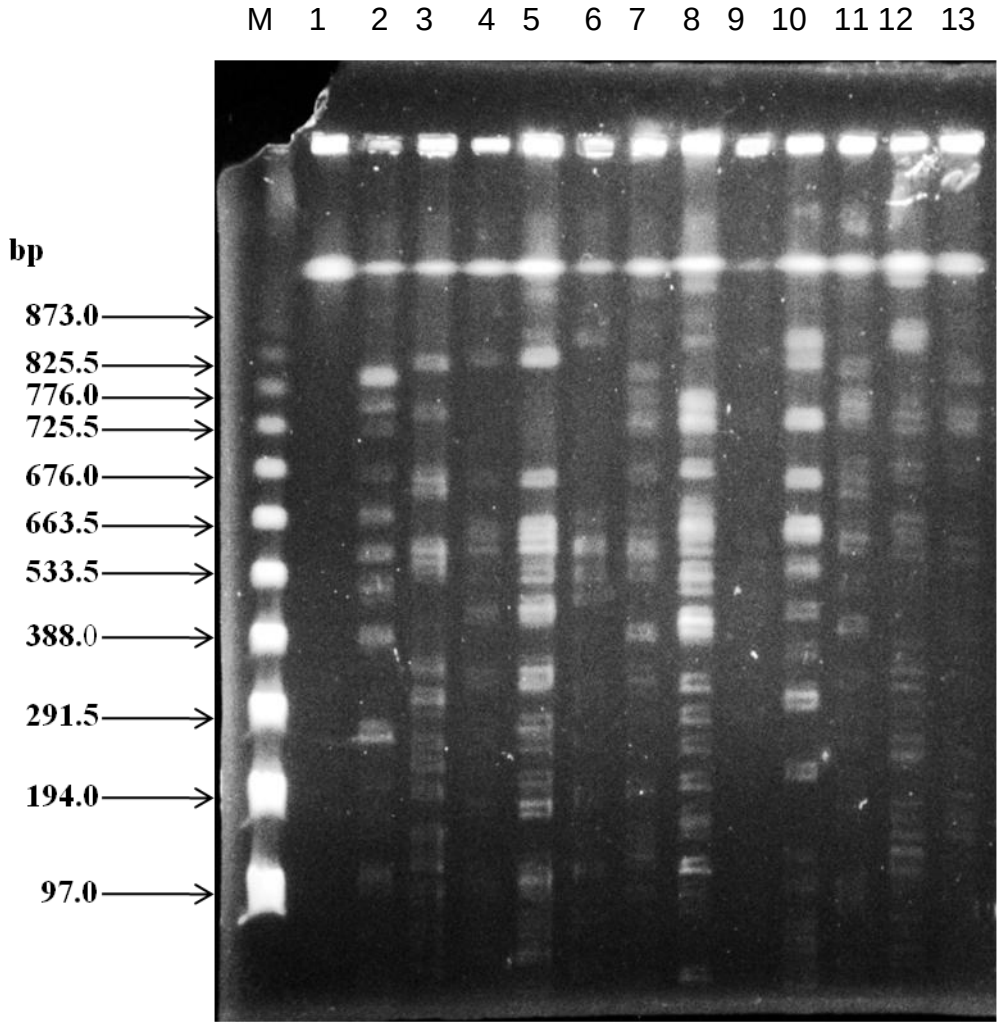

Plate 4. 4 ***Escherichia coli*** **isolates from urine showing diverse molecular weights**

Lane M, H9812 (Salmonella Braender-up standard for PFGE); Lane 1-13, represents *Escherichia coli* from urine cultures; (based on criteria 3-5 band differences), lane 1 and 9 were related strains; lanes 2 and 7 were related strains; lanes 4 and 6 were related. strains; lanes 10 and 12 were related strains; lanes 11 and 13 were related strains.

The Pulse field gel electrophoresis (PFGE) revealed that this collection of *E.coli* were genetically diverse as expected since the majority of isolates came from community-acquired infections where patients were unlikely to be related demographically (Table 4.6).

Table 4. 6 Chromosomal deoxyribonucleic acid molecular weights data analysis

Isolates	No of bands	Molecular weight	marker H9812	No of bands	Molecular weight
1	1	>873.0	M	11	97.0- 873.0
2	13	97.0-776.0	M	11	97.0- 873.0
3	16	97.0- 825.5	M	11	97.0- 873.0
4	10	194.0-776.0	M	11	97.0- 873.0
5	17	97.0- 873.0	M	11	97.0- 873.0
6	9	97.0- 873.0	M	11	97.0- 873.0
7	13	194.0-825.5	M	11	97.0- 873.0
8	19	97.0- 873.0	M	11	97.0- 873.0
9	1	>873.0	M	11	97.0- 873.0
10	16	97.0- 825.5	M	11	97.0- 873.0
11	10	97.0-725.5	M	11	97.0- 873.0
12	17	97.0- 873.0	M	11	97.0- 873.0
13	10	194.0-776.0	M	11	97.0- 873.0

Key –No's 1-13 (*E.coli*) isolates, M- marker-H9812, >

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

This is one of the few studies that has evaluated the magnitude of ESBL producing isolates and characterized the genetic profile among *E.coli* isolates obtained from patients suspected with UTI. Overall the prevalence of ESBL producing *E.coli* (14.6%) is of serious concern owing to the fact that, it predisposes risk factors of transferring resistant to other species of bacteria. The increasing prevalence of infections caused by antibiotic-resistant bacteria makes the empirical treatment of UTI difficult and outcome unpredictable. Despite the rise in the prevalence of ESBL producing *E.coli* in some countries (AitMhand *et al*., 2002) there are very few reports from Africa and especially Kenya (Kariuki *et al*., 2007; Blomberg *et al*., 2005). In developing countries, Kenya included, detection of ESBL is not commonly carried out in many microbiology units due to lack of resources and facilities for conducting ESBL identification. Thus the information on infections caused by ESBL producing organisms is limited particularly in our settings and many clinicians have not fully appreciated the immense significance of detecting ESBL.

In Kenyan hospitals, augmentin, ceftazidime and ceftriaxone are the most frequently prescribed β-lactams for UTI because of their availability in oral and intravenous formulations. In this study, ESBL producing isolates were significantly more resistant to ciprofloxacin, ceftriazone, nalidixic acid, augmentin, ciprofloxacin and ceftazidime ($p \leq 0.001$) as compared to non-ESBL producing Gram-negative isolates. A study conducted by Chun-Yu *et al*. (2008) reported that resistance of *E.coli* to ciprofloxacin

was still very low at less than 3%. But in Kenya uropathogenic *E.coli*, ESBL producers, are also resistant to various classes of antibiotics (Kariuki *et al*., 2007). Similar studies have reported cross-resistance to aminoglycosides, fluoroquinolones and trimethoprim/sulfamethoxazole in ESBL producing organisms (Procop *et al.*, 2003; Alhambra *et al.*, 2004). The high prevalence of co-resistance has led to the limited use of commonly available antibiotics, leading to the need for more expensive treatment options that have impacted negatively on clinical outcomes (Menashe *et al.*, 2001).

The present study has conclusively shown that the presence of TEM and SHV genes prevails in Mater hospital. The resistant levels of ESBL producers are a major threat to infection management as this may have contributed to the antimicrobial drug resistance reported in this study as compared to non-ESBL producers. One of the dilemma of ESBL producing organism is that they are frequently resistant to antibiotics other than beta lactams as they contain plasmids with genes that encode resistance to aminoglycosides, quinolones and cotrimoxazole. These genes are not only widespread in African countries but, in European and Asian countries including Northern Italy **(**Pagani *et al*., 2003), United Kingdom (Mushtaq *et al*., 2003) and India (Karim *et al*., 2001).

From the study,ESBLproducing *E.coli* isolates were preferably susceptible to nitrofurantoin and chloramph-enicol antimicrobial drugs. This finding concurred with a study done in Nigeria by Iroha *et al.* (2009) which advised limited use of any cephalosporin antibiotics on an ESBL positive *E.coli* infection. Use of fosfomycin and nitrofurantoin as the best choice for empiric treatment against ESBL producers was also

recommended in a study conducted in Spain by Tena *et al*. (2010). Since *E.coli* isolates showed high prevalence of resistance to various antibiotics, strategies to control the increase in resistant *E.coli* strains would be important. Although, the frequency of ESBL–producing isolates is increasing worldwide, the rate of infection can only be minimized by regular surveillance and monitoring in order to institute effective and credible treatment and management of UTI.

The confirmation of the ESBL production was performed using PCR. This method was able to determine the specific TEM and SHV genes although sequencing could have been the best tool for the analysis. The existence of the two dominant genes TEM and SHV presumes that these could have been the major cause of resistance in patients with UTI. The total number of isolates that had either TEM or SHV was twenty one (37%). The TEM genes were more common than SHV genes. Moreover, various non-TEM, non-SHV-type class A β-lactamases exhibiting extended-spectrum activities, including CTX-M-type were not captured in this study. On the other hand, PFGE restriction profiles of the twenty six isolates were digested using enzymes XbaI. The pattern consisted of 10 to 16 bands ranging from 97.0 to 873 kb. The PFGE revealed that this collection of *E.coli* strains were different as expected since the majority of isolates came from community-acquired infections where patients were unlikely to be related demographically.

The *E.coli* strains investigated in this study were genetically diverse and had come from different sources other than cross infection with in the hospital setting. This collection of *E.coli* isolates showed different number of bands as analyzed by pulse field gel

electrophoresis. The ESBL phenotype with multiple enzymes were found in this hospital in different strains of uropathogenic *E.coli* having similar patterns but with varying molecular weights. The findings also suggested that the resistance to most antibiotics was not spread by clonal dissemination but more likely occurred as an independent phenomenon in each strain as a response to antimicrobial pressure. It is also thought that multidrug resistant ESBL isolates spread their resistant through exchange of their genetic materials (TEM-1 and SHV) between Gram negative bacteria. In general, the resistant caused by ESBL producers indicates the failure of antibiotic treatment that results to enzyme production that are able to hydrolyze or inactivate the majority of the antimicrobial drugs rendering them ineffective. More studies are needed to establish other resistant ESBL genes which were not captured in this study and a further need to explain these findings.

5.1 Conclusion

The multidrug resistant ESBL producing *E.coli* isolates were present in this hospital with a prevalence of 14.6% that suggests the spread of resistant among the population of uropathogenic *E.coli* isolates. This prevalence appears to be low but may lead to treatment failure of UTI.

There were two predominant genes TEM and SHV among *E.coli* uropathogenic strains circulating in this hospital, presumably, this may have been the cause of resistance among these pathogens. Therefore, it is crucial to identify and prevent the spread of antimicrobial resistant in ESBL producing *E.coli* in hospital settings.

The antibiogram patterns, PCR and PFGE of uropathogenic *E.coli* showed that these pathogens were genetically diverse and pose a major challenge in the treatment UTI in this hospital.

5.2 Recommendations

The clinical laboratories should develop a habit of screening all uropathogenic *E.coli* to determine the ESBL producers in order to advise the hospital the way forward to managing the UTI patients.

The extended spectrum lactamase producers should not be treated with the third or fourth generation of cephalosporins, quinolones, fluoroquinolones and β lactams but instead use other therapeutic alternatives such as nitrofurantoin, chloramphenicol and gentamycin that are susceptible to ESBL producers.

Hospitals should have a monitoring and surveillance policy in case of any prevailing mutant strains in the hospital to minimize the spread of resistant among uropathogenic *E.coli.*

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APPENDICES

APPENDIX 1 Antimicrobial drugs and their sensitivity profiles

Antibiotics	Sensitive	Intermedite	Resistant
Penicillin			
Ampillicin (Amp)	≥17	14-16	≤13
β-lactam/β-lactamase inhibitors			
Amoxillin-clavulanic Acid (AUG)	≥18	14-17	≤13
Cephalosporin			
Cefuroxime (Cxm)	≥18	15-17	≤14
Ceftazidime (CAZ)	≥18	15-17	≤14
Ceftriaxone (CRO)	≥21	14-20	≤13
Cefepime (FEP)	≥18	15-17	≤14
Nitrofurantoin			
Nitrofurantoin (F)	≥17	15-16	≤14
Folate Pathway Inhibitors			
Trimethoprim/Sulphamethoxazole (SXT)	≥16	11-15	≤11
Monobactams			
Aztreonam (ATM)	≥22	16-21	≤15
Tetracycline			
Tetracycline (TE)	≥15	12-14	≤11
Aminoglycoside			
Gentamycin (CN)	≥15	13-14	≤12
Streptomycin (S)	≥15	12-14	≤11
Fluoroquinolones			
Ciprofloxacin (CIP)	≥21	16-20	≤15
Quinolones			
Nalidixic Acid (NA)	≥19	14-18	≤13
Phenicols			
Chloramphenicol(C)	≥18	13-17	≤12

Key - ≥ ≤

APPENDIX 2 Antimicrobial susceptibility tests for extended spectrum β-lactamase isolates

ORG No.	AUG	CXM	CAZ	FEP	CRO	CN	S	TE	CIP	NA	C	F	SXT	ATM	AMP	ENZYMES
16	R	R	I	R	R	R	S	S	S	S	S	I	R	I	R	ESBL
24	R	R	S	R	R	R	R	S	R	R	I	S	R	I	R	ESBL
27	R	R	R	R	R	S	S	R	R	R	R	I	R	R	R	ESBL
39	R	R	I	R	R	S	S	R	R	R	S	S	R	R	R	ESBL
104	R	R	I	I	R	R	R	R	S	S	R	S	R	R	R	ESBL
114	R	R	R	R	R	R	S	S	R	R	R	I	R	I	R	ESBL
128	R	R	R	S	R	R	S	R	R	R	S	S	R	I	R	ESBL
129	R	R	I	I	R	R	S	R	R	R	S	S	R	R	R	ESBL
131	R	R	R	S	R	R	R	R	R	R	S	S	R	I	R	ESBL
132	R	R	I	S	R	R	R	R	R	R	I	I	R	I	R	ESBL
143	R	R	R	S	R	S	I	R	R	R	R	S	R	I	R	ESBL
147	R	R	S	S	I	S	R	S	S	I	R	I	R	I	R	ESBL
158	R	R	R	S	R	R	R	R	R	R	R	S	R	I	R	ESBL
160	R	R	S	I	R	S	R	S	S	S	S	S	R	R	R	ESBL
207	R	R	R	I	R	S	R	R	R	R	R	S	R	R	R	ESBL
210	R	R	R	S	R	R	R	R	R	R	R	I	R	I	R	ESBL
216	R	R	R	R	R	R	R	R	R	R	S	S	R	R	R	ESBL
223	R	R	R	I	R	S	S	R	R	R	S	S	R	I	R	ESBL
224	R	R	R	R	R	R	R	R	R	R	S	S	R	I	R	ESBL
236	R	R	R	R	R	R	R	R	R	R	S	S	S	I	R	ESBL
237	R	R	R	R	R	R	I	R	R	R	S	S	S	I	R	ESBL
255	R	R	R	S	R	R	R	S	R	R	R	S	R	R	R	ESBL
275	I	R	R	I	R	R	R	R	R	R	I	R	R	I	R	ESBL
277	I	R	R	S	R	R	R	R	R	R	R	R	R	I	R	ESBL
278	I	R	I	S	R	R	R	R	R	R	R	R	R	R	R	ESBL
279	I	R	I	I	R	R	S	R	R	R	I	S	R	I	R	ESBL
280	S	R	I	S	R	R	S	R	R	R	R	R	R	I	R	ESBL
284	I	R	S	S	R	S	S	R	R	R	S	I	R	R	R	ESBL
286	R	R	I	S	R	R	S	R	R	R	R	I	R	R	R	ESBL
287	S	R	I	S	R	S	S	S	R	R	S	S	R	I	R	ESBL
289	R	R	R	I	R	S	S	R	R	R	I	R	R	R	R	ESBL
290	R	R	R	R	R	R	S	R	R	R	R	S	S	R	R	ESBL
291	I	R	R	I	I	R	R	S	R	R	S	R	R	I	R	ESBL
293	I	R	I	S	R	R	S	R	R	R	S	R	R	I	R	ESBL
295	I	R	R	I	R	S	S	R	R	R	I	I	R	I	R	ESBL
296	I	R	R	R	R	S	I	R	R	R	R	R	R	R	R	ESBL
299	R	R	R	S	I	R	R	R	R	R	I	S	R	I	R	ESBL
301	I	R	R	I	R	R	I	R	R	R	I	S	R	I	R	ESBL
303	I	R	I	S	R	R	S	R	R	R	I	S	R	I	R	ESBL
304	R	R	R	R	R	R	I	R	R	R	R	S	R	R	R	ESBL
305	R	R	S	S	R	R	S	I	R	R	S	S	S	I	R	ESBL
306	I	R	R	I	R	R	S	R	R	R	S	S	S	I	R	ESBL
307	I	R	I	S	R	R	S	R	R	R	R	R	R	I	R	ESBL
309	I	S	R	I	R	R	S	I	R	R	I	S	R	I	R	ESBL
318	R	R	R	R	R	S	S	R	R	R	R	S	R	R	R	ESBL
319	R	R	S	S	R	S	I	R	R	R	S	S	R	S	R	ESBL
320	R	R	R	R	R	R	I	R	R	R	S	R	R	I	R	ESBL
321	R	R	R	I	R	R	R	R	R	R	S	R	R	I	R	ESBL
323	R	R	I	R	R	S	R	R	R	R	S	R	R	I	R	ESBL
363	I	R	S	S	I	S	S	R	R	R	S	S	R	I	R	ESBL
365	I	R	R	I	R	S	S	R	R	R	S	R	R	R	R	ESBL
366	I	R	I	S	R	R	S	R	R	R	S	S	R	I	R	ESBL
376	R	R	I	I	I	S	S	R	R	R	S	S	R	I	R	ESBL
381	I	R	R	I	R	R	S	I	R	R	I	S	R	I	R	ESBL
382	R	R	I	I	R	S	R	R	R	R	S	R	R	I	R	ESBL
384	R	R	R	R	I	I	S	R	R	R	R	S	R	R	R	ESBL

Key- ORG, AUG, CXM, FEP, CRO, S, TE, CIP, C, F, SXT, ATM, AMP, ESBL, R, I, S

APPENDIX 3 Antimicrobial susceptibility tests for transconjugant isolates

ORG No	AUG	CXM	CAZ	FEP	CRO	TE	CIP	NA	C	F	SXT	AMP
ATCC 25922	S	S	S	S	S	S	S	S	S	S	S	S
J53	S	S	S	S	S	S	S	S	S	S	S	S
27	I	R	S	S	R	R	S	S	S	S	S	R
39	R	R	I	I	R	R	S	S	R	S	S	R
104	I	R	S	S	R	R	S	S	R	S	S	R
114	I	R	S	I	R	S	S	S	I	S	S	R
128	I	R	S	S	R	R	S	S	S	S	S	R
129	I	R	S	S	R	R	S	S	S	S	I	R
131	1	R	I	R	R	R	S	S	S	S	S	R
132	I	R	I	R	R	R	R	R	S	S	R	R
143	I	S	S	S	S	R	S	S	S	S	R	R
147	R	S	S	S	S	R	S	I	R	S	R	R
158	I	R	R	R	R	R	R	R	R	S	R	R
160	I	R	R	R	R	R	R	R	R	S	R	R
207	I	R	I	I	R	S	S	I	R	S	S	R
210	R	R	R	R	R	R	R	R	R	S	R	R
216	I	R	I	S	R	R	R	R	S	S	R	R
223	I	R	I	S	R	R	S	S	S	S	S	R
224	I	R	R	R	R	R	R	R	S	S	R	R
236	R	R	R	R	R	R	R	R	R	S	R	R
237	I	R	S	S	R	R	S	S	S	S	S	R
255	S	R	S	S	R	R	S	S	S	S	S	R
275	R	R	R	R	R	R	S	S	I	S	S	R
279	I	R	S	S	R	R	S	S	S	S	R	R
280	S	R	I	I	R	R	S	S	S	S	S	R
284	R	R	I	I	R	R	R	R	S	S	R	R
286	I	R	S	S	I	R	S	S	S	S	S	R
287	S	S	S	S	S	S	S	S	S	S	R	R
289	I	R	R	R	R	R	R	R	R	S	R	R
290	I	R	R	R	R	R	R	R	R	S	R	R
293	R	R	R	I	R	R	R	R	R	S	R	R
295	I	R	S	S	R	R	S	S	S	S	S	R
296	I	R	S	S	I	R	S	S	S	S	S	R
299	R	R	S	S	R	R	S	S	S	I	R	R
303	R	R	S	R	I	R	S	S	S	S	S	R
304	I	R	I	S	R	R	S	S	S	S	S	R
305	I	R	I	S	R	R	S	S	S	S	S	R
305	I	R	S	S	R	R	S	S	S	S	S	R
306	I	R	S	S	R	R	S	S	S	S	S	R
307	I	R	S	S	R	R	S	S	S	S	S	R
309	I	R	S	I	R	R	S	S	S	S	S	R
318	I	R	I	R	R	R	R	R	I	S	R	R
319	I	R	S	S	R	R	S	S	S	S	R	R
320	R	R	R	I	R	R	S	S	S	S	S	R
321	I	R	S	I	R	R	R	R	S	S	R	R
363	I	R	S	S	R	R	S	S	S	S	S	R
363	I	R	I	S	R	R	S	S	S	S	S	R
365	I	R	S	S	I	R	S	S	S	S	S	R
366	I	R	I	I	R	R	S	S	S	S	S	R
376	I	R	S	S	I	R	S	S	S	S	S	R
381	I	R	I	I	I	R	S	S	S	S	S	R
382	I	R	I	R	I	R	R	R	S	R	R	R
384	I	R	R	I	R	R	R	R		S	R	R

Key- ORG, AUG, CXM, FEP, CRO, S, TE, CIP, C, F, SXT, ATM, AMP, ESBL, R, I, S

APPENDIX 4