OCCURRENCE, ANTIBIOTICS AND DISINFECTANTS RESISTANCE OF EXTENDED SPECTRUM B-LACTAMASES PRODUCING E. COLI AND K. PNEUMONIA AND THE GENES ENCODING FOR THE RESISTANCE IN KENYA

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A thesis Submitted in Partial Fulfillments of the Requirements for the Award of Degree of Doctor of Philosophy (Medical Biochemistry) in the School of Pure and Applied Sciences, Kenyatta University

MAY, 2018
DECLARATION

I confirm that, this thesis report is my original work and has not been presented in any other university/institution for consideration. The thesis has been completed by referenced sources duly acknowledged where text, data, graphics, pictures or tables have been borrowed from other sources, including the internet. These are specifically accredited and reference cited in accordance with anti-plagiarism regulations.

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DEDICATION

This thesis is dedicated to my parents Magdaline Wangu and late Josephat Gathua. Also to my husband, James Kamau, my children, Moses Kagwi, Sarah Wangari and Magdaline Wangu without whose support I would not have had the ambition and inspiration to complete this degree.
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<td>AIDS</td>
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<td>AMP</td>
<td>Ampcillin</td>
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<td>API</td>
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<td>ARB</td>
<td>Antibiotic-resistant bacteria</td>
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<td>ASB</td>
<td>Antibiotic-susceptible bacteria</td>
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<td>ATCC</td>
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<td>BSI</td>
<td>Bloodstream infection</td>
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<td>CN</td>
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<td>ESBLs</td>
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<tr>
<td>MDR</td>
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<td>MP</td>
<td>Meropenem</td>
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<td>MRSA</td>
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<td>NBL</td>
<td>Non- β-lactamase</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>SAS</td>
<td>Sodium Alkyl Sulfate</td>
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<td>SHV</td>
<td>Sulphhydryl variable</td>
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<tr>
<td>SPSS</td>
<td>Statistical program for social sciences</td>
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<tr>
<td>SXT</td>
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<td>USA</td>
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<td>UTI</td>
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ABSTRACT

Extended spectrum beta lactamases (ESBLs) are enzymes that mediate resistance to β-lactam antibiotics by opening the beta-lactam ring of penicillin derived antibiotics, and other family of antibiotics, rendering them inactive against the bacteria they are intended to kill. The ESBLs were first identified in 1939 and since then, they have proliferated worldwide and have been found in a number of different bacteria, such as *Klebsiella pneumonia*, and *Escherichia coli* among others. These bacterial strains can cause severe and life-threatening infections, resulting in increased morbidity, mortality, and cost in treating these infections. Since these bacterial strains are not adequately identified in many Kenyan public health facilities, key information on prevalence, antibiotic and disinfectant susceptibility is inadequate. The main aim of this study was to determine the occurrence, antibiotic and disinfectant susceptibility in ESBLs producing *E.coli* and *K.pneumonia*, and genes encoding for the resistance. Analytical profile index (API) method was used to identify the presence of *E.coli* or *K. pneumonia* among the Gram negative bacteria. Double disc synergy method was used to test for the presence of ESBL enzymes in these two bacteria isolates. Disc diffusion method was used in Antimicrobial susceptibility testing while disinfectants susceptibility testing was done using classic method of successive dilutions. Polymerase chain reaction (PCR) was used to test for the presence of β-lactamases genes (*blaTEM, blaSHV, blaCTX-M* and *blaOXA*). Identification of the sub-types of the mother genes was done by sequencing method. Among the gram negative isolates identified, 21% were ESBL *E.coli* and 20% were ESBLs *K.pneumonia*. The study indicates that, antimicrobial resistance was high, Ampicillin (100%), sulfamethoxazole-trimethoprim combination (89%), and tetracycline (87%), cefuroxime (100%), ceftazidime (88%) and ceftriaxone (94%) and aztreonam (100%). Relatively lower resistance was recorded for gentamicin (56 %,) and meropenem (8% in *E. coli* and 18%, in *K.pneumonia*). All the six disinfectants were effective at recommended use concentration, losing affectivity with decreasing concentration. All the four ESBL genes (*blaCTX-M, blaTEM, blaSHV* and *blaOXA*) were identified from the study isolates. The most predominant enzyme in *E.coli* was CTX-M (29/36 while in *K. pneumonia*, SHV genes was the most predominant (22/34).Nine (9) sub-types of TEM (TEM-1, 186, 198, 219, 2018, 115, 154, 163, 169) were obtained while 5 sub-types of SHV (SHV-1, 12, 32,101 and 11) were obtained. Majority of both strains harbored*bla* combination of ESBL genes. Two of the identified sub-types TEM 208 and 219 are described for the first time. In phylogeny analysis, the different beta lactamase genes of *blaSHV* and *blaTEM* clustered separately with each forming a distinct cluster. Antimicrobial resistance to the ESBLs is high and this compares well with reports from other parts of the world. Updates of trends for regional epidemiological data on antimicrobial resistance are crucial in order to promote appropriate antimicrobial therapy as well as an effective infection control and clinical care management.
CHAPTER ONE

INTRODUCTION

1.1 Introduction and background

Beta-lactamases are proteins or enzymes that destroy the basic structure of the penicillin based antibiotic rendering them inactive against the organism they are intended to kill. In the 1960s, the first resistant gram-negative bacillus capable of producing beta-lactamase was discovered in Greece. This plasmid mediated resistant enzyme was given the name TEM after the name of the patient it was isolated from (Temoniera) (Canton et al., 2012). A closely related enzyme, TEM-2 was later discovered that was similar in biochemical characteristics to TEM-1. The two enzymes differed by a single amino acid (Canton et al., 2012).

TEM-1 and TEM-2 plasmid mediated enzymes are the most predominant resistant beta-lactamases in gram-negative bacilli, including *Pseudomonas aeruginosa, Enterobacteriaceae, Neisseria gonorrhoea and Haemophilus influenzae*. These enzymes, TEM-1 and TEM-2 are capable of hydrolyzing penicillin based antibiotics and first generation cephalosporins, such as cefazolin or cephalothin (Smith et al., 2000). Consequently they are not able to inactivate more stable newer cephalosporins such as ceftriaxone, ceftazidime, cefotaxime, or cefepime (Smith et al., 2000). When these antibiotics were first produced, they responded very well to a wide group of known resistant bacteria isolates. Another related but less common enzyme was discovered and named SHV, because sulfhydryl reagents had a variable effect on substrate specificity (Cloeckaert et al., 2007).
The emergence of these wide spectrum beta-lactamase (ESBL) bacteria, mainly gramnegative bacilli has caused a major health concern both in hospitals and community associated infections.

This is a class of bacteria that produces enzymes responsible for mediating resistance to extended-spectrum (third generation) cephalosporins such as ceftazidime and aztreonam but are not able to hydrolyze cephemycins and carbapenems (Sirot et al., 2001). The first report on ESBL-producing gram negative bacilli came from Europe in the 1980s which have since spread widely, causing a health concern worldwide. Recent reviews about these strains have started to recognize the laboratory aspects in detection and classification of ESBLs, however, they do not offer significant guidance about how to contain these organisms in a particular clinical scenario (Bejon et al., 2005). Due to this lack of clinical guidance, there has been notable increased mortality morbidity, and high cost in containing and managing the infections caused by these resistant bacteria isolates (Bejon et al., 2005)

*K. pneumonia* and *E. coli* are the most predominant bacteria known to produce the ESBL enzymes. They can live and survive in the gut of human without one being unwell or showing any signs or symptoms of infection (colonization). Invasive and infections such as urinary tract infections (UTI) and bacteremia caused by *Klebsiella* species and *E. coli* are among the most serious, with high mortality, especially in developing countries. Urinary tract infections and bacteremia are one of the most prevalent infectious diseases especially in children and the aged (Talon et al., 2004). The prevalence of multidrug- and pandrug-resistant strains belonging to these species especially in tertiary medical
institutions is a notable threat in treatment and containment of these serious infections (Olesen et al., 2004).

Previous studies reveal that, the proportion of strains showing antibacterial resistance has been increasing annually in community-acquired infections especially among extended-spectrum β-lactamase (ESBL)-producing *E. coli*, *Klebsiella*, methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *enterococci*, and penicillin-resistant *S.pneumonia* (Wang et al., 2003).

Since 1996, in an effort to contain the spread of these infections, several new antibiotics especially ones against β-lactamase enzymes have been produced, specifically designed to resist the destructive action of these enzymes. The upsurge of this drug resistance can be attributed to three causes; inappropriate use of antibiotics in the human population, animal population and spread of resistant strains between human or non-human sources (Hernández et al., 2005). Antibiotics use increases selective pressure in bacterial populations, selecting out a percentage of resistant bacteria which continue growing while only the vulnerable bacteria die. As the trend in antibiotics resistance is becoming more common, alternative treatments need to be considered. Calls for alternative antibiotic therapies have been issued, but development of new drugs is becoming rare (Hernández et al., 2005). However, when new classes of antibiotics are produced for treating these resistant bacterial infections, then, new beta lactam enzymes with stronger hydrolytic activity against the new drugs emerge (Philippon et al., 1989; Jacoby & Sutton 1991a; Jacoby 2009; Jacoby & Bush 2011).
This resistance is brought about by selective pressure arising from inappropriate use of these new antibiotics in management of individuals suffering from infections due to resistant bacteria. Not surprisingly, resistance to these expanded-spectrum β-lactam antibiotics due to continuous proliferation of these extended β-lactamases variants emerged quickly. The first of these enzymes capable of hydrolyzing the newer β-lactams, SHV –2, was found in a single strain of *Klebsiella ozaenae* (Philippon *et al*., 1989; Wang *et al*., 2003). To date, over 150 different ESBLs have been described (Monstein *et al*., 2008). Research done in Virginia, Common Wealth University showed that, approximately 15% of the isolates from 1.2% of patients were found to express ESBLs (Perez *et al*., 2007). Eight (8) different species of extended-spectrum β-lactamase producing bacterial strains were identified in this study and the patients included both in and outpatients. As provided in the National Committee for Clinical Laboratory Standards (Clinical Laboratory Standards Institute (CLSI, 2007), only 26% to 39% of the ESBLs producing isolates responded to ceftazidime (Bratu *et al*., 2005; Fritsche *et al*., 2008). In another survey done in Korea, 55% of 510 isolates of *K.pneumoniae* were β-lactamase producers (Cassi *et al*., 2014). ESBL-producing *E.coli* isolates are increasingly detected in nursing homes and community-based health-care facilities (Allerberger *et al*., 1993; Bass *et al*.; 1999a; Andreu & Planells, 2008; Bailey *et al*., 2011). Travel has also been incriminated as a vehicle in the spread of these infections by facilitating a potential influx of the resistant bacterial from the community to the health care settings (Allerberger *et al*., 1993; Bass *et al*., 1999a; Andreu & Planells, 2008; Bailey *et al*., 2011).
Bacterial strains harboring ESBLs enzymes that confer resistance to the beta lactam antibiotics threaten use of the limited available antibiotics. Infection control best practices and measures are essential in preventing outbreaks and spread of ESBL producing bacteria strains. However, bacteria have developed strategies against these infection control measures. Therefore, designing new measures to contain these resistant mechanisms is key. This may require proper laboratory identification of these bacterial isolates with well documented data that may help in discovery and design of new antimicrobial agents. The carbapenems group of antibiotics is widely regarded as the most potent drugs in management of these severe infections although comparative studies or clinical trial reports are inadequate.

Hence, routine laboratory identification of ESBL-producing bacteria and continuous review of infection control guidelines for community-onset blood infections associated with different infections are needed. Since these bacterial strains are not routinely identified in many Kenyan public health facilities, key information on prevalence, public health burden, antibiotic and disinfectants susceptibility are inadequate hence the rationale for the study.

A study done at Kenyatta National Hospital Nairobi, Kenya revealed that, nine (9) *K.pneumonia* isolates, from samples of blood and cerebrospinal fluid (CSF) of newborn infants, had a novel CTX-M-12 extended-spectrum β-lactamase. This enzyme has a more hydrolytic effect towards cefotaxime than ceftazidime (Kariuki et al., 2006).

Another study done at The Aga Khan University Hospital, Kenya showed that, the proportion of resistant to *Klebsiella pneumoniae* and *E. coli* due to ESBLs was greater than 80% for the antibiotics tested, with the exception of meropenem 1%, gentamicin
63%, and nitrofurantoin 23%. *K. pneumonia* had a higher proportion of resistant to gentamicin, ceftazidime, and nitrofurantoin (P < 0.05) than *E. coli* (Maina et al., 2013).

Another study, done at The Aga Khan University Hospital, Kenya to determine the genotype of ESBLs among 52 isolates of *K. pneumonia* and *E. coli* Indicated that, the distribution of the three genotypes detected among the 52 bacterial isolates was as follows: bla\textsubscript{SHV} 13 (25%), bla\textsubscript{CTX-M} 46 (88.5%), and bla\textsubscript{TEM} 18 (34.6%). The genotypes occurred singularly in 33 (63.5%) of the isolates and in several gene combinations among the remaining 19 (36.5%). bla\textsubscript{CTX-M} + bla\textsubscript{TEM} was the most frequent gene combination found in nine (17.3%) of the isolates followed by a combination of the three genes in six (11.5%) (Maina et al., 2011). There is therefore need to determine the occurrence and biological characteristics of ESBL in Kenya.

**1.2 Problem statement and Justification**

ESBLs related infections are usually resistant to 1\textsuperscript{st}, 2\textsuperscript{nd}and 3\textsuperscript{rd} generation cephalosporins, narrow and broad spectrum penicillins and aztreonam. These infections are characterized by serious treatment failures since they do not respond to many antibiotics for which the causal agents have become resistant. The choice of antibiotics therefore becomes extremely limited and this presents a major treatment dilemma. These bacteria are not routinely identified in many Kenyan hospitals and therefore they usually go unnoticed. Since there is no routine identification system of ESBLs in Kenya, this makes the clinicians start patient on ineffective antibiotics through empirical antibiotic prescription. Detection of these serious organisms therefore is of great importance to guide the clinician on selection of right antibiotic therapy first time.
There is therefore a need to first establish the types of resistant ESBLs present in Kenya and then the possibility of establishing routine testing of these organisms with a view to maintaining effective, affordable and available lines of treatments. Critical care units of hospital set ups are regarded as the main source of ESBL producing bacterial isolates. There is no adequate published data to indicate the magnitude of the problem in Kenyan hospitals. Over use of antibiotics is one of the mechanisms known for acquisition of an ESBL-producing organism. The actual burden of extended – spectrum β lactamase producing bacteria remain unknown in Kenyan health care facilities. Due to lack of information and surveillance data, national infection control guidelines on ESBLs-mediated resistance have also not yet been developed. It is not also known if these resistant strains of *E. coli* and *Klebsiella* strains also exhibit resistance or reduced sensitivity to detergents and disinfectants commonly used for infection control in health care facilities. There is therefore need to determine these factors in the Kenya environment.

1.3 Hypotheses

1. The occurrence of ESBLs producing *E.coli* and *Klebsiellapneumonia* in Kenyan health facilities is high.

2. The ESBLs producing *K. pneumonia E.coli* are resistant to most commonly used antibiotics and disinfectants.

3. The ESBLs genes in *E.coli* and *Klebsiella pneumonia* and their sub-types found in Kenya are not different from those in other countries.

4. The evolutionary relationships for genes coding for *E. coli* and *K. pneumonia* are different.
1.4 Objectives

1.4.1 General objective

To establish the prevalence of ESBLs producing *E. coli* and *K. pneumonia*, their antibiotic and disinfectants susceptibility, and genes encoding for the resistant enzymes.

1.4.2 Specific Objectives:

i. To determine the occurrence of *E. coli* and *K. pneumonia* that produce ESBLs enzymes.

ii. To determine the antibiotic and disinfectant susceptibility of *E. coli* and *K. pneumonia* that produce ESBL enzymes.

iii. To determine the gene subtypes encoding for β-lactamases enzymes for the *E. coli* and *K. pneumonia* isolates recovered in Kenya.

iv. To determine molecular evolutionary relationship of the genes encoding *E. coli* and *K. pneumonia*. 
CHAPTER TWO

LITERATURE REVIEW

2.1 Infections caused by *Klebsiella pneumonia* and *Escherichia coli*

The most serious among the pathogenic Klebsiella pneumonia and Escherichia coli bacterial strains are those that produce ESBLs enzymes that act on β-lactam antibiotics. These enzymes are able to hydrolyze the basic structure of β-lactam ring of antibiotics such as Penicillins, monobactams and cephalosporins and are widely distributed among the family *Enterobactericiae* (Sirot et al., 2001). These enzymes are known to mediate resistance to many gram negative antibiotics (Arlet et al., 1995; Bush et al., 1995).

They are important enzymes because they act as a mechanism of resistance to third generation cephalosporins such as cefotaxime, andaztreonam but do not hydrolyze cephemycins (such as cefoxitin) or carbapenems (such as meropenem) (Arlet et al., 1995; Bush et al., 1995). These enzymes are commonly inhibited by β-lactamase-inhibitors such as clavulanic acid, sulbactam and tazobactam (Thomson & Sanders 1992; Arlet et al., 1995; Jacoby & Bush 2011). ESBLs were first identified in 1939 and since then, they have been reported worldwide and have been isolated and identified in a number of different organisms, including *K. pneumonia, Klebsiella oxytoca, Morganella morganii, E. coli, Capnocytophaga ochracea, Proteousmirabilis, Shigella, dysenteriae, P. aeruginosa, Bacillus cepacia*, and *Salmonella* species (Hasman et al., 2005; Livermore et al., 2007; Coque et al., 2008; Jouini et al., 2010; Liu et al., 2010). Colonization or infections by bacteria strains that produces ESBLs have been known to show similar risks for infections. The attending physician should be cognizant of the presence of these risk factors and be able to put in place the appropriate
measures to contain the spread if the infection is known to occur as a result of bacterial isolate that produces ESBL enzyme.

Infections of the urinary tract and bacteremia and especially in pediatric wards are considered as main and important factors which cause mortality and diseases in the world, particularly in low resource countries (Coudron et al., 2003; Bejon et al., 2005; Jokinen & Scott, 2010). Sepsis is a serious infection especially among neonates and people who are immune-compromised. The rate of bacteraemia in low resource countries is 3 to 20 times higher than that in developed countries, and twice that of the paediatrics and neonatal intensive care units (NICUs) (Coudron et al., 2003; Bejon et al., 2005; Jokinen & Scott, 2010).

Documented information on epidemiology of bloodstream infection (BSI) in Africa is poor. An analysis of prospective studies of community-acquired BSI, identified 22 eligible studies (four in Southern Africa), where non-typhoidal *Salmonella*, *E.coli*, *Staphylococcus aureus* and *Streptococcus pneumonia* infection were the most identified (Petit et al., 1995; White, Jr. et al., 1997; Poirel et al., 2000; Musoke & Revathi 2000a).

Despite published data of community-acquired sepsis in African children (Springer & Horton 1969; Petit et al., 1995; White, Jr. et al., 1997; Musoke & Revathi 2000a; Musoke & Revathi 2000b; Poirel et al., 2000; Pitout et al., 2009) information on hospital-associated BSI, are extremely inadequate (Springer & Horton, 1969; White, Jr. et al., 1997). Published reports indicate that, healthcare-associated BSI may be responsible for 25000 deaths in Africa annually, majority of them being children (Berkley et al., 2005).
Overall, incidence rates of hospital-associated infection in developing countries are known to be at least twice that in developed high-income countries (Berkley et al., 2005). More research is needed on the epidemiology of health care-associated BSI to quantify the burden and better understand contributory factors in Africa. In light of globally increasing antimicrobial resistance, regional antimicrobial resistance prevalence and the efficacy of empiric antibiotic therapy, BSI in Sub-Saharan Africa also require evaluation. Information on upward trend prevalence of BSI isolates producing ESBLs, are of concern given the limited availability of appropriate antibiotics in many developing countries (Brent et al., 2006a; Kariuki et al., 2006; Chiller et al., 2009).

*K. pneumonia* and *E. coli* are also a major cause of infections of urinary tract, which are illnesses rated as the most predominant in community and health care associated bacterial infections. In individuals with anatomical or functional abnormalities, UTIs generally clears without antibiotic treatment but have a possibility of recurring. Younger and aged women and those with other complications of the genitor-urinary tract are more affected. These infections occur in the urinary tract and are divided into different groups according to the area they infect. The upper portion is comprised of the kidneys, renal, pelvis and ureter. The lower portion consists of the urinary bladder and the urethra (Arlet et al., 1995; Coque et al., 2008). UTI may cause premature birth for reasons such as: short time between pregnancies, multiple pregnancies infections, ascending genital tract infection, and admission to Neonatal Intensive Care Units (NICUs) (Arlet et al., 1995; Correia et al., 2003).

Infections such as diarrhea, meningitis, sepsis and urinary tract infections are among the main problems in hospitalized patients (Arakawa et al., 2000; Cambau et al., 2006;
Bogaerts et al., 2007). Some of the Klebsiella and E. coli strains causing these infections may be ESBLs producers and/or Multi-Drug resistant (MDR). Specific sub-populations at increased risks include small children, pregnant woman, the aged, patients with spinal injuries or catheterized patients with diabetes or multiple sclerosis, patients with acquired immunodeficiency syndrome/HIV and patients with underlying urologic abnormalities. Middle aged individuals are more affected than younger adults.

Children under the age of six are estimated to have a UTI prevalence of 7% among girls and 2% among boys (Deplano et al., 2005; Tacconelli et al., 2014, Kevin, 2005; Akram et al., 2007; Borsari et al., 2008). Urinary tract infections caused by use of catheter, are the most common among health care associated infections responsible for more than 1 million cases in health care settings and nursing homes in USA. The risk of UTI rises with prolonged period of catheterization. For visiting aged patients, infections of the urinary tract, are the second most predominant form of diseases in the USA responsible to nearly 25% of all illness (Deplano et al., 2005; Akram et al., 2007; Borsari et al., 2008). Risk factors specific to women for UTIs include:

- **Female anatomy.** Female anatomy increases the chances of UTI due to the short distance bacteria have to travel to reach the bladder.

- **Sexual activity.** Sexually active women are likely to have multiple sexual partners will therefore have more frequent UTIs than women who aren't sexually active.

- **Birth control gargets.** Birth control gargets eg diaphragms and spermicidal agents may also increase the risk, of UTI.
- **Menopause.** Changes in the anatomy of urinary tract decline the circulating estrogen especially after menopause, makes women more susceptible to infection. Blockages prevent passage of urine normally which make the urine to remain in the urethra therefore increasing the risk more. In addition, individuals who retain urine for a long time may give the bacteria a chance to attach to the bladder lining. This increases the risk of invasion of the urinary tract than those who often empty their bladder. Individuals with impaired immune system are at a great risk of infection due to invasion by many microbes as the body defense is weakened.

Uropathogens such as *E. coli* and *Klebsiella* have specialized virulence factors, such as adhesions, siderophores and toxins that enable them to infect and colonize the urinary tract. These pathogens are passed between individuals through contacts, via food and water. The upper tract infections originate in the urinary bladder and ascend through ureter to the kidneys. The vesico-urethral valve prevents reflux of urine from the urinary bladder in to the ureter. Individuals with urogenital anomalies’ or over distended urinary bladder form outflow obstruction, neurogenic malfunctions or pressure from an enlarged uterus during pregnancy are particularly susceptible to UTIs. Infection of the renal pelvis (pyelitis) and kidney (pyelonephritis) are the most common infections of the upper UTIs and can lead to septicemia (Anatoliotaki *et al.*, 2007; Bailey *et al.*, 2011). The UTIs are grouped as complicated or uncomplicated. Uncomplicated infections include acute cystitis or pyelonephritis in young women with underlying UTI or systemic disease (Altman *et al.*, 1975 Anatoliotaki *et al.*, 2007; Ho *et al.*, 2011). If diagnosis is cystitis, empirical antimicrobial therapy can be instituted and in many cases, the suspect organism is *E. coli*. Both urinalysis and urine culture should be performed for complicated cystitis.
and all cases of pyelonephritis. *E. coli* is responsible for almost all such infections. If polymorph nuclear neutrophils are absent, a culture should be performed before initiating therapy.

In non-obstructed, non-pregnant female adults, acute uncomplicated UTI is believed to be a benign syndrome. However, UTIs increase the risk of pyelonephritis, pre-mature birth and fetal mortality among pregnant women. It also causes renal impairment and end-stage renal disease in pediatric patients. In USA, community-acquired UTI is significant, costing the country about $1.6 billion per year (Altman *et al.*, 1975; Hill *et al.*, 2006a; Anatoliotaki *et al.*, 2007; Ho *et al.*, 2011).

Most UTI infections are generally self-limiting, and treatment using antibiotics may lead to rapid selection for resistant uropathogens and dislodging of the normal commensal which may have adverse effects on the gut and vaginal (Anatoliotaki *et al.*, 2007). Although these urinary tract infections may not require treatment with antibiotics as they tend to resolve a few days after exposure, there is need to closely monitor until they clear. However UTIs especially in pregnant women need to be treated appropriately due to lowered immune response. Infections of the urinary tract which have been identified have to be treated and antibiotics remains the solution for appropriate treatment (Ho *et al.*, 2011). The antibiotics recommended include, amoxicillin, cephalosporin, and Co-trimoxazole. There are a limited number of oral and intravenous antibiotics that are effective against these bacteria. The key is quick diagnosis so that any ineffective antibiotic treatment is stopped immediately. It is imperative that, that right antibiotics and correct dosages are prescribed for the appropriate length of time and only when required.
Drug charts are monitored regularly and any unnecessary antibiotics are stopped (Paton & Reeves 1988; Hooton et al., 2004; McIsaac et al., 2004).

Inappropriate use of antibiotics in treating different infections particularly UTIs increases the risk of emergence of resistant strains (Warren et al., 1999a; Naber 2000; Ronald 2002;). Since antibiotic use guidelines is rarely followed in most African countries including Kenya, and prescriptions are done without basing it on laboratory culture and susceptibility diagnostic reports, β-lactams are majorly going to be misused and these explains the presence of complex resistant phenotypes such as ESBLs among E. coli and Klebsiella strains. As these bacterial strains are increasingly becoming resistant to currently available antibiotics, more strategies need to be explored. In the USA, UTIs accounts for nearly 8-10 million out patients’ visits and 1 million emergency department visits, resulting in 100,000 hospitalizations (Warren et al., 1999a; Naber 2000; Ronald, 2002). Nearly 1-3 women will have had UTI requiring antimicrobial therapy by the age of 24 and therefore, there are chances that women are more likely to carry resistant strains than men (Warren et al., 1999a).

Inappropriate use of antimicrobials leads to acquisition of antibiotic resistance among bacteria which increases healthcare costs due to treatment complications. An antimicrobial becomes resistance when it becomes ineffective in controlling or killing a bacterial growth. In developing countries of the world, especially those with low resource health care systems, emergence of drug resistance is mainly driven by, ineffective treatments, self-medication practice, and the availability of counterfeit drugs in the market.
Many strains have acquired plasmid-conferring resistance to one or more drugs. Antimicrobial therapy should therefore be guided by antimicrobial sensitivity patterns. Multiple-drug-resistant (MDR) bacteria are difficult to treat. Continued selective pressure has resulted to the development of MDR bacteria and MDR resistance is an inevitable genetic response to misappropriate exposures of pathogen populations to antibiotics (Akindele & Rotilu, 1997b; Arredondo-Garcia & Amabile-Cuevas, 2008; Baldy-Chudzik & Stosik, 2007).

Treatment of *Escherichia coli* and *Klebsiella pneumonia* infections has become more challenging because of increasing resistance to extended-spectrum cephalosporin resulting from the generation of ESBL enzyme (Akindele & Rotilu 1997b; Bejon et al., 2005; Baldy-Chudzik & Stosik, 2007). Long-term hospitalization, admission to critical care unit (CCU) and the use of invasive medical devices were considered as factors causing infection and colonization with organisms that produces ESBL enzyme (Arlet et al., 1997a; Bae et al., 2007a; Al-Hasan et al., 2009; Tunwa et al., 2011).

Since CTX-M ESBL have become more predominant ESBL type than TEM and SHV mutants in Europe, the epidemiology of infections by ESBL-producing bacteria has changed from predominantly healthcare-associated risk factors to community-associated risk factors, and from *K. pneumonia* to *E. coli* as the main hosting pathogen (Allerberger et al., 1993; Bass et al., 1999a; Andreu & Planells, 2008; Bailey et al., 2011). This worldwide spread of ESBL-producers into the community possibly requires new approaches for the surveillance and prevention of ESBL-producing *E. coli* and *K. pneumonia* in health care settings. Better infection control practices using effective disinfectants and detergents may also help to stop the spread of
resistant strains (Borer et al. 2002, 1993; Andreu & Planells, 2008; Bayraktar et al., 2010).

2.2. Emergence of bacterial resistance
The first plasmid-mediated penicillinase ESBL, were TEM and SHV that emerged as a result of mutations by substitutions of one or more amino acid (Arredondo-Garcia & Amabile-Cuevas, 2008). These changed enzymes confer resistance to all oxyimino-cephalosporins by causing disfiguring of ESBL active site which allows the deformation of the oxyimino group reducing the attack efficiency on the β-lactam ring. The exception to these are oxyimino group α-methoxy-cephalosporins (cephamycins) or carbapenems. (Arredondo-Garcia & Amabile-Cuevas, 2008) (Azucena & Mobashery 2001; Bailey et al., 2010). TEM and SHV ESBLs have over 200 members known to date (http://www.lahey.org/studies).

Another ESBL group includes the CTX-M enzymes that are organized in five major CTX-M groups: 1, 2, 8, 9, and 25 (Jacoby & Sutton, 1991b; Bonomo & Szabo, 2006). The CTX-M enzymes, which was discovered after TEM and SHV is becoming more prevalent than its ancestors (TEM and SHV) and comprises a rapidly growing ESBLs group which is distributed widely and among a large group of microbes of epidemiological significance (Jacoby & Sutton, 1991b; Bonomo & Szabo, 2006). ESBLs can lead to the therapeutic failure of â-lactam antimicrobials.

Wide spread of plasmid associated ESBLs can occur when these plasmids are disseminated across species. These plasmids often carry genes encoding co-resistance and therefore can confer resistance to other antibiotics such as aminoglycosides, chloramphenicol, sulfamethoxazole–trimethoprim, tetracycline and fluoroquinolones to
name a few. Concomitant β-lactam, fluoroquinolones and aminoglycoside resistance is increasingly being reported (Arlet et al., 1995; Gootz & Marra, 2008; Barriga-Angulo et al., 2009).

2.2.1 Types of ESBLs
TEM–1 or 2 and SHV-1 genes are derivatives of most of ESBLs genes. These β-lactamases are plasmid-mediated, and confer antimicrobial resistance to penicillin and cephalosporins. Sequencing and genotyping molecular technologies have identified 26 and 50 unique derivatives of TEM and SHV β-lactamases, respectively (Jacoby et al., 2006; Jacoby 2009).

Most of the bacterial strains that produces ESBLs enzymes come from hospitalized in-patients and have frequently caused hospital associated outbreaks, primarily due to Klebsiella pneumonia strain that often have associated resistance to amino glycosides. A single amino acid mutation or a combination of mutations on the mother OXA-1, TEM-2, TEM-1 or SHV-1 is sufficient to change an enzyme from a non-β-lactamase (NBL) to an expanded spectrum β-lactamase. Such mutant enzymes are able to break and destroy third generation cephalosporins as well as monobactams. However, these enzymes are not active against clavulanic acid and have little or no activity on cefoxitin and carbapenems (Bush et al., 1995).

2.2.1.1 CTX-M
The ‘CTX’ denotes the elevated potency of its activity against cefotaxime. While they are remarkably active towards cefotaxime, their ability to degrade ceftazidime differs from one enzyme to another (Poirel et al., 2002a). Among them, CTX-M-15 has an excellent
hydrolytic activity to ceftazidime and may hydrolyze fourth generation cephalosporins such as cefepime, particularly well (Tzouvelekis et al., 2000). CTX-M-ESBLs are however sensitive to inhibition by clavulanic acid and tazobactam (Bush et al., 1993). The co-production of CTX-M and other β-lactamases such as OXA-1 and/or TEM-1 may mask the standard phenotype conferred by CTX-Ms (Yan et al., 2000) and may allow the bacteria to resist β-lactamase inhibitors (Canton et al., 2008). Cefotaximases enzymes, such CTX-M have replaced TEM and SHV as the most predominant type of ESBLs, especially in community-acquired infections caused by Klebsiella and E. coli (Savard et al., 2013).

The CTX-M-type ESBLs which are mainly found in Gram negative bacteria are over 160 enzymes (http://www.lahey.org/Studies/other.asp). They have been reported worldwide and have evolved to the following sub-types; CTX-M-1, CTX-M-15, CTX-M-14, CTX-M-3, CTX-M-2, and CTX-M-9. In the last couple of years, CTX-M-15 has become the most predominant enzyme among E. coli strains (Pitout et al., 2005a). They are divided into five different groups based on amino acid identities: group CTX-M-1 comprising CTX-M-1, -3, -10, -12, -15, -28, -30 and FEC-1; group CTX-M-2 which includes CTX-M-2, -4, -5, -6, -7, -20, and Toho-1; group CTX-M-8 which includes only CTX-M-8; group CTX-M-9 includes CTX-M-9, -13, -14, -16, -17, -19, -21, -24, -27, and Toho-2, and finally; group CTX-M-25 includes CTX-M-25 and CTX-M-26 (Pitout et al., 2005a). Gram negative bacterial, producing CTX-M enzymes occurs widely and have been reported worldwide, which includes
2.2.1.2 TEM

These enzymes are originally from TEM-1 and TEM-2. The first TEM ESBL enzyme was TEM-3 identified in *K. pneumonia* isolates in France and was borne on a plasmid (Sirot *et al.*, 1987). Other examples of TEM-derived ESBLs include the TEM-12 that was identified from a plasmid of *Klebsiella oxytoca* (Du Bois *et al.*, 1995). The TEM-52 ESBL has been studied into details and is found in high prevalence in the United States (Doi *et al.*, 2007). This enzyme has also been reported in Europe in countries such as Belgium (Cloeckaert *et al.*, 2007). The *bla*<sub>TEM-52</sub> is another TEM-type ESBL that is frequently found in the genetic context of old group of transposons.

2.2.1.3 SHV

The first SHV-type ESBL was SHV-2, obtained from a *K. ozaenae* isolated in Germany in 1983. These ESBLs have now been reported in many countries, in *Pseudomonas* and *Acinetobacter* thus indicating a global dissemination pattern (Paterson *et al.*, 2003). While SHV-12 has been reported in Europe (Livermore *et al.*, 2007), SHV-2 and SHV-5 has been detected in *K.pneumonia* from South Africa (Pitout *et al.*, 1998a). In Kenya, CTX-M-12 (GenBank accession number [AF305837](https://www.ncbi.nlm.nih.gov/nuccore/AF305837)), is the only enzyme reported only once previously from an isolate in Kenya (Kariuki *et al.*, 2001). Testing for the production of ESBLs enzymes is not usually carried out in many diagnostic laboratories in Kenya public health facilities and therefore, information about treatment failure when these infections are treated with penicillin and cephalosporins is not adequately documented.
2.2.1.4 IRTs β-lactamases

Point mutations on the TEM-1 may give rise to TEM-type ESBLs but may also give rise to the inhibitor resistant TEMs (IRTs). The IRT enzymes also originated from both TEM-1 and TEM-2 (Knox, 1995). IRT-producing isolates are sensitive to piperacillin/tazobactam combinations, cephalosporins, cephemycins, and carbapenems. However they do not respond to ampicillin/sulbactam and amoxicillin/clavulanic acid inhibitor combinations (Chaibi et al., 1996). Although these resistant enzyme type are less prevalent than the ESBLs, they are of great clinical importance because they result in therapeutic failure when inhibitor-based antimicrobials are prescribed without determining susceptibility profiles of the pathogen (Henquell et al., 1994).

2.2.1.5 OXA-1

Besides OXA-1 that is a narrow spectrum β-lactamase, OXA enzymes have broad-spectrum activity. Such enzymes are frequently detected in *P. aeruginosa*. clavulanic acid and EDTA poorly inhibit these enzymes. With the advent of inhibitor-based treatments, these enzymes have gained recognition because of their poor sensitivity to these agents (Randegger & Hachler 2001). The OXA ESBL-type have mainly been isolated and 54 patients reported in *P. aeruginosa* in France and Turkey but the geographical distribution of such enzymes remains poorly understood (Bush et al., 1995). OXA-1 is particularly important because it confers resistance to clavulanic acid, an important agent used against ESBL-producers.
2.3 Global epidemiology of ESBLs

Some studies of hospital associated isolates of *K. pneumonia* from Europe shows that 14% to 16% produce ESBLs ((Jacoby *et al.*, 2006; Jacoby, 2009). In France the prevalence increased from less that 1% in 1985 to 15% by 1988.

A study done showed that, most infections caused by *Klebsiella*, occurs during hospital stay and are responsible for 5 to 7% of all hospital associated infections (Sirot *et al.*, 1987). *Klebsiella* bacteremia infections account for 20% to 50% in more than 50% *Klebsiella pneumonia* infections. Of major concern, is the burden of *Klebsiella* infections in neonatal wards. Other types of ESBLs include CTX-M and inhibitor resistant β-lactamase.

Bacterial organisms, producing ESBL-enzymes were originally detected in Europe (Pitout *et al.*, 1998a). Though the original reports about ESBLs enzymes were from England and Germany, the majority of the documented information in the early years after the discovery of ESBL was from France (Sirot *et al.*, 1987; Philippon *et al.*, 1997; Pitout *et al.*, 1998). In 1986, a large outbreak caused by an ESBL producing bacteria occurred in France. This clinical incidence, which was first to be reported, affected a large group of patients where fifty four (54) patients in three critical care units got the infection which then spread to four other wards in the same hospital (Sirot *et al.*, 1987; Philippon *et al.*, 1997; Pitout *et al.*, 1998b). The proliferation of ESBLs in France was quite dramatic.

In the United States, the first report about the organisms producing ESBLs occurred in 1988 (Ambroziec *et al.*, 2007). In the following years, in 1989, TEM – 10 associated
infections due to ESBL producing *Klebsiella pneumonia* was documented in Chicago by Quinn and colleagues (Bratu et al., 2005). Other early ESBLs related infections outbreaks, mainly of the TEM – type ESBLs (particularly TEM–10, TEM –12 and TEM – 26) were reported. Infections caused CTX–M ESBLs have been documented recently in Canada (Bratu et al., 2005). Though several outbreaks caused by *Klebsiella* that produces ESBLs have been documented from South Africa, no National Surveillance Data have been internationally published (Ferraz et al., 2000a; Paterson et al., 2003; Kariuki & Hart, 2005; Brent et al., 2006b; Boyle et al., 2011a).

Studies have shown that 36.1% of *K. pneumonia* obtained from a South Africa health care facility were ESBL producers (Poirel et al., 2002b; Nelson et al., 2003; Weldhagen et al., 2003; Chaudhary et al., 2004). Reports from Nigeria and Kenya have shown that, infections of *Klebsiella* due to bacteria resistant to 3rd generation cephalosporins have occurred but without documentation of ESBL production testing ((Kariuki et al., 2001; Kasap et al., 2010).

A novel (CTX–M–12) was isolated and identified in Kenya (Kariuki et al. 2001; Kasap et al., 2010). In Australia, the first ESBL to be detected in Australia were from strains of Gentamycin resistant *Klebsiella* species which were collected between 1986 and 1988 from Perth hospital (Mulgrave, 1990). In the ten years, bacteria capable of producing ESBLs have been identified all over Australia and in the Northern Territory with outbreaks of this infection occurring inpatients of all age groups including children. Studies indicate that, the proportion of *K. pneumonia* strain capable of producing ESBL enzymes in Australian health care facilities is estimated to be 5% (Vonberg et
al.,2008). *Kpneumonia* isolates, carrying SHV – 2 were reported from China in 1988 (Jacoby et al., 1991). In 1998 and 1999, a report comprising few numbers of bacterial isolates showed that, 24.5% of *E. coli* and 30.7% of *K. Pneumonia* isolates, were strains capable of producing ESBLs enzymes (Vonberg et al., 2008). Other National surveillance data have documented the presence of ESBLs producing bacteria in 5 to 8% of *E.coli* isolates from Malaysia, Japan, Singapore and Korea but 12 to 14% in Philippines, Indonesia, Thailand, and Taiwan, (Melzer et al., 2007).

In Asia, the rate of ESBLs producing *K.pneumonia* have been 20 to 50% while in Japan, it has been as low as 5% (Mulgrave, 1990). After the discovery of (CTX–M–12) in Kenya, (Kariuki et al., 2001) it was imperative to determine the epidemiology of ESBLs associated infections and the clinical burden by carrying out more studies.

According to the studies described and documented, and following the classification of beta lactamases, there are about 119 ESBLs identified. These different ESBLs have different hydrolytic patterns which will show different antibiotic response patterns. Molecular characterization of these organisms is imperative such that a more precise identity of the enzymes can be obtained.

Patients who are at high risk of acquiring infections caused by ESBL–producing bacteria, or even colonization, are those who are very ill and who stays for long in hospitals and nursing homes. Those in hospitals are likely to be in critical care units where invasive medical devices eg central venous lines, urinary catheters and endotracheal tubes are used on patients for a long period. Bacteria that produce ESBL enzymes are likely to be isolated 11-67 days after the patient’s admission to the hospital (Asensio et al., 2000).
Other risk factors discovered in individual studies, includes the presence of nasogastric tubes (Asensio et al., 2000). Recent surgery, poor nutritional status and jejunostomy tubes have also been incriminated as possible risks factors (Akram et al., 2007; D’Agata et al., 2007). Over use of antibiotic has also been found as a major risk factor. Researches have shown a close association of acquisition of ESBL producing bacterial strains and use of third generation cephalosporins (Bradford PA, 2001).

Evidence based reports have shown a causal association between development of antimicrobial resistance due to ESBL enzymes and antimicrobial usage in health care facilities (Arpinet et al., 2003; Savardet al., 2013). In health care settings, ESBLs producing bacteria isolates, encounters simultaneous or consecutive selective pressure with different penicillin based antibiotic molecule and therefore it is possible that, this fluctuating selective pressure will result in ESBL variants of several antibiotic rather than a single one (Rice et al., 1996). Nursing homes may serve as epicenter for introduction of the organisms producing ESBL- into critical care units in hospitals. Conversely, patients discharged form hospitals with colonization or infection with the ESBLs may return to the nursing homes with ESBLs and become the source of infection to that health care facility (Fukuda et al., 1997). Infections with ESBL carrying organisms especially those caused by E. coli and Klebsiella are mainly obtained from hospital and may include abscesses, peritonitis, intra-abdominal urinary tract infections, ventilation associated pneumonia, cholangitis, and central line associated bacteria ((Berkley et al., 2005). Although these bacterial strains may cause a variety of hospital associated infection, it is also imperative to rule out colonization before initiation of antibiotic treatment. This is because, these resistant bacterial due to ESBL–enzyme have anatural liking to colonize the skin and
upper respiratory track of sick patients (Mbori-Ngacha 1997; Alvarez et al., 2004; Talon et al., 2004; Nordmann et al., 2009; Canton et al., 2012).

Although much recognition has been put on these serious infections by ESBL–producing bacterial isolates, there have been no published guidelines on management of such infections. Therefore, for optimal therapy on in vitro effectiveness of antibiotic regimens, prospective observational studies and case series are essential (Alvarez et al., 2004; Canton et al., 2012). In study looking at the prevalence of ESBLs at a Chicago nursing home, 46% of individuals were carrying ESBLs-producing bacteria and all were E. coli (Wiener et al., 1999). It was found that, these individuals had been continuously admitted at nursing home for a period of more than 6 months. Like any other hospital setting, use of antibiotics in nursing homes, is a determinant for colonization with bacteria producing the ESBL enzymes due to frequent over use of antibiotics. The above statement is supported by a recent study that indicated that, 38% patients in a nursing home s had been treated with a systemic antibiotic for a period of one month continuously (McArthur et al., 2000). In some studies (Rice et al., 1996) treatment with third generation cephalosporins has been identified as a factor for colonization and infection with ESBLs infections but not all studies (Rice et al., 1996).

In contrast, (Rice et al., 1996), in critical care units of hospitals, use of antibiotics that are administered orally eg trimethoprim-sulfamethoxazole, and ciprofloxacin may also be a predisposing factor for colonization (Rice et al., 1996). Due to the nature of Nursing home settings, patients may have several other determinants for infection or colonization with these resistant bacteria because hand washing practice in nursing home personnel is poor (Smith et al., 2000).
In nursing homes use of catheters and decubitus ulcers are frequent and have been incriminated with colonization the resistant bacterial strains brought about by ESBL enzymes (Smith et al., 2000).

2.4 Spread of ESBL producing organism

The hands of health care workers are presumably colonized, by contact with the skin of patients colonized with the organism (Kamatchi et al., 2004). It is important to recognize that many patients may have asymptomatic colonization with ESBL-producing organisms without signs of overt infection. These patients represent an important reservoir of organisms. For every patient with clinically significant infection with an ESBL-producing organism, at least one other patient exists in the same unit with gastrointestinal tract colonization with an ESBL producer.

2.5 Managements of ESBL E. coli and K. pneumonia infections

Because of the ability of ESBL-producing bacteria to break open the basic molecular structure (β–lactam ring) of many penicillin based antibiotics including cephalosporins, the antibiotic choice for treating these infections is seriously minimized. Bacteria with plasmids carrying genes encoding these enzymes will sometimes have genes resistant to trimethoprim/sulfamethoxazole and aminoglycosides (Maya et al., 2011). The carbapenems, eg meropenem, are still regarded antibiotics of choice for managing infections with these resistant E. coli and K. pneumonia (Mammeri et al., 2005). It has been reported that, >98% of the P. mirabilis, K. pneumonia and E. coli producing ESBLs respond well to this class of antibiotic (Maya et al., 2011).
In Hong Kong, ESBL-producing *E. coli* strains were shown to respond to fosfomycin which is an old drug that was used to treat these types of infections (Ho *et al.*, 2010). Another choice which can be used for the treatment of ESBL producing bacterial infections is colistin. This drug is considered a reliable alternative at the present moment since there are no new anti-antibiotics that have been successfully produced beside carbapenems for the treatment of these infections.

Colistin is used in the treatment of other non ESBLs multi drug resistant infections caused by carbapenem resistant *A. baumannii* and *P. aeruginosa* (Maya *et al.*, 2011). The figure below provide guideline for the management of suspected cases of ESBL strains (Figure 2.1).
Figure 2.1: Guideline for the management of suspected cases of ESBL producing organisms
2.5.1 Indications for treatments

2.5.1.1 Bacteremia

The first line treatment for bacteremia caused by ESBLs producing bacteria is Carbapenem while second-line treatment is Fluoroquinolones. This is mainly because, their ability to kill the resistant organisms is not affected by these ESBL enzymes in vitro (Bush et al., 1995).

2.5.1.2 Hospital acquired pneumonia

Diagnosing hospital acquired pneumonia infection may be a challenge. Laboratory diagnosis and identification of ESBL-producing bacteria from a specimen of sputum or bronchial aspirate does not necessarily show that the patient has pneumonia because the positive culture may be as a result of colonization which does not require antibiotic treatment (Johnson et al., 1990; Biedenbach et al., 1999). However, these can be considered if clinical signs such radiological changes, fever and consolidation are present where treatment of choice is carbapenem while second-line treatment is fluoroquinolones.

2.5.1.3 Intra-abdominal infection

For management of this type of infection, a combination of antibiotics with agents expanded against gram-negative aerobic and anaerobic bacteria to achieve empiric coverage of likely pathogens. Treatment of choice is carbapenem while second-line treatment is fluoroquinolones (Bush et al., 1995).
2.5.1.4 Infections of urinary tract

Laboratory isolation and identification of organism carrying ESBL enzyme from a urine specimen of patient with catheter in the absence of clinical symptoms or signs may also indicate colonization rather than infection, hence no antibiotic treatment required. The figure below (Figure 2.2 and Table 2.1) provides information on antimicrobial agents for treatment of acute uncomplicated and complicated UTI (Lewis et al., 1990).

2.5.2 Antimicrobial agents for treatment of acute uncomplicated UTI.

A patient present with acute urinary tract infection

No fever, no flank pain, ability to take oral medicine

No

YES

The following is recommended based on availability, allergy history and tolerance.

- Nitrofurantoin, 100mg twice per day for 5 days or
- Trimethoprim/sulfamethoxazole, 160/800 twice a day for three days or
- Fosfomycin 3-g single dose

Take alternative diagnosis such as complicated UTI and treat accordingly

Figure 2.2: Antimicrobial agents for treatment of acute uncomplicated UTI ((Lewis et al., 1990).
Table 2.1: Treatment of complicated urinary tract infections. (Mulvey et al., 2009).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Place in therapy</th>
<th>Key advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrofurantoin: 100 mg oral q6h for 7 days minimum</td>
<td>Treatment of complicated and uncomplicated lower UTI</td>
<td>widely available</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rarely resistant in E. coli</td>
</tr>
<tr>
<td>Fosfomycin 3 g sachet oral once every 3 days for 14 days for c complicated UTI</td>
<td>Treatment of complicated and uncomplicated lower UTI</td>
<td>Resistance rare even in Spain where it is used extensively, oral capsules and iv formulation also available</td>
</tr>
<tr>
<td>Gentamycin 3–5 mg/kg intravenous daily in divided doses or 5–7 mg/kg iv once daily (consult local guidelines)</td>
<td>Treatment of complicated UTI</td>
<td>Resistance relatively uncommon</td>
</tr>
</tbody>
</table>

2.6. Approaches in determination of antimicrobial susceptibility testing

2.6.1 Broth dilution tests.

This procedure involved preparing two-fold dilutions of antibiotics (such as 1, 2, 4, 8, and 16 µg/mL) in a liquid growth medium dispensed in test tubes (Johnson et al., 2003). The antibiotic-containing tubes are inoculated with a standardized bacterial suspension of 1–5×10⁵ CFU/mL. Following overnight incubation at 35°C, the tubes are then examined for
visible bacterial growth as evidenced by turbidity. The lowest concentration of antibiotic that prevented growth represents the minimal inhibitory concentration (MIC). The precision of this method is considered to be plus or minus 1 two-fold concentration, due in large part to the practice of manually preparing serial dilutions of the antibiotics [3]. The advantage of this technique is the generation of a quantitative result (MIC). The principal disadvantages of the macro dilution method is the tedious, manual task of preparing the antibiotic solutions for each test, the possibility of errors in preparation of the antibiotic solutions, and the relatively large amount of reagents and space required for each test.

2.6.2 Antimicrobial gradient method.

The antimicrobial gradient diffusion method uses the principle of establishment of an antimicrobial concentration gradient in an agar medium as a means of determining susceptibility. The E-test (bio-Mérieux AB BIODISK) is a commercial version available in the United States. It employs thin plastic test strips that are impregnated on the underside with a dried antibiotic concentration gradient and are marked on the upper surface with a concentration scale. As many as 5 or 6 strips may be placed in a radial fashion on the surface of an appropriate 150-mm agar plate that has been inoculated with a standardized organism suspension like that used for a disk diffusion test. After overnight incubation, the tests are read by viewing the strips from the top of the plate. The MIC is determined by the intersection of the lower part of the ellipse shaped growth inhibition area with the test strip.
2.6.3 Disk diffusion method

The disk diffusion susceptibility method (Nelson-Filho et al., 2015) is simple and practical and has been well-standardized. The test is performed by applying a bacterial inoculum of approximately 1–2×10⁸CFU/mL to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. Up to 12 commercially-prepared, fixed concentrations, paper antibiotic disks are placed on the inoculated agar surface. Plates are incubated for 16–24 h at 35°C prior to determination of results. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug are interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS) [13] or those included in the US Food and Drug Administration (FDA)-approved product inserts for the disks. The results of the disk diffusion test are “qualitative,” in that a category of susceptibility (such as, susceptible or resistant) is derived from the test rather than an MIC.

2.7 Infection control implication of ESBL producing organism

Hospital environment can be a major source of spread for organism producing ESBL and can spread easily within. Hands of health care workers especially where hand washing practice is poor, can serve as a good mechanism of transmitting infections from one patient to another. Environmental sources such as baths tabs, blood pressure cuffs, sinks, bronchoscopes, and ultrasound gel are also good mechanism for spread of infection in health care facilities. (Aiken et al., 2011).
Small hospital outbreaks can occur especially in high risk health care units such as the neonatal units, CCU, and hematology-oncology units caused by a single clone of particular bacteria while large outbreaks usually involve many circulating bacterial strains. Multidisciplinary approach for effective infection control is required to contain the menace (Romao et al., 2005). The main focus when instituting these infection control measures is by enforcing proper hand wash practices to prevent patient to patient spread. Another key aspect in preventing the spread of this organism is by screening all patients on admission to hospitals and those being transferred from hospitals to nursing and residential homes (Gupta, 2007).

2.7.1 Infection control using detergents and disinfectants

Disinfection and sterilization practices in a health care setting is key in ensuring that medical and surgical equipments are free of any contaminating microbes that may transmit infectious pathogens to patients. The selection, preparation, storage and use of disinfectants, are important factors for maximum effectiveness of disinfectants. This is important since, environmental surfaces, medical and surgical instruments which the disinfectant are supposed to sterilize, can be a good mechanism for spreading infections from one patient to another within the hospital setting (Wellington et al., 2013). It is good to appreciate the fact that, different strategies have been formulated to control resistance both in antimicrobial drugs and disinfectants in hospitals and the community set ups. Also efforts have been put to embrace and practice good infection control practices (Agarwal et al., 2015).

Multiple studies have documented lack of compliance by the health care providers to adhere to established guidelines for disinfection and sterilization (Agarwal et al., 2015).
Failure to follow or enforce the use of infection control guidelines has resulted to high impact disease outbreaks. Alcohols, chlorine-based products, phenols and hydrogen peroxides are some of the most important disinfectants used in hospitals today and may control transmission of pathogenic strains between patients and healthcare workers and among patients (Agarwal et al., 2015; Tosic et al., 2016).

2.7.1.1 Alcohol.

In a hospital environment, alcohols are usually classified as bactericidal rather than bacteriostatic which are active against vegetative forms of bacteria. They are also active against viruses, fungi and mycobacterial tuberculosis but do not inactivate bacterial spores (Nelson-Filho et al., 2015). Their effectiveness reduces sharply when concentration goes below 50%. The optimum performance concentration is 60%–90% solutions in water (volume/volume) (Agarwal et al., 2015; Luo et al., 2015; Tosic et al., 2016).

The mechanism of activity by alcohol is proteins denaturation and work more quickly when mixed with water (Nelson-Filho et al., 2015; Tuuli et al., 2016). Ethyl alcohol is active against M. tuberculosis at a concentration of 95%, in the sputum specimen or water suspension within 15 seconds. Many studies have shown that, Ethyl alcohol at a concentration of 70%, is very effective in killing the tissue phase of C. immitis, H. capsulatum B dermatitidis, C. neoformans, and the culture phases of the latter three organisms aerosolized onto various surfaces (Nelson-Filho et al., 2015; Tuuli et al., 2016).
2.7.1.2 Chlorine and Chlorine Compounds

2.7.1.3 Hypochlorite

Some companies use a mixture of chlorine and water (hypochlorite), rather than using chlorine gas, also known as bleaching powder. Hypochlorites are affected by light, physical energy, and temperature, before they are able destroy the pathogens in water. Hypochlorites and chlorine gas have the same mechanism of activity in killing the pathogens they are designed to kill (Rutala et al., 1999). When mixed with water, it forms hypochlorous acid which is the active compound of the disinfectant. Disinfection by chlorine gas and hypochlorites is done in a similar way. The difference occurs in the way chlorine is added into the water, how it is handled and stored (Greenberg et al., 1992).

2.7.2 Hydrogen Peroxide

Published reports describe hydrogen peroxide as an effective disinfectant with bactericidal, virucidal, sporicidal, and fungicidal properties (Nelson-Filho et al. 2015; Luo et al. 2015; Tuuli et al., 2016). Hydrogen peroxide works best at a concentration of 6% to 25%. The product is sold as a ready to use compound that contains 7.5% hydrogen peroxide and 0.85% phosphoric acid to maintain a low pH (Luo et al., 2015; Tuuli et al., 2016).

The mechanism of action by hydrogen peroxide is by releasing hydroxyl free radicals that are known to be destructive. They attack and destroy many essential cell components including DNA and membrane lipids. Some bacteria that can only grow in presence of oxygen, plus other facultative anaerobes possess cytochrome, which has ability to produce the enzyme catalase. This enzyme catalase has the ability degrade hydrogen peroxide gas in to water and oxygen there by protecting the cells form metabolically
producing hydrogen peroxide. Achievement of this defense depends on the concentrations used (Luo et al., 2015; Tuuli et al., 2016). Hydrogen peroxide has the ability to eliminate a wide range of microorganisms, including yeasts, viruses, bacteria, fungi, and spores (Luo et al., 2015; Tuuli et al., 2016).

2.7.3 Phenolics

Phenol microbiocidal characteristics occur when an active group such as alkyl and phenyl replaces one of the hydrogen atoms on the aromatic ring. Phenol, in high concentrations, can actively poison protoplasmic by entering and destroying the cell wall and fixing the cell proteins. At low concentrations and higher molecular-weight, phenol derivatives has the ability to destroy the bacterial cell by dismantling the essential enzyme systems causing leakage of cells essential metabolites from the cell wall (Luo et al., 2015; Nelson-Filho et al., 2015; Tosic et al., 2016). Published reports on the efficacy of commonly used phenolics showed that, they are active against mycobacteria, viruses, bacteria fungi (Luo et al., 2015; Nelson-Filho et al., 2015; Tosic et al., 2016).

2.8 Methods for determining the Effectiveness of Antiseptics and Disinfectants

2.8.1 Disk-Diffusion Method

This method involves applying different chemicals to separate, sterile filter paper disks (Jeong et al., 2005). The disks are then placed on an agar plate that has been inoculated with the targeted bacterium and the chemicals diffuse out of the disks into the agar where the bacteria have been inoculated. As the “lawn” of bacteria grows, zones of inhibition of microbial growth are observed as clear areas around the disks. The diameter across each zone is measured in millimeters.
2.8.2 Use-Dilution Method

Other methods are also used for measuring the effectiveness of a chemical agent in clinical setting (Nelson-Filho et al., 2015). The use-dilution test is commonly used to determine a chemical’s disinfection effectiveness on an inanimate surface. For this test, a cylinder of stainless steel is dipped in a culture of the targeted microorganism and then dried. The cylinder is then dipped in solutions of disinfectant at various concentrations for a specified amount of time. Finally, the cylinder is transferred to a new test tube containing fresh sterile medium that does not contain disinfectant, and this test tube is incubated. Bacterial survival is demonstrated by the presence of turbidity in the medium, whereas killing of the target organism on the cylinder by the disinfectant will produce no turbidity.

2.8.3 In-use Test

An in-use test can determine whether an actively used solution of disinfectant in a clinical setting is microbially contaminated (Bayraktar et al., 2010). A 1-mL sample of the used disinfectant is diluted into 9 mL of sterile broth medium that also contains a compound to inactivate the disinfectant. Ten drops, totaling approximately 0.2 mL of this mixture, are then inoculated onto each of two agar plates. One plate is incubated at 37 °C for 3 days and the other is incubated at room temperature for 7 days. The plates are monitored for growth of microbial colonies. Growth of five or more colonies on either plate suggests that viable microbial cells existed in the disinfectant solution and that it is contaminated. Such in-use tests monitor the effectiveness of disinfectants in the clinical setting.
2.9 DNA Amplification and sequencing Methods

2.9.1 Loop mediated isothermal DNA amplification method (LAMP)

LAMP is a specific, simple, rapid and cost-effective isothermal nucleic acid amplification method (Fakruddin, 2011). LAMP has an improved simple visual amplicon detection system. LAMP relies on the auto-cycling strand displacement deoxyribonucleic acid (DNA) synthesis which is carried out at 60-65°C for 45-60 min in the presence of Bacillus stearothermophylus DNA polymerase, deoxyribonucleotide triphosphate (dNTPs), specific primers and the target DNA template. The LAMP method employs a DNA polymerase with high strand displacement activity and a set of four specially constructed primers (two inner and two outer primer) that recognize six distinct sequences on the target DNA. The mechanism of the LAMP amplification reaction includes three steps: Production of starting material, cycling amplification and elongation and recycling High-level of precision can be attained without expensive equipments. There are fewer and simpler sample preparation steps compared with conventional PCR and real-time PCR. Substantial alteration of the fluorescence of the reaction tube can be visualized without costly specialized equipment as the signal recognition system is highly sensitive. LAMP is a one-step amplification reaction taking only 30-60 min. LAMP is more resistant to various inhibitory compound (Nagamine et al., 2002). By combination with reverse transcription (RT), LAMP can amplify ribonucleic acid (RNA) sequences with high efficiency. It is highly sensitive and able to detect DNA at as few as six copies in the reaction mixture (Notomi et al., 2000) LAMP has the potential to be helpful in basic research on medicine and pharmacy, environmental hygiene, point-of-care testing and cost-effective diagnosis of infectious diseases(Nagamine et al., 2002). LAMP is as
suitable for DNA sequencing as PCR, in terms of both Sanger sequencing and Pyrosequencing. (Fakruddin et al., 2012).

2.9.2 Nuclei acid Sequenced based Amplification (NASBA)

NASBA, also known as 3SR (Guatell et al., 1990) and transcription mediated amplification (Gill and Ghaemi, 2008) is an isothermal transcription-based amplification system. NASBA specifically designed for the detection of RNA targets. In some NASBA systems, DNA can also be amplified. The complete amplification reaction is performed at the predefined temperature of 41°C. Throughout the amplification reaction, constant temperature is maintained allowing each step of the reaction to proceed as soon as amplification intermediate formed. The exponential kinetic of the NASBA process is attributed by multiple transcription of RNA copies from a given DNA product, is intrinsically more efficient than DNA-amplification methods limited to binary increases per cycle (Sooknanan, 1995). This amplification system uses a consortium of three enzymes (avian myeloblastosis virus reverse transcriptase, RNase H and T7 DNA dependent RNA polymerase) leading to main amplification product of single-stranded RNA (Sooknanan, 1995). NASBA RNA product can be sequenced directly with a dideoxy method using RT and a labeled oligonucleotide primer. The length of the target sequence to be amplified efficiently is limited to approximately 100-250 nucleotides. High-level of precision can be acquired without expensive equipment. NASBA amplicon detection step has significantly improved, incorporation of the use of enzyme-linked gel assay, enzymatic bead-based detection and electro chemilumine scent (ECL) detection, molecular beacon technology and fluorescent correlation spectroscopy (Sergentet et al., 2008)In clinical use and pathogen detection, NASBA pose theoretically higher analytical
sensitivity than reverse transcription-polymerase chain reaction RT-PCR making it an established diagnostic tool (Manojkumar et al., 2006)It has potential for detection and differentiation of viable cells through specific and sensitive amplification of messenger RNA, even against the background of genomic DNA (Fakruddin et al., 2012)

2.9.3 Methods of DNA Sequencing

2.9.3.1 Sanger Method

The Sanger method relied on a primer that would bind to a denatured DNA molecule and initiate the synthesis of a single-stranded polynucleotide in the presence of DNA polymerase enzyme, using the denatured DNA as a template (Sanger and Coulson, 1997). In most circumstances, the enzyme would catalyze the addition of a nucleotide. A covalent bond would therefore form between the 3’ carbon atom of the deoxyribose sugar molecule in one nucleotide and the 5’ carbon atom of the next. Dye-terminator sequencing utilizes labelling of the chain terminator ddNTPs, which permits sequencing in a single reaction, rather than four reactions as in the labelled-primer method. In dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labelled with fluorescent dyes, each of which emit light at different wavelengths.

Owing to its greater expediency and speed, dye-terminator sequencing is now the mainstay in automated sequencing. Its limitations include dye effects due to differences in the incorporation of the dye-labelled chain terminators into the DNA fragment, resulting in unequal peak heights and shapes in the electronic DNA sequence trace chromatogram after capillary electrophoresis (Sanger and Coulson, 1997).
This problem has been addressed with the use of modified DNA polymerase enzyme systems and dyes that minimize incorporation variability, as well as methods for eliminating "dye blobs". The dye-terminator sequencing method, along with automated high-throughput DNA sequence analyzers, is now being used for the vast majority of sequencing projects.

2.9.3.2 Methods of phylogenetic analysis

There are two major groups of analysis to examine phylogenetic relationship between sequences. Phenetic methods where trees are calculated by similarities of sequences and are based on distance methods. The resulting tree is called a dendrogram and does not necessarily reflect evolutionary relationships. Distance methods compress all of the individual differences between pairs of sequences into a single number (Kidd and Laura, 1971).

Cladistic methods where trees are calculated by considering the various possible pathways of evolution and are based on parsimony or likelihood methods. The resulting tree is called a cladogram. Cladistic methods use each alignment position as evolutionary information to build a tree (Kidd and Laura, 1971)

2.9.3.3 Phenetic methods based on distances

Starting from an alignment, pairwise distances are calculated between DNA sequences as the sum of all base pair differences between two sequences (the most similar sequences are assumed to be closely related). This creates a distance matrix. All base changes can be considered equally or a matrix of the possible replacements can be used (Adams, 1972)
Insertions and deletions are given a larger weight than replacements. Insertions or deletions of multiple bases at one position are given less weight than multiple independent insertions or deletions. It is possible to correct for multiple substitutions at a single site. From the obtained distance matrix, a phylogenetic tree is calculated with clustering algorithms. These cluster methods construct a tree by linking the least distant pair of taxa, followed by successively more distant taxa (Farris, 1971)

- Unweighted Pair Group Method using Arithmetic averages (UPGMA) clustering: this is the simplest method
- Neighbor joining: this method tries to correct the UPGMA method for its assumption that the rate of evolution is the same in all taxa.
CHAPTER THREE
MATERIALS AND METHODS

3.1 Testing site
Preparations of cultures, testing and analysis were carried out at Centre for Microbiology Research (CMR) in Kenya Medical Research Institute (KEMRI) and International livestock Research Institute (ILRI), Kenya.

3.2 Preparation of cultures for use in investigations
Five hundred and eighteen (518) bacterial strains of gram negative bacilli, were kindly provided by KEMRI, CMR and sub cultured for the detection of *E. coli* and *K. pneumonia*, the isolates of interest. To transfer the bacterial isolates from the stock cultures, a small amount of bacteria colony was scraped off the nutrient agar slope of a stock culture, using a sterile wire loop. The bacterial colony was put in a small volume of sterile nutrient broth and put in to the incubator for 18-24 hours at 37 °C. After incubation, revive isolates were sub-cultured on MacConkey agar to obtain pure bacterial colonies for analysis.

3.3 Bacterial identification and susceptibility testing
3.3.1 Bacterial identification by API 20E method
Among all the pathogens, MDR *E. coli* and *K. pneumonia* have emerged as one of the world's greatest health threats in past two decades. The nosocomial infections caused by these ESBL producing MDR *E. coli* and *K. pneumonia* complicated the therapy and limit treatment options hence the reason for their selection as the isolate to be studied. To identify for *E. coli* and *Klebsiella pneumonia* among the gram negative bacilli, API-20E test kit (bio Merieux, Inc., Hazelwood, MO) was used. The method provides an easy way
to identify bacteria belonging to members of the family *Enterobacteriaceae* specific species. API-20E is a testing system comprising 20 individual miniaturized test tubes or cupules each containing different chemical products used to determine the metabolic characteristics of gram negative bacteria, and ultimately, the genus and species of the bacteria.

Pure culture of the test organism in pure saline suspension was inoculated in the plastic strip holding the twenty mini-test tubes. Sterile oil was added into the ADH, LDC, ODC, H2S and UREA compartments to create anaerobic environment. Some water was added to the tray to prevent the compartment from drying. The strips were then marked with the identification of the patient and the initials of the person doing the test (Figures 3.1a and b). The strips were then incubated for 24-48 hours to allow the biochemical reactions to take place. After incubation, some compartments were read immediately but for some, additional reagents were added according to manufactures’ instructions. Results were interpreted using API reading scale as per manufactures’ instructions (Figure c).

**Figure 3.1:** API method for the identification of *E.coli* and *K.pneumonia*.

(a) Inoculation of bacterial culture (b) inoculated strips before incubation (c) inoculated strips after incubation
3.3.2 Antimicrobial susceptibility testing

The Kirby-Bauer and Stokes' antibiotic susceptibility methods are usually recommended. For this study, Kirby-Bauer method was used as one of the methods recommended by the NCCLS (Nijs et al., 2003) (Figures 3.2 a, 3.2 b and 3.2 c). Good laboratory practice and maintaining a standard set of procedures was followed to ensure accuracy and reproducibility of this method.

A pure colony of the test organism was picked from macConkey agar plate with a sterilize wire loop and inoculated in a suspension of sterile normal saline. The pure colony was added until it reached a turbidity of 0.5 McFarland standards. Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile non-toxic swab on an applicator was dipped into the adjusted suspension. The swab was rotate several times by pressing firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. This was then inoculated on the dried surface of a Muller-Hinton agar plate by streaking the swab over the entire sterile agar surface. This procedure was repeated two more times, as the plate was rotated at 60° each time, to ensure an even distribution of inoculum. The plates were left on the bench for 3 to 5 minutes to allow any excess surface moisture to be absorbed before applying the antibiotic disks. The appropriate disks were evenly placed (no closer than 24 mm from center to center) on the surface of the agar plate by use of a sterile forceps. Care was taken not to move the antibiotic disc once it has come in contact with the agar surface since some of the compound diffuses almost instantaneously.
The plates were clearly labeled with an identity number, Inverted and placed in an incubator at 35°C within 15 minute after inoculation. Incubation was done aerobically for 16-18 hours after which, each plate was examined and the diameter of the zone of inhibition including the diameter of the disks measured using a ruler. The zone sizes were interpreted by referring to the manufacturer provided standard table and the organism was reported either as susceptible, or resistant. Quality control organism of *E. coli* ATCC 25922 was used to validate the accuracy of the procedure.

![Image](image.png)

**Figure 3.2: Antibiotic susceptibility testing by Kirby-Bauer method:**

a) Inoculation of the test isolate  
 b) Antibiotic discs added  
 c) Zones of inhibition

### 3.3.3 Identification of ESBL production

The presence of ESBL enzymes was tested in all the isolates *E. coli* and *K. pneumonia* that were identified in this study. The production of these enzymes was analyzed by Modified Double Disc Synergy Test (MDDST) using antibiotic sensitivity disc of amoxicillin-clavulanate (20/10 μg) together with three other cephalosporins; ceftriaxone, cefuroxime and ceftazidime. This test permits to evaluate the inhibition of ESBL activity by Clavulanic acid.
A pure colony of the test organism was picked from mac Conkey agar plate with a sterilize wire loop and inoculated in a suspension of sterile normal saline. The pure colony was added until it reached a turbidity of 0.5 McFarland standards. Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile non-toxic swab on an applicator, was dipped into the adjusted suspension. The swab was rotate several times by pressing firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. This was then inoculated on the dried surface of a Muller-Hinton agar plate by streaking the swab over the entire sterile agar surface. A disc containing amoxicillin-clavulanate (30/10 μg) was put in the middle of the plate followed by placing two discs of cephalosporins, (ceftriaxone, cefuroxime and ceftazidime) 15mm apart, center to center to that of the amoxicillin-clavulanate disc (Jouini et al., 2010). The plates were incubated at 37°C aerobically for 18-24 hours. An increase in zone or change in pattern of inhibition towards amoxicillin-clavulanate was considered positive for the production of ESBL enzyme. K. pneumonia ATCC® 700603 was used as a positive control for ESBL production and E. coli ATCC® 25922 was used as a negative control (Olesen et al., 2004).

The disc on the left is cefotaxime (30 mg): the disc in the centre is amoxicillin-clavulanate(20/10 mg): the disc on the right is ceftazidime (30 mg). Note the expansion of the zones around the cefotaxime and ceftazidime discs adjacent to the amoxicillin-clavulanate (Jouini et al 2010)
3.4 Disinfectants susceptibility to ESBLs

3.4.1 Disinfectants used in the study and rationale for their selection

Disinfectants and antiseptics play a key role in management of bacterial infections. It was imperative to test the disinfectants together with the antibiotic to check whether the disinfectants used to clean area of possible contamination are also effective against the ESBL producing isolates. To facilitate on the selection of the types of the disinfectants to be tested, a visit was done to several health care facilities to know the type commonly used. During the visits six disinfectants/antiseptic were selected (Table 3.1)

This study will provide information on proper selection of antimicrobial agent for hospital use and management of hospital and community acquired infections (Hawkey, 1998). In order to find the commonly used disinfectants in practice, and to determine the criteria for selection, several health facilities were visited (Table 3.1). A convenience sampling method was used to select 20 isolates (10 *K.pneumonia* and 10 *E.coli*) which were tested against the disinfectants.
<table>
<thead>
<tr>
<th>Health facility</th>
<th>Disinfectants used</th>
<th>Disinfectants/antiseptic usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Kiambu District Hospital</td>
<td>Sodium hypochlorite (1 in 5 parts)</td>
<td>- Used in the lab</td>
</tr>
<tr>
<td></td>
<td>Chlorhexidine Gluconate (Savlon)</td>
<td>- Laundry work</td>
</tr>
<tr>
<td></td>
<td>Hydrogen peroxide (6%W/V)</td>
<td>- Used for general cleaning of floors</td>
</tr>
<tr>
<td></td>
<td>Sodium hypochlorite (1 in 5 parts)</td>
<td>- Sterilization of Theatre equipments and other instruments</td>
</tr>
<tr>
<td></td>
<td>10% formalin</td>
<td>- In sterilizing laboratory safety cabinets</td>
</tr>
<tr>
<td></td>
<td>5% phenol</td>
<td>- Used in the TB laboratory</td>
</tr>
<tr>
<td>2 Nairobi hospital</td>
<td>Chlorhexidine Gluconate (Savlon) (1.5% v/v Chlorhexidine and 15% w/v Cetrimide).</td>
<td>- For general cleaning of floors</td>
</tr>
<tr>
<td></td>
<td>Sodium hypochlorite (1 in 5 parts)</td>
<td>- Sterilization of Theatre equipments and other instruments</td>
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</tr>
<tr>
<td></td>
<td>5% phenol</td>
<td>- Used in the TB laboratory</td>
</tr>
<tr>
<td>3 National HIV reference lab</td>
<td>Sodium hypochlorite (1 in 5 parts)</td>
<td>- General disinfection of the lab and glass.</td>
</tr>
<tr>
<td>4 National Microbiology reference lab</td>
<td>Sodium hypochlorite (1 in 5 parts)</td>
<td>- General disinfection of the lab and glass.</td>
</tr>
<tr>
<td>5 Mercy light clinic (kiambu)</td>
<td>Chlorhexidine Gluconate (Savlon), sodium hypochlorite (6% w/v)</td>
<td>- Used for sterilising medical equipments, and other genera areas, sterilization of gauze for dressing wound</td>
</tr>
<tr>
<td>6 Dr macharia clinic (Thika)</td>
<td>Sodium hypochlorite (1 in 5 parts)</td>
<td>- Used for all sterilization purposes</td>
</tr>
</tbody>
</table>
3.4.2 Testing methodology for the disinfectants

The following disinefectants were selected for testing against the ESBL s isolates of *E. coli* and *K. pneumonia* (Table 3.2).

**Table 3.2: List of the Disinfectants Selected**

<table>
<thead>
<tr>
<th>Disinfectants</th>
<th>User concentration</th>
<th>Tested concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen peroxide (6% W/V)</td>
<td>3%</td>
<td>1:2, 1:4, 1:8, 1:16 and 1:32</td>
</tr>
<tr>
<td>Chlorhexidine Gluconate (Savlon) (1.5% v/v Chlorhexidine and 15% w/v Cetrimide).</td>
<td>2%</td>
<td>1:2, 1:4, 1:8, 1:16 and 1:32</td>
</tr>
<tr>
<td>Sodium hypochlorite</td>
<td>5%</td>
<td>1:2, 1:4, 1:8, 1:16 and 1:32</td>
</tr>
<tr>
<td>Formalin</td>
<td>10%</td>
<td>1:2, 1:4, 1:8, 1:16 and 1:32</td>
</tr>
<tr>
<td>Phenol</td>
<td>5%</td>
<td>1:2, 1:4, 1:8, 1:16 and 1:32</td>
</tr>
<tr>
<td>Ethanol</td>
<td>70%</td>
<td>1:2, 1:4, 1:8, 1:16 and 1:32</td>
</tr>
</tbody>
</table>

The effectivenes of the six commercial disinfectants against the isolates was tested using a method described by (Meseret et al., 2014). Briefly, seven tubes were arranged in a row and labelled as number 1-7. One (1 ml) of sterile nutrient broth was added in to all tubes, except tube number 1. This was followed by adding 1 ml of known concentration of a disinfectant to tubes number 1 and 2. Tube 2 was then well mixed and 1 ml of its content transferred into tube 3. The content of tube 3 was again mixed and 1 ml taken and added to tube 4. The same was repeated for tube 5. After this, 1 ml of tube 5 content was discarded. Lastly, 0.1 ml suspension of a pure culture of the isolates equivalent to turbidity of 0.5 McFarland standards was added to all tubes, except tube number 7. Tube number 6, which contained nutrient broth + test organism was used as positive control while tube number 7 which contained nutrient broth + distilled water (without the bacteria) as a negative control.
All the tubes (1-7) were then incubated 18-24 hours at 35-37°C. After incubation, the contents of the tubes was sub-cultured on to culture plates containing nutrient agar and incubated again at 35-37°C for 24 hour. The plates were then observed for bacterial growth. The bactericidal concentration was considered as the concentration of the tube in which no growth was observed.

### 3.5 Identification of β-lactamase genes by Polymerase Chain Reaction (PCR)

The aim of the PCR analysis was to determine the genes, encoding for ESBLs in *E. coli* and *K. pneumonia*. The analysis was done by PCR assay for detection of *blaCTX-M-1*, *blassHV*, *blaOXA-1* and *blaTEM* genes, using the primers described by Kiiru *et al.*, (2012).

#### 3.5.1 DNA extraction

Seventy (70) bacteria strains of *K. pneumonia* (34) and *E. coli* (36) phenotypically identified as positive for ESBL production were analyzed. Bacterial DNA extract was obtained using boiling method (Pitout, *et al.*, 2010) as follows; DNA was prepared from overnight pure cultures of tests isolates grown on Mueller Hinton agar plates. Few colonies of pure culture of test organism was suspended in 1ml sterile distilled water or pure deionized sterile water and boiled at 100°C for 5 minutes. Upon boiling, the preparation was spun using a centrifuge at 14,000 rpm for 5 minutes.

The supernatant was then stored at -20°C until further use while cell debris was discarded.

#### 3.5.2 PCR amplification

The DNA extract was used as template in the specific PCR amplifications for identification of selected *bla* genes, (*bla-OXA-1*, *bla-TEM*, *bla-CTX-M* and *bla-SHV*). PCRs screening for these genes were performed using consensus sequence primers previously...
published for bla\textsubscript{SHV} and bla\textsubscript{TEM, OXA-1} and CTX-M\textsubscript{M} (Arlet \textit{et al.}, 1997b), bla\textsubscript{CTX-M}\textsubscript{M} (Lartigue \textit{et al.}, 2004) and bla\textsubscript{OXA-1} (Olesen \textit{et al.}, 2004) Table 3.3.

**Table 3.3: List of primers used for PCR screening and sequencing of β-lactamase genes**

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer name</th>
<th>5'-3' sequence</th>
<th>Annealing temperature</th>
<th>Expected product size (bp)</th>
<th>Gene accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{blaTEM}</td>
<td>TEM–F</td>
<td>GCGGAACCCCTATTTTG</td>
<td>50</td>
<td>964</td>
<td>EF125012 related</td>
</tr>
<tr>
<td></td>
<td>TEM–R</td>
<td>TCTAAAGTATATGAGTAACCTTGTTGCTGAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{blaSHV}</td>
<td>SHV–F</td>
<td>TTCGCCCTGTGATATCTCCCTG</td>
<td>50</td>
<td>854</td>
<td>AF148850 related</td>
</tr>
<tr>
<td></td>
<td>SHV–R</td>
<td>TTAGCCTGTCCAGTGYTCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{blaCTX-M}</td>
<td>CTX-F</td>
<td>ATGTGCAGYTACAGTARGTKATGGC</td>
<td>60</td>
<td>593</td>
<td>Y10278-related</td>
</tr>
<tr>
<td></td>
<td>CTX-R</td>
<td>ATGTGCAGYTACAGTARGTKATGGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{blaOXA-1}</td>
<td>OXA-1F</td>
<td>ATGAAACACACAATACATATCAACTTCG</td>
<td>62</td>
<td>820</td>
<td>JQ2967-related</td>
</tr>
<tr>
<td></td>
<td>OXA-1R</td>
<td>GTGTGTAGAATGGTGATCAGTATT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Source: Kiiru \textit{et al.}, 2012.

The \textit{bla} genes were detected using a PCR procedure demonstrated by (Kiiru \textit{et al.}, 2012). In the method, gene to be detected was amplified in a total reaction volume of 25μl containing 5μl of 10pmol each of primer, 0.5μl of 20mM of dNTPs, 2.5μl 10mM Tris-HCl (pH 8.8), 25mM MgCl\textsubscript{2} and 1.25μl Taq DNA Polymerase (ThermoFischer Scientific, Rockford, IL, USA). This was followed by adding 2μl of template DNA. Nuclease free DNA was added to make a final reaction volume of 25μl master mixture. The mixture was then placed in MJ-mini Bio-Rad thermal cycler (Bio-Rad, USA). The PCR amplification cycle was carried out starting with an initial denaturation temperature of 95°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds. Annealing
conditions were programmed based on the gene under analysis for 1 minute (see table above) and an extension temperature of 72°C for 1 minute 30 seconds. The final extension conditions were set at temperatures 72°C for 10 minute. The presence and sizes of amplicons were detected on 1.2% agarose gels using GeneRuler 100 bp DNA Ladder Plus as a size marker (Hansen et al., 2012). After electrophoresis DNA fragments was visualized by Bio-Rad Gel documentation system (Bio-Rad, USA).

3.5.3 PCR product gel extraction and purification using Qiagen kit

Fragments of DNA obtained from the PCR analysis were pieced from the agarose gel with a sharp, clean and sterile scalpel blade and weighed. One volumes of gel was added to three volumes of buffer (provided in the test kit) and incubated at 50°C for 10 minutes or until the gel slice has melted completely. After the gel slice has completely dissolved, 1 volume of isopropanol was added to the sample and mixed thoroughly. QIAquick spin column was used to bind DNA by applying the sample to the QIAquick column, and centrifuged for 1 minute. The flow-through was discarded and 0.5 ml of Buffer QG added to QIA quick column and centrifuged for 1 minute. This step was to remove all traces of agarose. To wash, 0.75 ml of Buffer PE was added to QIAquick column and centrifuge for 1 minute and the flow-through discarded and the QIAquick column centrifuged for an additional 1 minute at 13,000 rpm. QIAquick column was then placed into a clean 1.5 ml micro centrifuge tube and the DNA eluted by adding 50 µl of Buffer EB (10 mM TrisHCl, pH 8.5) to the center of the QIAquick membrane and centrifuged the column for 1 minute. the products were then kept in 1.5ml eppendorff tubes at -20 to 70°C awaiting sequencing.
3.6 DNA Sequencing

Sequencing of $bla$ genes was commercially done at International Livestock Research Institute (ILRI) done using the bi-directional strategy. Bi-directional sequencing of the PCR products was done using the DyeDeoxy chain termination method in ABI3730 Sanger sequencer (PE Biosystems, Foster City, Calif). Sequencing of the entire gene fragment was done using the same set of primers used for amplification.

3.6.1 Phylogenetic analyses

The DNA sequences of $bla_{TEM}$ and $bla_{SHV}$ were obtained from ABI3730 Sanger sequencer. The sequences were processed on SeqTrace 0.9.0 (Stucky, 2012) to build consensus sequences and to trim off bases that were incorrectly called. The sequences were aligned using Clustal Omega on SeaView version 4 (Gouy et al., 2010).

Phylogenetic tree was obtained using the maximum likelihood method and the tree inferred by bootstrap test of 1000 replicates using PhyML in SeaView version 4 (Gouy et al., 2010) and MEGA 6 (Tamura et al., 2013). Other published beta lactamase genes sequences available in GeneBank were appropriately incorporated in the $bla_{TEM}$ and $bla_{SHV}$ phylogenetic analysis to infer the closest relatives. The generated phylogenetic trees were visualized on FigTree version 1.4.1. (http://tree.bio.ed.ac.uk/software/figtree/)

3.7 Data Analysis

The raw data was stored in a computer and processed using statistical program for social sciences (SPSS /PC Microsoft TM). The data was recorded in Microsoft excel and databases created in Microsoft access and cleaned by ensuring consistency of variables before analysis using IBM SPSS version 20 software. The data was kept confidential by
use of unique identifiers in a password-protected Ms. Access database. Descriptive statistics were used to compute frequencies and proportions of phenotypic resistance, and susceptibility to various antimicrobials and for the presence of resistance genes among isolate. Susceptibility data was analyzed using WHONET 5.6 software and further processed on Microsoft Excel. Differences in ESBL carriage and differences in resistance among isolates were determined using 2-tailed Fisher’s exact test or the Chi-square tests (X2) with GraphPad Prism© software.

Fisher’s test (non-parametric) was used where the dataset was not normally distributed whereas Chi-square (parametric) was used on normal distributed datasets. Tests values were set at 95% confidence intervals (CI) and a $p$ value of greater than 0.05 was not considered significant.

Prevalence of each gene was obtained using the same program. Description of sequences was done using bioinformatics tools obtained at the website of the National Center of Biotechnology Information on http://www.ncbi.nlm.nih.gov while alignment of amino acid sequences to analyze for mutations based on the wild-type gene was done using the ClustalW program on http://www.ebi.ac.uk(Thompson et al. 1994).
CHAPTER FOUR

RESULTS

4.1 Prevalence of ESBLs in *E. coli* and *K. pneumonia*

Five hundred and eighteen (518) gram negative bacterial isolates, provided from Research Pathology were cultured for the presence of *K. pneumonia* and *E.coli*, the isolates of interest. Upon identification, 172 isolates were found to be *E.coli* and *K. pneumonia*. Ninety three, (93) were *E. coli* (54%) and 79, were *K. pneumonia* (46%). Out of 172 isolates of *K. pneumonia* and *E.coli*, 70 (41%) were ESBLs positive (21% ESBLs *E. coli* and 20% *K. pneumonia*) (Table 4.1).

<table>
<thead>
<tr>
<th>Bacteria type</th>
<th>Number of isolates obtained</th>
<th>ESBLs Negative</th>
<th>ESBLs Positive</th>
<th>% ESBL positive n=172</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E.coli</em></td>
<td>93</td>
<td>57</td>
<td>36</td>
<td>21</td>
</tr>
<tr>
<td><em>K.pneumonia</em></td>
<td>79</td>
<td>46</td>
<td>34</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>172</td>
<td>102</td>
<td>70</td>
<td>41</td>
</tr>
</tbody>
</table>

4.2 Antibiotic susceptibility for *K. pneumonia* and *E.coli*

Among the *E. coli* strains, highest resistances were recorded for ampicillin (81%), sulfamethoxazole-trimethoprim combination (80%), and tetracycline (79%). Among cephalosporins, highest resistances were recorded against cefuroxime (74%), ceftazidime (63%) and ceftriaxone (63%). Relatively lower resistant was recorded for gentamicin (44%), cefepime (44%)
In general the resistance prevalence of the *K. pneumonia* strains were similar to those of *E. coli*. There were no statistical significant differences between resistance in *K. pneumonia* and *E. coli* (p=0.5686, OR=1.226, CI=0.7062-2.146). Similar to resistance patterns observed in *E. coli*, high resistant was recorded for Ampicillin (95%), sulfamethoxazole-trimethoprim combination (92%), and tetracycline (87%). Resistance profiles of these isolates to cephalosporins were also close to those of *E. coli* with high resistant being recorded for cefuroxime (80%), ceftazidime (68%) and ceftriaxone (76%). Relatively lower resistant were recorded for gentamicin (42%), cefepime (57%) and lowest for meropenem (9%) (Figure 4.2).

**Figure 4.1: Antibiotic susceptibility for *E.coli***

In general the resistance prevalence of the *K. pneumonia* strains were similar to those of *E. coli*. There were no statistical significant differences between resistance in *K. pneumonia* and *E. coli* (p=0.5686, OR=1.226, CI=0.7062-2.146). Similar to resistance patterns observed in *E. coli*, high resistant was recorded for Ampicillin (95%), sulfamethoxazole-trimethoprim combination (92%), and tetracycline (87%). Resistance profiles of these isolates to cephalosporins were also close to those of *E. coli* with high resistant being recorded for cefuroxime (80%), ceftazidime (68%) and ceftriaxone (76%). Relatively lower resistant were recorded for gentamicin (42%), cefepime (57%) and lowest for meropenem (9%) (Figure 4.2).
Figure 4.2: Antibiotic susceptibility for K. pneumonia

4.3 Antibiotic susceptibility for ESBLs K. pneumonia and E.coli

The ESBL producing E.coli and K. pneumonia were significantly more resistant to antimicrobials compared to non-ESBL strains (P<0.0001, OR 8.2254, CI: 3.637 – 18.60).

ESBL strains recorded high resistance prevalence to all antimicrobials (above 60%) except for gentamicin which was 56% for K. pneumonia. Meropenem recorded lower resistance, (8% E. coli and 18% K. pneumonia) (Figure 4.3 and 4.4). For both species, ESBL-producers were also more likely to exhibit resistance to other classes of antimicrobials than non-ESBL strains. (p=0.02, OR=33.46, CI=1.44-778).
Figure 4.3: Antibiotic susceptibility for ESBL producing E. coli and K. pneumonia
Figure 4.4: Antibiotic resistance for ESBL and non-ESBLs producing *E. coli* and *K. pneumonia*
4.4 Susceptibility to disinfectants

Twenty (20) isolates were also picked (10 ESBLs producing *E. coli* and 10 ESBLs producing *K. pneumonia*) and their susceptibility to 6 disinfectants was determined. The disinfectants were phenol, hydrogen peroxide, Sodium hypochlorite and Chlorhexidine Gluconate (Savlon), ethanol and formalin.

The results indicated that, 10% formalin showed the highest effectiveness with 100% susceptibility at user concentration (neat), 1:2 and 1:4 dilutions. The susceptibility reduces as the concentrations decrease. This was followed by hydrogen peroxide and phenol with 100% susceptibility at user concentration and 1:2 dilutions. The susceptibility also decreases with decrease in concentrations. The other four disinfectants were only effective at the user recommended concentration (Table 4.2).

Table 4.2 shows the number of tested strains that grew at a given dilution. The user concentration (neat) is the undiluted sample of the test disinfectant at the concentration recommended for use.
Table 4.2: Susceptibility of ESBLs producing strains to disinfectants

<table>
<thead>
<tr>
<th>Dilutions</th>
<th>10% formalin (%)</th>
<th>5% phenol (%)</th>
<th>Hydrogen peroxide (%)</th>
<th>Savlon (%)</th>
<th>Sodium hypochlorite (Jik) (%)</th>
<th>70% Ethanol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>User dilution * (recommended concentration for use)</td>
<td>20(100)</td>
<td>20(100)</td>
<td>20(100)</td>
<td>20(100)</td>
<td>20(100)</td>
<td>20(100)</td>
</tr>
<tr>
<td>1:2</td>
<td>20(100)</td>
<td>19(95)</td>
<td>20(100)</td>
<td>13(65)</td>
<td>6(30)</td>
<td>15(75)</td>
</tr>
<tr>
<td>1:4</td>
<td>20(100)</td>
<td>15(75)</td>
<td>19(95)</td>
<td>15(75)</td>
<td>4(20)</td>
<td>2(10)</td>
</tr>
<tr>
<td>1:8</td>
<td>19(95)</td>
<td>6(30)</td>
<td>19(95)</td>
<td>6(30)</td>
<td>2(10)</td>
<td>0(0)</td>
</tr>
<tr>
<td>1:16</td>
<td>19(95)</td>
<td>0(0)</td>
<td>12(60)</td>
<td>0(0)</td>
<td>1(5)</td>
<td>0(0)</td>
</tr>
<tr>
<td>1:32</td>
<td>13(65)</td>
<td>0(0)</td>
<td>9(45)</td>
<td>0(0)</td>
<td>0(0)</td>
<td>0(0)</td>
</tr>
</tbody>
</table>

* Recommended concentration for routine use. Number in the bracket indicates % susceptible
4.5 Amplification of ESBLs genes in *K. pneumonia* and *E. coli*

Table 4.3: Patterns of amplified ESBLs gene types identified in *K. pneumonia* and *E. coli*

<table>
<thead>
<tr>
<th>Gene type</th>
<th>Number of isolate amplified</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. coli</em> (N= 36)</td>
</tr>
<tr>
<td><strong>blaTEM</strong></td>
<td>26</td>
</tr>
<tr>
<td><strong>blaSHV</strong></td>
<td>5</td>
</tr>
<tr>
<td><strong>blaCTX-M</strong></td>
<td>29</td>
</tr>
<tr>
<td><strong>blaOXA-1</strong></td>
<td>10</td>
</tr>
</tbody>
</table>

4.5.1 Carriage of combination of *bla* genes in *E. coli* and *K.pneumonia*

The isolates were tested for the carriage of bla gene combinations. Among the *E. coli* strains, the most ESBLs genes combination was *blaCTX-M* + *blaTEM* accounting for 20/36 followed by *blaCTX-M*+*blaTEM*+*blaOXA-1* (12/36), *blaTEM* + *blaSHV* (9/35), *blaCTX-M* + *blaTEM* + *blaSHV* (6/36) and *blaCTX-M* + *blaSHV* (4/36). In *K. pneumonia*, the most common combination is *blaTEM* + *blaSHV* accounting for 12/34 followed by *blaCTX-M* + *blaSHV* (2/34),*blaCTX-M* + *blaTEM* + *blaSHV* (2/34), *blaCTX-M*+*blaTEM*+*blaOXA-1* (1/34) and *blaCTX-M* + *blaTEM* (1/34) (Table 4.4).
Table 4.4: Occurrence of ESBLs genes occurring in combination in *E.coli* and *K. pneumonia*

<table>
<thead>
<tr>
<th>Two or more genes in combinations</th>
<th><em>E.coli</em></th>
<th><em>K.pneumonia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N= 36</td>
<td>N=34</td>
</tr>
<tr>
<td><strong>bla</strong>CTX-M + <strong>bla</strong>SHV</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td><strong>bla</strong>CTX-M + <strong>bla</strong>TEM</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td><strong>bla</strong>TEM + <strong>bla</strong>SHV</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td><strong>bla</strong>CTX-M+<strong>bla</strong>TEM+<strong>bla</strong>OXA-1</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td><strong>bla</strong>CTX-M + <strong>bla</strong>TEM-<strong>bla</strong>SHV</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

### 4.6 Sequencing of **bla**SHV and **bla**TEM

#### 4.6.1 Identified sub-types of **bla**TEM and **bla**SHV by sequencing method

Sequence analysis was done on the DNA of 10 ESBLs isolates that had **bla**TEM and 10 isolates that had **bla**SHV. A total of 20 ESBLs isolates were sequenced. The results revealed 9 sub-types of **bla**TEM and 5 sub-types of **bla**SHV. The most common sub-type of **bla**TEM was TEM-1 (6) followed by TEM-115 (3) and one each of other sub-types (186, 198, 219, 208 and 154) (1). For **bla**SHV, the sub-type obtained was **bla**SHV-1 (2) and (12, 32, 101 and 11) (1). (Table 4.5)
Table 4.5: ESBL genes Sub-types identified in TEM and SHV

<table>
<thead>
<tr>
<th>TEM - types</th>
<th>Number identified in each sub-type (N=10)</th>
<th>SHV- types</th>
<th>Number identified in each sub-type (N=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM -186</td>
<td>1</td>
<td>SHV- 1</td>
<td>2</td>
</tr>
<tr>
<td>TEM – 198</td>
<td>1</td>
<td>SHV-12</td>
<td>1</td>
</tr>
<tr>
<td>TEM -219</td>
<td>1</td>
<td>SHV - 32</td>
<td>1</td>
</tr>
<tr>
<td>TEM – 208</td>
<td>1</td>
<td>SHV- 101</td>
<td>1</td>
</tr>
<tr>
<td>TEM – 1</td>
<td>6</td>
<td>SHV-11</td>
<td>1</td>
</tr>
<tr>
<td>TEM – 115</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM- 154</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM -163</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM -169</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.7 Phylogenetic analyses for blaSHV and blaTEM

Seaview and MEGA 6 generated identical phylogenetic trees. Sequences of blaOKP (A and B) and blaLEN available on Gene Bank were included in the phylogenetic analysis. Sequences of blaOKP available in Gene Bank are only from Klebsiella pneumonia. Sequences of blaLEN from different bacteria species were also incorporated in the phylogenetic analysis. The different beta lactamase genes blaSHV, blaOKP and blaLEN clustered separately with each forming a distinct cluster (Figure 4.5). It was noted that, blaLEN are the closest relative to blaSHV compared to blaOKP.
Figure 4.5: Phylogenetic tree for SHV
Rooted phylogenetic tree based on nucleic-acid sequences of near full length $bla_{\text{SHV}}$ genes and $bla_{\text{OKP}}$ (A/B) as the out group. Branch length values are percentage bootstrap values. Tip labels are aligned for clear visualization. Branch labels in blue font represents isolates from this study.
while those in black represents isolates from GeneBank in the format (Genebank accession number, Isolate name, Gene name).

Sequences of blaTEM (TEM-1, TEM-2, TEM-29, TEM-104 and TEM-190) of different bacteria species available on Gene Bank were randomly selected to represent different species in the phylogenetic analysis. Sequences of genes blaOKP (A/B) from Gene Bank were included as the out group. The blaTEM gene sequences of isolates from this study cluster together with blaTEM gene sequences of different species from Gene bank (Figure 4.6).
Figure 4.6: Phylogenetic tree for TEM

Rooted phylogenetic tree based on nucleic-acid sequences of near full length blaTEM genes. Branch length values represent relative phylogenetic distances. Tip labels are aligned for clear visualization. Labels in red font represent isolates from this study.
5.1 Discussion

Multidrug resistance and production of ESBLs by gram negative bacteria in hospitals and community continue to be a matter of scientific concern. Infections caused by these types of bacteria have been associated with high mortality (Berkley et al., 2005). This study describes the prevalence and antimicrobial resistance profiles in K. pneumonia and E. coli isolates from stock cultures of gram negative bacteria. These infections results in poor treatment outcomes as a result of empirical treatment protocols not supported by laboratory antibiotic sensitivity testing report. Further there is no adequate data regarding the prevalence and antimicrobial susceptibility of ESBL-producing K. pneumonia and E. coli in Kenya. This study found that, the prevalence of ESBLs was 41% (E. coli 21% and K. pneumonia was 20%). The epidemiology of ESBLs is quite complicated. Initially, there are certain levels to consider: the wider geographical area, the country, the hospital, the community, and the host (in most cases a single patient or a healthy carrier). Moreover, these are bacteria (E. coli is more endemic, and K. pneumoniae is more epidemic) and their mobile genetic elements, usually plasmids. Additionally, there are various reservoirs, including the environment (e.g. soil and water), wild animals, farm animals, and pets.

The final component entails transmission from food and water, and via direct or indirect contact (person to person) (Carattoli, 2008). The first ESBL to be identified was found in Germany in 1983, but it was in France in 1985 and in the United States at the end of the 1980s and the beginning of the 1990s that the initial nosocomial outbreaks occurred (Rice
et al., 1990). Soon thereafter, it was discovered that many of the *K. pneumonia* strains that caused nosocomial infections in France in the early 1990s were ESBL producers (Sirot et al.). A study conducted at the National Public health laboratory (NPHL), Kathmandu, Nepal reported that 31.57% of *E. coli* were confirmed as Extended Spectrum á-lactamase producers, these isolates further exhibited co-resistance to several antibiotics (Thakur et al. 2013).

Multidrug resistance has been reported among ESBL producing bacteria. The study showed that, the isolates were highly resistant to most of the antibiotic tested. Among the non-ESBL producing *E. coli* strains, highest resistances were recorded for ampicillin (81%), sulfamethoxazole-trimethoprim combination (80%), and tetracycline (79%). Among cephalosporins, highest resistances were recorded against cefuroxime (74%), ceftazidime (63%) and ceftriaxone (63%). Relatively lower resistant was recorded for gentamicin (44%), cefepime (44%) and lowest for meropenem (5%). The resistance pattern for the non-ESBL producing *K. pneumonia* strains were similar to those of non-ESBL producing *E. coli*. As for *E. coli*, high resistant was recorded for Ampicillin (95%), sulfamethoxazole-trimethoprim combination (92%), and tetracycline (87%). Resistance profiles of these isolates to cephalosporins were also close to those of *E. coli* with high resistant being recorded for cefuroxime (80%), ceftazidime (68%) and ceftriaxone (76%). Relatively lower resistant were recorded for gentamicin (42%), cefepime (57%) and lowest for meropenem (9%).

Majority of the isolates were resistant to antimicrobials used as first and second lines of treatment such as trimethoprim, cephalosporins, and tetracycline and to different
generations of aminoglycosides. While the epidemiologic and clinical basis of emergence of such resistances is not obvious, trimethoprim and sulfamethoxazole (co-trimoxazole) have been widely used as a first-line antimicrobial and for prophylaxis against opportunistic infections among HIV positive patients in Kenya (Chiller et al., 2009).

The use of this combination has been linked to increased resistance to sulphur-potentiated antimicrobials in related studies (Chiller et al., 2009). Another possible explanation on the basis of emergence of such resistances is the low proportion of patients treated based on laboratory data to support prescriptions.

A research done in Western Kenya (Shapiro et al., 1999) showed that 74% of Kenyan patients, even those with diarrhoea, frequently receive antimicrobials to which their isolates were not susceptible. Such antibiotic-use practices are likely to promote proliferation of MDR strains encoding resistance to sulphonamides and trimethoprim and to other antimicrobials whose resistance genes could be physically linked to those of these two antimicrobials (Cocchi et al., 2007). Other studies also shows that the proportion of strains resistant to fluoroquinolones, aminoglycosides and carbapenems may be prevalent among hospitalized patients (Cocchi et al., 2007). The study shows that only a small proportion of these strains remain susceptible to cephalosporins. The high resistance to cephalosporins, including third generation cephalosporins indicates that, a significant proportion of patients may not be treated with these antibiotics. Resistance to β-lactam and β-lactamase inhibitor such as augmentin was relatively high (64% among E. coli and 72% among K. pneumonia). This is particularly worrying
because inhibitor combinations are also frequently used to treat infections caused by ESBL-producers (Blazquez et al., 1993).

A significant number of isolates in this study remain susceptible to cefepime, which is a 4th generation cephalosporin (56% Bla and 43% K. pneumonia). Meropenem remains the most effective β-lactam antibiotic against these resistant isolates. Despite the hope given by this apparently effective β-lactam antibiotic, its empiric application for treatment of infections caused by isolates with expanded-spectrum resistance to β-lactam antibiotics is not recommended due to the possibility of treatment failure. Treatment failures using such agents normally arise from inducible β-lactamase production which may increase the chances of patient death (Melzer & Petersen 2007). Given these limitations, only few therapeutic options that include carbapenems remain for the treatment of this patient population.

Though this study did not look at the source of the clinical specimens, reports in other studies that show that, organisms with increased antimicrobial resistance are isolated more frequently from individuals with complicated urinary tract infection (Gupta et al., 2006; Hill et al., 2006b). This is a consequence of acquisition of organisms following health care exposures, together with repeated previous antimicrobial courses for many patients with persistent abnormalities who experience recurrent infection. This is more common among women (Gupta et al., 2006; Hill et al., 2006b). Most women with frequent recurrent acute uncomplicated urinary tract infection also receive repeated antimicrobial courses, which may promote the emergence of antimicrobial-resistant E. coli in the gut flora (Gupta et al., 2006; Hill et al., 2006b). However, the increasing antimicrobial resistance reported in community-acquired bla infection has many other
potential sources. Uropathogenic *E. coli* are usually acquired from environmental exposures including sexual partners, household members, pets, food and, frequently, during travel.

There is widespread dissemination of resistant uropathogenic *E. coli* clones globally (Guerra *et al.*, 2003; Hansson *et al.*, 2002; Herndon 1992). Related studies relates emergence and spread of these resistant strains with medical travel to countries such as India and those in the Middle East (Grenet *et al.*, 2004; Gupta *et al.*, 2006; Agarwal *et al.*, 2015).

Recent international guidelines recommend empirical treatment with three days of trimethoprim/sulfamethoxazole (TMP/SMX), five days of nitrofurantoin, a single dose of fosfomycin, or three to five days of pivmecillinam (Ensor *et al.*, 2006; Mathai *et al.*, 2008). For this empirical approach to be effective, practitioners must have knowledge of the local prevalence of antimicrobial resistance to the potential first-line empirical antimicrobials. If a certain antimicrobial has a resistance of >20%, to *E. coli* strains, then that antimicrobial is not considered to be appropriate for empirical therapy including uncomplicated urinary tract infection (Guerra *et al.*, 2003). Considering that, most prescriptions in Kenya and in Africa are not backed by laboratory data, it is highly likely that, these "blind" treatments only serve as a mechanism for emergence and spread of MDR strains in hospitals and community settings. We speculate that the high resistance profiles observed in our isolates is associated with poor prescription practices in Kenya.

Resistance from blood isolates is also of immense clinical and epidemiologic importance worldwide (Kariuki *et al.*, 1993; Kramer *et al.*, 2006). Yet, associated changes in total
burden of disease and the dynamic interaction between MDR and antibiotic-susceptible bacteria (ASB) have not been accurately quantified. Bacterial infections of the bloodstream occurs when immune system fail to contain infection at a focal site resulting in dissemination of the disease in the body making it a major cause of morbidity and mortality (Kramer et al., 2006).

The occurrences of these infections, their distribution pattern, and the responsible organisms have changed with the evolution of medical care especially when the emergence of increasingly ill and immune-compromised population of hospitalized patients who are often heavily dependent on medical support is considered. These infections account for mortality rate of 35%, 24 additional hospital days, resulting in excess hospital costs of $40,000 per survivor (Aiken et al. 2011; Arlet et al., 1995; Herindrainy et al., 2011). Although this study did not determine the underlying conditions of the patients, it is possible that some of the patients with invasive infections are immuno-compromised or predisposed by various unknown factors. Other studies on blood infections have found that the frequent use of antibiotics in hospitalized patients creates an ecological niche for MDR that replace ASB, without increasing the total burden of disease. Alternatively, MDR and ASB may not compete, and, thus, increasing infection rates caused by ARB. A combination of ASB and MDR as etiologic agents only serves to increase the disease burden.

Since the isolates from this study have been shown are to be highly resistant to antibiotics, antimicrobial interventions may not be the best strategy to curb the spread of MDR strains and therefore, this study demonstrates the need to put in place measures that ensure prudent infection control measures (Arlet et al 1995; Aiken et al., 2010;
Herindrainy et al., 2011). The practical solution to this immense problem is to design and enforce infection control strategies in Kenyan health care facilities to minimize the spread of these resistant organisms. Awareness and behavior change through education are the cornerstones of improving compliance with infection control strategies.

This study clearly indicates the presence of ESBL-mediated resistance enzyme among E. coli and Klebsiella strains causing infections in health care facilities in Kenya. The upsurge of multi-drug resistant bacteria to extended-spectrum cephalosporins threatens the effort to proper manage infections caused by K. pneumonia and E. coli. The most commonly encountered species of such resistances have been found and reported in a number of bacteria species. However, most of these bacteria isolates capable of producing ESBL includes K. pneumonia, K. oxytoca and E. coli. Despite the increasing trend of ESBL producing bacteria in these isolates in many countries, there are very few reports from Africa (Ferraz et al., 2000b; Hima-Lerible et al., 2003; Berkley et al., 2005; Fam et al., 2011; Mandomando et al., 2010). Although only a few studies have reported on the prevalence of ESBL producers in public hospitals of Kenya, ESBL isolation and identification is not routinely done. The unique pattern of resistance among the isolates studied indicated that, probably, hospitals may be the perfect settings where ESBL-strains and strains resistant to multiple antimicrobials evolve. Majority of isolates in this study that were ESBL-positive belonging to both species were significantly more resistant to antimicrobials compared to non-ESBL strains (P<0.0001, OR 8.2254, CI: 3.637 – 18.60). For both species, ESBL-producers showed resistance to other classes of antimicrobials than non-ESBL strains. This study therefore emphasizes the benefits of analyzing co-
resistance among hospital isolates because this could aid issuance of prescriptions and notification of strains that can easily result to treatment failure.

Some studies also showed that, treatment of infections by these organisms with extended-spectrum cephalosporins or aztreonam may result in treatment failure even when the organisms responsible for the infection appear to be susceptible by routine antibiotic susceptibility testing (Warren et al., 1999b; Lolans et al., 2005; Nelson-Filho et al., 2015; Luo et al., 2015; Tosic et al., 2016). In addition, other studies recommend that patients carrying these organisms in their guts (colonization) or those who are infected should be placed under close monitoring to prevent hospital transmission; (Lolans et al., 2005; Nelson-Filho et al., 2015; Luo et al., 2015; Tosic et al., 2016).

Management of infections caused by bacterial bacteria that produce ESBLs can overburden healthcare systems especially in poor resource countries considering that, these organisms account for a significant proportion of critical care infections. Furthermore, infections by ESBLs producing bacteria are associated with expensive and limited treatment options, which can impact negatively on treatment outcomes (Nelson-Filho et al. 2015; Luo et al., 2015).

In this study, resistance to 3rd generation cephalosporins was found to co-occur with resistance to two or more other classes of antibiotics such as ampicillin, cotrimoxazole, ciprofloxacin and gentamicin which agrees with reports from related studies (Ambroziec et al., 2007; Gomi et al., 2001; Aggarwal et al., 2003). Similar studies show that many ESBL-producing bacteria also mediate resistance to other classes of antibiotics, like, aminoglycosides and fluoroquinolones (Adrian et al., 2000; Bonomo & Szabo 2006;
Giamarellou et al., 2008). Based on these findings, it is clear that most ESBL-producers exhibit an MDR phenotype (Adria et al., 2000; Bonomo & Szabo 2006; Giamarellou et al., 2008).

Heavy use of broad spectrum antibiotics, prolonged hospital stay, indwelling medical devices and severe underlying diseases are some of the factors associated with the spread of ESBL-producers and challenges in management of severe infections globally (Arlet et al., 1995; Carson & Naber 2004). The clinical relevance of ESBLs has been well documented by numerous published reports describing clinical failure with antimicrobial agents supposed to be effective (Padmini et al., 2004). Thus, the choice of antimicrobial agents effective against ESBL-producing organisms is currently very limited to a few classes of antibiotics, such as carbapenems, that are often the recommended treatment of complicated urinary tract infections caused by ESBL E. coli. However, carbapenemase-producing Enterobacteriaceae are increasingly being reported and are becoming a clinical health threat in some countries such as Morocco (MacKenzie et al., 2000).

The antimicrobial susceptibility results show that imipenem and carbapenem remain the most efficacious drugs against ESBLs associated urinary tract infections. Nevertheless, there remains a need for continuous surveillance and judicious use of this family of antibiotics to prevent the emergence of carbapenem-resistant E. coli isolates in our region.

Laboratory diagnosis of organisms producing ESBLs would be important for a successful epidemiological investigation of these resistant isolates. Until now, ESBL genes have been studied in different parts of the world but very limited studies on the same have
been done in Kenya. This study detected genes for four different types of ESBLs which included, $\text{bla}_{\text{CTX-M}}, \text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}}$ and $\text{bla}_{\text{OXA-1}}$. Among ESBLs producing $E.\text{coli}$, the most predominant $\text{bla}$ ESBL enzyme was $\text{bla}_{\text{CTX-M}}$ (29/36) followed by $\text{bla}_{\text{TEM}}$ (26/36), $\text{bla}_{\text{OXA-1}}$ (10/36) and $\text{bla}_{\text{SHV}}$ (5/36). This is a dramatic turnaround from the situation in the 1990s when SHV and TEM ESBLs were the most predominant and CTX-M types were rarely recognized. The distribution of ESBL-producing genes in our series, with a large predominance of CTX-M, is similar to those reported in several countries including Argentina, the UK, Spain, France and India (Hernández et al., 2005; Ryoo et al., 2008,) among others countries where CTX-M producing $\text{Enterobacteriaceae}$ have been described to be endemic. On the other hand, the most predominant $\text{bla}$ genes in $K.\text{pneumonia}$ were $\text{bla}_{\text{SHV}}$ (22/34) followed by $\text{bla}_{\text{TEM}}$ (12/34), $\text{bla}_{\text{CTX-M}}$ (4/34) and $\text{bla}_{\text{OXA-1}}$ (2/34). Therefore, the two species were distinctly different gnomically in which case, $\text{bla}_{\text{SHV}}$ were the predominant $\text{bla}$ genes in $K.\text{pneumonia}$ and $\text{bla}_{\text{CTX-M}}$ in $E.\text{coli}$.

In class-A ESBLs, CTX, SHV and TEM types, are the most widespread and clinically relevant worldwide (Perez et al., 2007). In Morocco, $\text{Enterobacteriaceae}$ that are capable of producing ESBL enzymes have been isolated from samples collected in different hospitals (Tsang et al., 2000) and (Courvalin, 1997) and the ESBL-positive strains frequently expressed $\text{bla}_{\text{CTX-M}}$ among other ESBLs such as $\text{bla}_{\text{TEM}}, \text{bla}_{\text{SHV}}, \text{bla}_{\text{DHA}}$ and $\text{bla}_{\text{OXA-1}}$ type (Chaves et al., 2001). CTX-M ESBLs are the most commonly described ESBL types in Tunisia (Heritage et al., 2003) and Algeria (Poirel et al., 2003) and (Wang et al., 2003). This also illustrates the large spread of ESBL-producing $E.\text{coli}$ in the North African countries.
Originally, ESBLs were mostly associated with hospital linked infections. However, reports from several studies indicate that ESBLs are becoming common among community-acquired pathogens, especially among *E. coli*. In particular, the CTX-M family of ESBLs has been reported to be the most prevalent among the ESBL-produced enzymes in community-onset UTIs worldwide (Naumovskiet al., 1992).

The study also found out that the two isolates (*K. pneumonia* and *E. coli*) carried different combinations of genes. Although *bla*SHV gene was more common among *Klebsiella* strains as a sole *bla* gene, more isolates of *E. coli* carried the *bla*SHV gene in combination with *bla*TEM, compared to a corresponding carriage of this combination among *Klebsiella* strains. (Naumovski et al., 1992). The most predominant gene combination *blain E. coli* was *bla*CTX-M + *bla*TEM, (20/36) while in *Klebsiella* the most prevalent combination was *bla*TEM + *bla*SHV (12/34). These results therefore show that *bla*CTX-M and *bla*OXA-1 are not significantly implicated in resistance in *Klebsiella* strains, while *bla*SHV is significantly more important in this species than in *E. coli*. These results are similar to those published in other countries showing that carriage of different *bla* genes may confer similar phenotypes among *E. coli* and *Klebsiella* species (Paterson et al., 2003).

Some isolates of *E. coli* from this study carried a number of *bla*TEM genes that had previously been described only in *Klebsiella* such as *bla*TEM-115 and 198 (Heritage et al., 2003). We also identified inhibitor resistant TEM enzymes from *K. pneumonia* (TEM-169 and 163) that had only been described in *E. coli* (Goussard et al., 1999). These enzymes mediate combined resistance to penicillins and augmentin (Rice et al 1993). A number of isolates bore a complex mutant TEM (CMT), *bla*TEM-154 that mediates
resistance to most third generation cephalosporins and augment in but do not to hydrolyze carbapenems and cephamycins. These results further show that, the diversity of bla genes among these species is high and this possibly reflects a high antimicrobials selection pressure in the hospital set up. Such environments have been reported to promote mechanisms that enhance emergence and transmission of MDR strains (Kariuki et al., 2001; Kasap et al., 2010). Such bla combinations apparently increase the spectrum of resistance to β-lactam antibiotics and inhibitor-based antimicrobials as observed among isolates carrying a combination of bla_{CTX-M} +bla_{OXA} + or bla_{1}. A past study implicates bla_{OXA-1} in the resistance to β-lactamase inhibitors (Karisik et al., 2006; Clinical and Laboratory Standards Institute, 2007). Isolates of clonal complex ST131 have also been associated with other types of β-lactamases, as well as ciprofloxacin-resistant \textit{E. coli} isolates that do not have ESBLs (Bogaerts \textit{et al.}, 2007; Clermont \textit{et al.}, 2009; Grami \textit{et al.}, 2016). Considering the fact that most of our isolates that bore CTX-M were also resistant to fluoroquinolones and aminoglycosides, it is highly likely that such strains belong to ST131 but this remains to be determined.

Infection control is critical in hospitals and is useful in curtailing the spread of highly resistant strains. This study tested the effectiveness of selected detergents and other surface cleaning agents and found that for all agents tested, the recommended user-concentrations are effective enough to decontaminate surfaces. Since these isolates are highly MDR, the use of these antimicrobials agents and detergents offer a viable option for reducing contaminations of hospitals surfaces and hand sanitation. This will in turn reduce chances of cross contamination between the patients and the health-care workers and limit transmission of MDR strains from one ward to another.
A key factor in prevention of MDR infections is the consequent implementation and compliance with effective hygiene measures (Tamma et al., 2012). Targeted surface disinfection is a major measure of standard infection control. The surface disinfectants must be effective against the targeted pathogens. Surfaces near patients and high-touch surfaces must be effectively disinfected (Tamma et al., 2012).

Surface disinfectant cleaners (SDCs) that are often used for this purpose and can be used in the immediate proximity of patients are usually based on surface-active ingredients such as quaternary ammonium compounds (QACs). Some data indicate that adaptation or resistance to QACs can develop and QACs have greater activity against gram-positive bacteria compared to gram-negative bacteria (Boyce and Pittet 2002). Literature reports on sodium alkyl sulfate (SAS) efficacy are conflicting. Some studies found that SAS are not sufficiently effective against gram-negative bacteria or that efficacy is lower against gram-negative than against gram-positive bacteria (Block et al., 1991). Other studies found that SAS are effective (Shimizu et al., 2002). Outbreaks of contaminated SAS disinfectant solutions have been reported (Weber et al., 2007). Some strains, particularly biofilm-forming species, survive or even multiply in SAS disinfectants at concentrations at which they are normally used and this can result in infections such as septicemia (Reiss et al., 2000).

The study showed that, all the disinfectants tested were 100% effective against the 20 isolates. 10% formalin showed the highest effectiveness with 100% susceptibility at user concentration (neat), 1:2 and 1:4 dilutions. The susceptibility reduces as the concentrations decrease. This was followed by hydrogen peroxide and phenol with 100%
susceptibility at user concentration and 1:2 dilutions. The susceptibility also decreases with decrease in concentrations. The other four disinfectants were only effective at the user recommended concentration.

The link between lower susceptibility to SAS and antibiotic resistance is not conclusively established (Russell et al., 1998). No resistance breakpoints have been defined for biocides so defining resistance to these compounds is difficult. In addition, reversible adaptations to an active ingredient versus stable resistance must be distinguished (Pagedar et al., 2011). SAS are used for multiple applications, e.g., in the cosmetic, pharmaceutical, and food industries. Adaptation and resistance have been shown for different species. For gram-negative bacteria, cross-resistance to different antibiotics and to different types of SAS, and selection for antibiotic-resistant strains has been found (Soumet et al., 2012). Antibiotic resistance and resistance to biocides can have the same molecular mechanisms, although biocides generally show a broader activity because their mode of action is nonspecific (Chuanchuen et al., 2001). The association between antibiotic-resistance and biocide-resistance seen in gram-negative bacteria can be explained by a link between the genes for both resistance mechanisms (Kampf et al., 2013).

Phylogeny analysis was done to determine the genetic relatedness of the ESBL-producing isolates of *K. pneumonia* and *E. coli* strains. While the origin of TEM-genes remains unknown, it is almost certain that plasmid-borne SHV, which have given rise to ESBL phenotype, comes from the chromosome of the gram negative bacilli, *K. pneumonia* (Haeggmanet al., 1997). While some reports show that *K. pneumonia* carries the SHV-1
gene on their chromosome (Haeggman et al., 1997; Thompson et al. 1994), other findings have shown SHV-negative *K. pneumonia* that carries one of two other chromosomal β-lactamase genes with similar degrees of resistance to broad spectrum penicillins, namely *bla*<sub>LEN</sub> and *bla*<sub>OKP</sub>.

These three genes (*bla*<sub>SHV</sub>, *bla*<sub>LEN</sub> and *bla*<sub>OKP</sub>) which are *bla* for β-lactamase are closely related gene families and the diversification from a common ancestor has presumably emerged as a long-term evolutionary process. Accordingly, *K. pneumonia* strains carry either a chromosomally located *bla*<sub>SHV</sub>, *bla*<sub>LEN</sub> or *bla*<sub>OKP</sub> gene (Ambler et al., 1991). This is an important clinical issue to be considered when using *bla*<sub>SHV</sub> genotyping analysis by means of PCR amplification. Since nucleotide sequences differs between these phylogenetically different subgroups, a primer based on the *bla*<sub>SHV</sub> sequence that is meant to be universal for all *K. pneumonia* might not be universal if some *K. pneumonia* actually possess *bla*<sub>LEN</sub> or *bla*<sub>OKP</sub> genes (Ambler et al., 1991). Phylogenetic analysis of *K. pneumonia* producing SHV isolates from our study supports findings of other studies as they formed three different clusters (SHV, OKP and LEN) but originating from the same ancestors.

Isolates TEM 115 and TEM 198 *K. pneumonia* from this study have *bla*<sub>TEM</sub> gene sequences on a common node and hence closely related to each other and they are also closest to the gene sequences of the isolates in Gene Bank. Most *bla*<sub>TEM</sub> gene sequences of isolates from this study cluster together with the exception of TEM 163 *E. coli* and TEM 219 *K. pneumonia* that are on a node that is split from the rest of the analyzed isolates.
5.2 Conclusion

The prevalence of *E. coli* and *K. pneumonia* capable of producing ESBLs enzymes in Kenya is relatively high (41%). Antibiotic resistance for ESBLs *E. coli* and *K. pneumonia* was also found to be high with over 60% resistant except for meropenam and gentamycin. According to the study, meropenem appear to be the drugs of choice in management ESBLs related infections. The high antibiotic resistance rate can lead to decrease in therapeutic options. All disinfectants tested against the ESBLs producing *E. coli* and *K. pneumonia* were effective against the isolates at the recommended user-concentrations. Care should be taken during preparations to ensure correct quantities are used as low incorrect quantities can lower affectivity as the results indicate. The most common ESBLs genes among *E.coli* and *K. pneumonia* isolates were *bla* CTX-M and *bla* SHV respectively. More than genes occurring in a single isolate common. Sequencing results revealed 9 sub-types of *bla TEM* and 5 sub-types of *bla SHV*. In phylogeny analysis, the *bla TEM* gene sequences of isolates from this study clustered together with *bla TEM* gene sequences of different species from Gene bank while *bla SHV* clustered very differently from other similar genes.

5.3 Recommendations

Based on the data presented in this study, we recommend the following;

- ESBLs determinations should be carried out routinely alongside antibiotic susceptibility tests for bacteria recovered from in-patients prior to application of treatment regimes
• Studies be done to determine the relationships between the identified genes and the antibiotics resistance.

• Epidemiological data on antimicrobial resistance be compiled in order to promote appropriate antimicrobial therapy as well as an effective infection control and clinical case management.

5.3.1 Recommendations for future study

• Studies be carried out to establish the relationship between ESBIs and the conditions of the patients from whom the isolates are obtained.

• Studies be done on to determine the genetic relatedness among isolates from different clinical backgrounds

• Studies be carried out on the genetic basis for disinfectant resistance observed and the relationship between this resistance and that targeted to antibiotics.
REFERENCES


Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing : seventeenth informational supplement. [M100-S17=v.27,no.1].


Courvalin, P. (1997). Personal communication. TEM-14 has been entirely sequenced and is indistinguishable from TEM-3.


Wayne, PA. Clinical Laboratory Standards Institute (CLSI) (2007). *Performance standards for antimicrobial susceptibility testing: 15th informational supplement (M100-S15)*.


Multiple antibiotic-resistant Klebsiella and Escherichia coli in nursing homes

APPENDICES

APPENDIX I

Sample gel electrophoresis showing a PCR amplification of $bla_{\text{TEM}}$ in *Klebsiella* (1-4) and *E. coli* (5-18). NC: negative control of isolate known not to carry this gene, PC: positive control strain carrying this gene. M: molecular weight marker

Sample gel electrophoresis showing a PCR amplification of $bla_{\text{OXA}}$ in *Klebsiella* (A, 2-3) and *E. coli* (A, 4-7). Lane 1 and lane 8, on figure A): negative control of isolate known not to carry this gene.

Sample gel electrophoresis showing a PCR amplification of $bla_{\text{SHV}}$ in *Klebsiella* (2-7) and *E. coli* (8-13). NC: negative control of isolate known not to carry this gene, PC: positive control strain carrying this gene. M: molecular weight marker