

**Antibiotic Susceptibility Patterns and Detection of Genes Responsible for
Resistance of *Klebsiella* species and *Escherichia coli* Isolated from
Environmental Sources around Nairobi, Kenya**

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DECLARATION

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

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DEDICATION

To Lucy Wangari Njugu, Rose Wanjiku Njugu, Calvin Mwihuri Njugu and George Ngondi Michuki for enabling and facilitating me to achieve all I have.

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LIST OF ABBREVIATIONS AND ACRONYMS

AACs	Aminoglycoside Acetyltransferases
ANTs	Aminoglycoside Nucleotidyltransferases
APHs	Aminoglycoside Phosphotransferases
CDC	Centre for Disease Control and Prevention
cGMP	Cyclic guanosine monophosphate
CLSI	Clinical Laboratory Standards Institute
CMR	Center for Microbiology Research
CSC	Center Scientific Committee
DDD	Double disc diffusion
DEC	Diarrheagenic <i>Escherichia coli</i>
DNA	Deoxyribonucleic acid
EAEC	Enteraggregative <i>Escherichia coli</i>
EC	Enteric <i>Escherichia coli</i>
ECP	<i>E. coli</i> Common Pilus
EHEC	Enterohaemorrhagic <i>Escherichia coli</i>
EIEC	Enteroinvasive <i>Escherichia coli</i>
EPEC	Enteropathogenic <i>Escherichia coli</i>
ERC	Ethical Review Committee
ESBL	Extended Spectrum Beta Lactamase
ETEC	Enterotoxigenic <i>Escherichia coli</i>
GI	Gastro Intestinal

H₂S	Hydrogen sulfide
ICU	Intensive Care Unit
<i>Int</i>	Integrase gene
KEMRI	Kenya Medical Research Institute
KIA	Kligler Iron Agar
KCN	Potassium cyanide
MDR	Multidrug Resistant
MIC	Minimum inhibitory concentration
MRVP	Methyl Red Voges- Proskauer
ONPG	O-nitrophenyl-beta-D galactopyranoside
PCR	Polymerase Chain Reaction
PLA	Pyogenic Liver Abscess
RI	Resistance Integrins
SC	Simmon's citrate
SHV	Sulfhydryl variable
SI	Super Integrins
SSC	Scientific Steering Committee
ST	Shiga-like Toxin
TEM	TEMoneira
TSI	Triple Sugar Iron
UTI	Urinary Tract Infections
UV	Ultra violet Light
XLD	Xylose lysine deoxycholate

ABSTRACT

The Enterobacteriaceae are a large family of Gram negative bacteria which inhabit the intestines of man and animals. Members of this family are not only found in the gastrointestinal tract but are also in soil and water and in the respiratory tracts of human and animals where they cause a variety of septic and urinary tract infections. Many of these organisms harbor antibiotic-resistance genes, usually inserted into genetic mobile platforms (plasmids, transposons, and integrons) able to spread among water and soil bacterial communities. The organisms have developed various mechanisms of drug resistance which include extended spectrum β -lactamases (ESBLs) production, Ambler Class C β -Lactamases (*AmpC*) β -lactamase production, efflux mechanisms and porin deficiency.

To determine susceptibility patterns and resistance mechanisms in environmental *Klebsiella* and *Escherichia coli* (*E. coli*), bacteria strains were isolated from streams/rivers, sewage dams and bore holes in Nairobi and its environs. Isolates were identified by biochemical typing. Susceptibility to aminoglycosides, nucleic acids inhibitors, fluoroquinolones and β -lactamases antibiotics was done using the disk diffusion method. Polymerase Chain Reaction (PCR) was used to screen for the presence of aminoglycoside modifying enzymes (*ame*) and integron 1 and 2 genes (*int1* and *int2*). Presence of plasmid mediated resistance was also screened by isolation of plasmids from multi resistant isolates and the plasmids were characterized according to their sizes. Ability for bacteria to transfer resistance plasmid was determined by conjugation experiments with *E. coli* C600. Chi square

or Fisher's Exact test was used as appropriate to determine any significant association of data that can be put into tables with mutually exclusive and exhaustive cells. The prevalence of *E. coli* (40.1%) was significantly higher than *Klebsiella* spp. (29.1%) ($p < 0.05$). Resistance to nucleic acid inhibitors was the highest (57%) compared to aminoglycosides (27%), beta lactams (17%) and lastly fluoroquinolones (10%) ($p < 0.05$; chi-squared for independence). Presence of *Int1* was significantly lower in *E. coli* (47.4%; [18/38]) than in *Klebsiella* spp (73.0% [27/37]), ($p < 0.05$; chi-squared for independence). Integron 2 was not detected in any of the isolates. Aminoglycoside modifying enzyme gene *aac(6')-lb-cr* was detected in 18.2% (2/11) of *E. coli* and 25% (5/20) of *Klebsiella* species. Two *E. coli* and three *Klebsiella* spp transferred ampicillin resistance to *E. coli* C600. In this study, *E. coli* and *Klebsiella* spp isolated from water samples obtained from boreholes, streams/rivers, sewage sources showed resistance to the four main groups of antibiotics tested namely; quinolones, aminoglycosides, nucleic acid inhibitors and beta-lactam antibiotics tested. Resistance was plasmid mediated in *E. coli* and *Klebsiella* spp from environmental sources and due to production of *int1* and *ame* genes enzymes.

CHAPTER ONE

1.0 INTRODUCTION

The Enterobacteriaceae are a large family of Gram negative bacteria which inhabit intestines of man and animals and contain some of the most medically significant genera. Certain genera such as *Salmonella*, *Shigella* and *Yersinia* species are important pathogens of the gastrointestinal (GI) tract. Others, such as *Escherichia* and *Enterobacter*, are typically considered normal flora of the intestine but may also cause disease if given the opportunity (Houndt and Ochman, 2000). Other than causing infections of the GI tract, *Klebsiella* also causes community-acquired pneumonia (Keynan and Rubinstein, 2007).

Klebsiella pneumoniae (*K. pneumoniae*) and *Escherichia coli* (*E. coli*) are found in soil and water but are also found in the gastrointestinal and respiratory tracts of human and animals where they cause a variety of septic and urinary tract infections (Keynan and Rubinstein, 2007). Among the gram-negative bacteria implicated in nosocomial blood stream infections, *Klebsiella pneumoniae* and *Escherichia coli* (*E. coli*) are the most prevalent (Podschun and Ullmann, 1998).

Contemporary populations of enteric bacteria, when compared with those from the pre-antibiotic era, display a higher tolerance in their nonspecific responses to several antibiotics (Houndt and Ochman, 2000). Within a few years of the introduction of the first cephalosporins, *K. pneumoniae* isolated within hospitals were noted to produce β -lactamases capable of inactivating this novel antibiotic. These β -lactamases which

turned out to be Extended Spectrum β -Lactamases (ESBLs), were largely restricted to the hospital environment and were almost exclusively of the Temoneira (TEM) and Sulfhydryl variable (SHV) enzyme variants (Kollef *et al.*, 1999). ESBLs are capable of conferring bacterial resistance to the penicillins, first- generation, second-, and third-generation cephalosporins, and aztreonam (but not the cephamycins e.g, cefoxitin or carbapenems such as meropenem). ESBLs confer resistance to these antibiotics through their ability to hydrolyse the β -lactam ring (Bush *et al.*, 1995). Many ESBL-producing outbreak strains of *Klebsiella* spp are isolated most frequently from critically ill patients, within specialist units. Bacteria carrying ESBLs are thus clinically important because these resistant pathogens cause nosocomial infections that commonly appear in outbreaks and are associated with treatment failure, prolonged hospital stay, increased health expenditure as well as a possible increase in mortality (Kollef *et al.*, 1999). In addition to ESBLs production reports, Ambler Class C β -Lactamases (AmpC β -lactamases) which are plasmid mediated enzymes are being reported increasingly among *E. coli* and *Klebsiella pneumoniae* isolates worldwide. In Ireland AmpC mediated resistance in *Klebsiella* within a tertiary care hospital was found to be as a result of transfer of a mobile genetic element rather than the spread of a single clone. This result suggested a need for continuous surveillance of the prevalence and evolution of AmpC enzymes among enteric bacteria (Roche *et al.*, 2008).

Away from clinical settings, *Enterobacteriaceae* from treated and untreated urban sewage waters in Portugal have been characterized for their antibiotic resistance phenotype. Among the isolated bacteria *Klebsiella pneumonia* from treated sewage

presented a high level resistance to amoxicillin. The results obtained for *K. pneumonia* isolates suggest that wastewater treatment is accompanied by an increase in resistance to the other antibiotics tested. With the exception of amoxicillin and cephalothin, similar results were observed for isolates for *Shigella* spp. (Miguel *et al.*, 2007). Two treated wastewater isolates belonging to this genus were simultaneously resistant to amoxicillin, sulfamethoxazole/trimethoprim, tetracycline and ciprofloxacin or gentamicin. A comparison of the antibiotic resistance frequency in *Escherichia* spp. from raw wastewater vs. treated wastewater revealed that, for the antibiotics amoxicillin, ciprofloxacin, tetracycline and cephalothin, the resistance prevalence increased by 5–10% in treated wastewater (Miguel *et al.*, 2007).

The results thus showed that wastewater treatment was accompanied by a generalized increase in antibiotic resistance and multiresistance phenotype in *Escherichia* spp. (Miguel *et al.*, 2007). However, further studies are needed to provide some insights into the prevalence of multiple-resistant isolates from environmental setups (Stelling *et al.*, 2005). It is assumed that commensal and environmental bacteria will present comparatively lower antibiotic resistance prevalence than their counterparts isolated from clinical samples, but it can be observed from many studies that the resistance prevalence for sulfamethoxazole/trimethoprim, ciprofloxacin and amoxicillin are of the same order of magnitude in clinical and environmental isolates (Stelling *et al.*, 2005). The present study aimed at investigating the prevalence of resistance in *Escherichia coli* and *Klebsiella* species isolated from the environment from Nairobi, Kenya and environs.

1.1 Statement of the problem

In Kenya limited studies have been carried out to evaluate the mechanism of resistance by opportunistic pathogens such as *Klebsiella* and *Escherichia coli* which are the most observed in studies elsewhere. Opportunistic pathogens (*Klebsiella* and *E. coli*) have been shown to be overburdening due to resistance to antibiotics and the resistance mechanisms have been identified and interventions recommended and implemented in European countries. In Kenya the prevalence of resistance has been determined in very limited studies most of them being clinical and mechanisms of resistance have rarely been identified. However, limited information is available about Kenya on whether environmental isolates harbor resistant strains and hence a possible source of resistant opportunistic infections. It is therefore, difficult to recommend any possible interventions such as appropriate antibiotics therapies and water treatment remedies against environmental *Klebsiella* and *E.coli* strains.

1.2 Justification

Multidrug-resistant (MDR) *E. coli* and *Klebsiella* species are increasingly being reported as a cause of suppurative infections, bacteremia and a substantial percentage are involved in nosocomial infections and contribute a huge burden of disease in hospitals and the community. In addition, antimicrobial resistance among these microorganisms has risen dramatically worldwide and this current trend presents a great public health challenge. High prevalence of ESBL producing organisms is being reported in many parts of the world including limited clinical reports in Kenya and thus, there is need to determine the prevalence in Kenya so as to develop interventions. Some studies have shown that use of β -lactam/ β -lactamase inhibitor

combinations have been followed by successful reduction in ESBL-producing *K. pneumoniae*. In order to apply that recommendation in Kenya there was need to determine the susceptibility patterns of *E. coli* and *Klebsiella* species to β -lactam/ β -lactamase inhibitor combinations in Kenya. Production of AmpC β -lactamases and aminoglycoside modifying enzymes has also been shown to be a course of resistance to a variety of drugs. It is therefore, of much importance to determine the prevalence of *E. coli* and *Klebsiella* species producing the enzymes in strains isolated locally. This study was carried out to determine the prevalence and mechanism of resistance of these isolates to antibiotics and determine their ability to transfer resistance in environmental isolates obtained from Nairobi, Kenya and hence provided information that will be helpful in formulation of intervention policies and recommendations applicable locally.

1.3 Hypotheses

Klebsiella species from environmental sources have similar susceptibility patterns and resistance genes to *E. coli* from environmental sources.

1.4 Objectives

1.4.1 General Objective

1. To determine the antibiotic susceptibility phenotypes among bacterial strains of *E. coli* and *Klebsiella* spp isolated from various environmental setups, sewage systems and waste effluents within Nairobi.

1.4.2 Specific Objectives

1. To determine the presence of *E. coli* and *Klebsiella* in various environmental setups, sewage systems and waste effluents within Nairobi.
2. To determine the antibiotic susceptibility patterns of environmental *E. coli* and *Klebsiella* spp against commonly used classes of antibiotics, beta lactams, aminoglycosides and fluoroquinolones.
3. To test for the presence of plasmids, Integron1, Integron2 and genes encoding Aminoglycoside Modifying Enzymes.
4. To determine the ability of multi-drug resistant organisms to transfer resistance to *E. coli* C600.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Enterobacteriaceae Infections

Enterobacteriaceae is a group of Gram-negative bacteria that can cause infections of the digestive tract or other organs of the body. The group *Enterobacteriaceae* includes the bacteria *Enterobacter*, *Escherichia*, *Klebsiella*, *Morganella*, *Proteus*, *Providencia*, *Salmonella*, *Serratia*, *Shigella*, and *Yersinia* (<http://www.merck.com/mmhe/sec17/ch190/ch190h.html>).

2.1.1 *Escherichia coli* (*E. coli*) characteristics

Escherichia coli is a Gram negative bacterium of *Enterobacteriaceae* family of gamma-proteobacteria. It forms rod-shaped cells of 2.0-6.0 µm in length and 1.1-1.5 µm in width with rounded ends (Orskov *et al.*, 1984). These organisms are non-spore-forming, gram-negative bacteria, usually motile by peritrichous flagella although some are non-motile (Ewing, 1986). They are facultative anaerobes and produce gas from fermentable carbohydrates. The methyl red reaction is positive but the Voges- Proskauer reaction is negative. Most strains of this species promptly ferment lactose or give a positive o-nitrophenyl-beta-dgalactopyranoside (ONPG) reaction. They produce indole, do not hydrolyse urea and fail to grow in Møller's potassium cyanide (KCN) broth. Hydrogen sulphide production is not detectable on triple sugar iron (TSI) agar or Kligler's iron agar (KIA), phenylalanine is not deaminated and gelatin is not liquefied. Most strains decarboxylate lysine and utilize

sodium acetate, but do not use citrate as a sole carbon source (Orskov *et al.*, 1984; Ewing, 1986). In addition to being oxidase negative, all members of this family are glucose fermenters and nitrate reducers (Ewing, 1986).

Most *E. coli* strains are harmless, but some, such as serotype O157:H7 can cause serious food poisoning in humans, and are occasionally responsible for costly product recalls (Vogt and Dippold, 2005). In most cases, the pathogenicity of a particular enteric bacterium can be determined by its ability to metabolize lactose. Non-utilizers are usually pathogenic while the lactose utilizers are not pathogenic (<http://medic.med.uth.tmc.edu/path/00001500.htm>).

Escherichia coli are commonly found in the lower intestine of warm-blooded organisms (endotherms). They are not always confined to the intestine, and their ability to survive for brief periods outside the body makes them an ideal indicator organism to test environmental samples for fecal contamination (Feng *et al.*, 2002). The bacteria can also be grown easily and its genetics are comparatively simple and easily-manipulated or duplicated through a process of metagenics, making it one of the best-studied prokaryotic model organism, and an important species in biotechnology and microbiology. The harmless strains of *E. coli* are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K₂, (Bentley and Meganathan, 1982) or by preventing the establishment of pathogenic bacteria within the intestine (Reid *et al.*, 2001).

2.1.2 *Escherichia coli* Infections

Although *Escherichia coli* normally inhabits the intestines, certain strains of *E. coli* can cause intestinal infections that produce bloody, watery, or inflammatory diarrhea (traveler's diarrhea). In children, diarrhea caused by certain strains of *E. coli* O157 may lead to destruction of red blood cells and kidney failure (hemolytic-uremic syndrome). *E. coli* can also cause urinary tract infections (particularly in women) and bacteremia and meningitis in newborns (particularly premature newborns) (<http://www.merck.com/mmhe/sec17/ch190/ch190h.html>).

Members of genera belonging to the *Enterobacteriaceae* family have earned a reputation placing them among the most pathogenic and most often encountered organisms in clinical microbiology. They are usually associated with intestinal infections, but can be found in almost all natural habitats. They are the causative agents of such diseases as meningitis, bacillary dysentery, typhoid, and food poisoning. Among the Enterobacteriaceae *E. coli* and *Klebsiella* have found major importance in both nosocomial and community-acquired infections (Meyer *et al.*, 1993).

Enteric infection caused by *E. coli* can be due to at least five different pathotypes operating through different mechanisms and thus leading to different symptoms. Enteric *E. coli* (EC) are classified on the basis of serological characteristics and virulence properties (<http://www.textbookofbacteriology.net/e.coli.html>). The pathotypes include: Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli*

(EPEC), Enterohemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC) and Enteroinvasive *E. coli* (EIEC) (Nataro and Kaper, 1998).

2.1.2.1 Enteroaggregative *E. coli* (EAEC)

It is found only in humans. So named because they have fimbriae which aggregate tissue culture cells, EAEC bind to the intestinal mucosa to cause watery diarrhea without fever. EAEC are non-invasive. They produce a hemolysin and a Shiga-like Toxin (ST) similar to that of ETEC. EAEC has been shown to have significant resistance to trimethoprim-sulfamethoxazole and ampicillin (Wanke *et al.*, 1998).

2.1.2.2 Enteropathogenic *E. coli* (EPEC)

It is a causative agent of diarrhea in humans, rabbits, dogs, cats and horses. Like ETEC, EPEC also causes diarrhea, but the molecular mechanisms of colonization and etiology are different. EPEC lack fimbriae, Shiga-like Toxin (ST) and Labile Toxin (LT), but they utilize an adhesin known as intimin to bind host intestinal cells. This pathotype has an array of virulence factors that are similar to those found in *Shigella*, and may possess a shiga toxin. Adherence to the intestinal mucosa causes a rearrangement of actin in the host cell, causing significant deformation (Knutton *et al.*, 1987). EPEC adhere to the human intestine and produce typical attaching and effacing lesions which are characterized by dissolution of the brush border membrane and destruction of microvilli (Knutton *et al.*, 1987) and which correlate with a positive fluorescent actin staining test (Knutton *et al.*, 1989).

EPEC cells are moderately-invasive (i.e. they enter host cells) and elicit an inflammatory response. Changes in intestinal cell ultrastructure due to “attachment and effacement” are likely the prime cause of diarrhea in those afflicted with EPEC. A variety of antibiotics may be used but antibiotic resistance is common among EPEC (Nataro and Kaper, 1998).

2.1.2.3 Enteroinvasive *E. coli* (EIEC)

It is found only in humans. EIEC infection causes a syndrome that is identical to Shigellosis, with profuse diarrhea and high fever. Although little work has been done to characterize EIEC virulence factors by molecular typing, it is assumed that they are virtually identical to those of *Shigella* species (Nataro and Kaper, 1998). Resistance of EIEC to trimethoprim-sulfamethoxazole and ampicillin is common, whereas resistance to quinolones is low (Oldfield and Wallace, 2001).

2.1.2.4 Enterohemorrhagic *E. coli* (EHEC)

It is found in humans, cattle, and goats. The most famous member of this pathotype is strain O157:H7, which causes bloody diarrhea and no fever. EHEC can cause hemolytic-uremic syndrome and sudden kidney failure. It uses bacterial fimbriae for attachment (*E. coli* common pilus, ECP) and is moderately-invasive and possesses a phage-encoded Shiga toxin that can elicit an intense inflammatory response (Rendón *et al.* 2007).

2.1.2.5 Enterotoxigenic *E. coli* (ETEC)

It is a causative agent of diarrhea (without fever) in humans, pigs, sheep, goats, cattle, dogs, and horses. ETEC uses fimbrial adhesins (projections from the bacterial cell surface) to bind enterocyte cells in the small intestine (Nataro and Kaper, 1998).

ETEC can produce two proteinaceous enterotoxins:

- the larger of the two proteins, LT enterotoxin, is similar to cholera toxin in structure and function.
- the smaller protein, ST enterotoxin causes Cyclic guanosine monophosphate (cGMP) accumulation in the target cells and a subsequent secretion of fluid and electrolytes into the intestinal lumen.

ETEC strains are non-invasive, and they do not leave the intestinal lumen. ETEC is the leading bacterial cause of diarrhea in children in the developing world, as well as the most common cause of traveler's diarrhea. Each year, ETEC causes more than 200 million cases of diarrhea and 380,000 deaths, mostly in children in developing countries (http://www.who.int/vaccine_research/diseases/diarrhoeal/en/index4.html).

2.1.3 *Klebsiella* Species Characteristics

Klebsiella is a Gram-negative member of the Enterobacteriaceae family, is non-motile, encapsulated, lactose fermenting, facultative anaerobic, rod shaped bacterium found in the normal flora of the mouth, skin, and intestines (Ryan and Stanley, 2004). It was recognized over 100 years ago as a cause of community-acquired

pneumonia. *Klebsiella pneumonia* is clinically the most important member of the *Klebsiella* genus of Enterobacteriaceae; it is closely related to *Klebsiella oxytoca* from which it is distinguished by being indole-negative and by its ability to grow on both melezitose and 3-hydroxybutyrate.

The genus *Klebsiella* was originally divided into 3 main species based on biochemical reactions. Today, seven species with demonstrated similarities in DNA homology are known. These are (1) *Klebsiella pneumoniae*, (Kp) (2) *Klebsiella ozaenae*, (3) *Klebsiella rhinoscleromatis*, (4) *Klebsiella oxytoca*, (5) *Klebsiella planticola*, (6) *Klebsiella terrigena*, and (7) *Klebsiella ornithinolytica*. *Klebsiella pneumoniae* is the most medically important species of the group. *Klebsiella oxytoca* and *K. rhinoscleromatis* have also been demonstrated in human clinical specimens. In recent years, *Klebsiella* spp. have become important pathogens in nosocomial infections (Nordmann *et al.*, 2009). They naturally occur in the soil and about 30% of strains can fix nitrogen in anaerobic conditions (Fouts *et al.*, 2008). Most clinical isolates of *K. pneumoniae* possess a well defined polysaccharide capsule that appears to be a critical virulence factor (Domenico *et al.*, 1982).

Typically, members of the genus *Klebsiella* express 2 types of antigens on their cell surface. The first is a lipopolysaccharide (O antigen); the other is a capsular polysaccharide (K antigen). Both of these antigens contribute to pathogenicity (<http://www.emedicine.com/med/TOPIC1237.HTM>). Some capsular serotypes are isolated at significantly higher frequency than others (Xiong *et al.*, 2002). K antigen,

a capsular polysaccharide, is considered to be an important virulence factor of *K. pneumonia* (Lin *et al.*, 2004). According to these capsular polysaccharides, *K. pneumonia* can be classified into 77 serological K antigen types.

2.1.4 *Klebsiella* species Infections

The incidence of community-acquired pneumonia attributed to *K. pneumonia* has decreased over the years, however its role as a common healthcare-associated pathogen causing infections of the urinary tract, bloodstream, pneumonia and intra-abdominal infections has become exceedingly common (Keynan and Rubinstein, 2007). *Klebsiella pneumoniae* also causes suppurative infections, bacteremia, and a substantial percentage of nosocomial infections, pneumonia, urinary tract infections, septicemia, ankylosing spondylitis, and soft tissue infections (Bryan *et al.*, 1983; Meyer *et al.*, 1993). *Klebsiella pneumoniae* is an opportunistic pathogen mainly involved in infections of the urinary and respiratory tract of patients with underlying conditions. The bacterium appears to rapidly develop antibiotic resistance, and it is frequently involved in outbreaks in hospital settings (Podschun and Ullmann, 1998).

Infection with *Klebsiella* organisms occurs in the lungs, where they cause destructive changes. Necrosis, inflammation, and hemorrhage occur within lung tissue, sometimes producing thick, bloody, mucoid sputum described as currant jelly sputum (Podschun and Ullmann, 1998). The illness typically affects middle-aged and older men with debilitating diseases such as alcoholism, diabetes, or chronic bronchopulmonary disease. This patient population is believed to have impaired

respiratory host defenses. The organisms gain access after the host aspirates colonizing oropharyngeal microbes into the lower respiratory tract (Podschun and Ullmann, 1998).

Klebsiella have also been incriminated in nosocomial infections. Common sites include the urinary tract, lower respiratory tract, biliary tract, and surgical wound sites. The spectrum of clinical syndromes includes pneumonia, bacteremia, thrombophlebitis, urinary tract infection (UTI), cholecystitis, diarrhea, upper respiratory tract infection, wound infection, osteomyelitis, and meningitis. The presence of invasive devices, contamination of respiratory support equipment, use of urinary catheters, and use of antibiotics are factors that increase the likelihood of nosocomial infection with *Klebsiella* species. Sepsis and septic shock may follow entry of organisms into the blood from a focal source. Rhinoscleroma and ozena are two other infections caused by *Klebsiella* species. These diseases are rare. Rhinoscleroma is a chronic inflammatory process involving the nasopharynx, whereas ozena is a chronic atrophic rhinitis characterized by necrosis of nasal mucosa and mucopurulent nasal discharge (<http://www.emedicine.medscape.com/article/219907-overview>).

Klebsiella oxytoca has been implicated in neonatal bacteremia, especially among premature infants and in neonatal intensive care units. Increasingly, the organism is being isolated from patients with neonatal septicemia. Extensive use of broad-spectrum antibiotics in hospitalized patients has led to both increased carriage of *Klebsiella* spp. and subsequently, the development of multidrug-resistant strains that

produce extended-spectrum beta-lactamase (ESBL). These strains are highly virulent, have capsular type K55, and have an extraordinary ability to spread. Most outbreaks are due to a single clone or single gene; the bowel is the major site of colonization with infection of the urinary tract, respiratory tract, and wounds. Bacteremia and significant increased mortality have resulted from infection with these species (<http://www.emedicine.medscape.com/article/219907-overview>).

Klebsiella pneumoniae resistance to third-generation cephalosporins is typically caused by production of extended-spectrum β -lactamases with the ability to hydrolyse broad-spectrum cephalosporins, monobactams and penicillins (Gales *et al.*, 2002). Emergence of ESBL production is associated with prior use of antimicrobials, and varies widely between continents and countries, with highest rates reported from Latin America (Gales *et al.*, 2002). These enzymes are frequently carried on plasmids that also house separate genes that encode resistance to aminoglycosides and sulfonamides and many of these organisms also possess high-level resistance to fluoroquinolones (Du Bois *et al.*, 1995). Thus, ESBL producing *Klebsiella pneumoniae* in hospitals and ICU settings are commonly multidrug resistant, complicating antimicrobial therapy.

Strains of *Klebsiella pneumoniae* that are resistant to multiple antibiotics, including the newer cephalosporins, have emerged and the infections caused by them are frequently epidemic in nature and have complicated chemotherapy significantly (Bryan *et al.*, 1983; Meyer *et al.*, 1993). For instant carbapenems are the treatment of choice for serious infections due to ESBL-producing organisms, yet carbapenem-

resistant isolates have recently been reported (Paterson and Bonomo, 2005). Community-acquired primary pyogenic liver abscess (PLA) caused by *Klebsiella pneumoniae* is an emerging disease receiving increasing attention in the last 20 years. Patients with this infection can present with or without septic metastatic complications. Most cases have been reported from Taiwan and studies revealed that K1 was the predominant serotype causing liver abscess (Chuang *et al.*, 2006). The entity has also been described in other parts of Asia, North America and Europe (Ko *et al.*, 2002).

2.2 Antibiotics resistance

Antimicrobial resistance is emerging as an important public health problem in both the hospitals and the community. Untreatable infections are being recognized more frequently and, as important bacterial pathogens become increasingly resistant, the lack of new or alternative antimicrobial agents makes serious outbreaks a possibility (Cohen, 1994). Human and animal pathogenic and potentially pathogenic bacteria are constantly released with wastewater into the water environment. Many of these organisms harbor antibiotic-resistance genes, eventually inserted into genetic mobile platforms (plasmids, transposons and integrons) able to spread among water and soil bacterial communities (Alonso *et al.*, 2001).

In such systems, nonpathogenic bacteria could serve as a reservoir of resistance genes where the habitats act as platforms for genetic exchanges. Moreover, the introduction (and progressive accumulation) in the environment of antimicrobial

agents, detergents, disinfectants, and residues from industrial pollution, as heavy metals, contributes to the evolution and spread of such resistant organisms in the water and other environmental systems. The heavy use of prophylactic antibiotics in aquaculture is particularly relevant (Cabello, 2006). Additionally, environmental bacteria act as an unlimited source of antibiotic resistance genes that can be passed to pathogenic organisms. Note that many of these genes are not primarily resistance genes, but belong to the hidden 'resistome' (D'Costa *et al.*, 2006) the set of genes able to be converted to antibiotic-resistance genes upon adoption into genetic expression machinery such as integrons and transposons. Human health risk assessment protocols for antibiotic and resistant bacteria in water are only emerging now (Kim and Aga, 2007). Antibiotic-resistant organisms from humans and animals are released into the sewage by contaminated sites including urine, feces, eventually corpses and manure. In particular, wastewater from hospitals and intensive farming facilities (under concentrated animal feeding operations) is probably a major source of pathogenic and antibiotic-resistant organisms and antibiotic-resistance genes that are released into the environment (Baquero *et al.*, 2008).

Water bacteria might be indigenous to aquatic environments, or exogenous, transiently and occasionally present in the water as a result of shedding from animal, vegetation, or soil surfaces. More than 90% of bacterial strains originating from seawater are resistant to more than one antibiotic, and 20% are resistant at least to five antibiotics (Alonso *et al.*, 2001). The study of antibiotic resistance in indigenous

water organisms is important, as it might indicate the extent of alteration of water ecosystems by human action.

For instance, *Aeromonas* strains from Portuguese estuarine water carry less frequently β -lactamase genes than Enterobacteriaceae (10% versus 78%) (Henriques *et al.*, 2006). In water reservoirs half of *Aeromonas* strains might present multiple antibiotic resistances (Blasco *et al.*, 2008). Estuarine water-borne *Aeromonas* strains carry almost as frequently as Enterobacteriaceae class 1 integron platforms carrying antibiotic-resistance genes (Henriques *et al.*, 2006). The persistence of such genetic structures cannot probably be explained solely by antibiotic selection, suggesting that activities resulting in antibiotic resistance might have other physiological roles, or that they are placed in multifunctional plasmids. The most frequent gene cassette found involves aminoglycoside- resistance genes, rarely under positive selection and there is a suspicion that some other resistance genes, as integron *sul* genes, might provide benefits for the bacteria, unrelated with resistance. However, some of these mobile gene cassettes in *Aeromonas* might involve important mechanisms of resistance, as Qnr, involved in fluoroquinolone resistance, which might be horizontally propagated by IncU-type plasmids (Cattoir *et al.*, 2008).

Certainly the dense bacterial populations in sewage treatment plants favor genetic exchange among bacterial populations and communities, integrons predating transposons and plasmid dissemination. Multiresistance plasmids of broad host-range are consistently recovered in sewage (Schlüter *et al.*, 2007). Interestingly, antibiotic-

resistance genes from manure influence the lagoons and groundwater gene pool, but this pool also contains antibiotic-resistance genes from indigenous bacteria (Koike *et al.*, 2007).

2.2.1 Environmental damage mediated by antibiotics in water environments

Pharmaceuticals are introduced in the environment from human and veterinary applications at volumes comparable with total pesticide loadings (Brain *et al.*, 2008). Antibiotic resistance is not the only possible adverse effect of antibiotic release in water environments hence, ecotoxicity tests are starting to be introduced to document additional effects (Yamashita *et al.*, 2006). Antibiotics may act, at very low concentrations, as signaling agents (a kind of hormone) in microbial environments (Linares *et al.*, 2006; Fajardo and Martínez, 2008). Common receptors have been identified in plants for a number of antibiotics and disinfectants affecting chloroplast replication (fluoroquinolones), transcription–translation (tetracyclines, macrolides, lincosamides, aminoglycosides, pleuromutilins), folate biosynthesis (sulfonamides, and probably trimethoprim), fatty acid synthesis (triclosan), and sterol biosynthesis (azoles, statins) (Brain *et al.*, 2008). During the past years the environmental consequences of the release of triclosan in freshwater environment has been considered (Capdevielle *et al.*, 2008). Ciprofloxacin affects stream microbial communities, including those colonizing senesced leaf materials (Maul *et al.*, 2006).

For the non aquatic organisms, obviously the density of antibiotic-resistance organisms and antibiotic-resistance genes in fresh water varies with the proximity to

areas with increased antibiotic consumption, metal pollution, and between seasons, being more frequently found in rainy seasons (Peak *et al.*, 2007). Alterations in microbial ecosystems, either produced by antimicrobial release or by the unexpected effective dispersal in water environments of resistant pathogenic organisms (Quinteira and Peixe, 2006) might be relevant for public health.

2.3 Mechanisms of Antibiotics Resistance

The various mechanisms of drug resistance in Gram-negative bacilli include ESBLs production, AmpC β -lactamase production, efflux mechanisms and porin deficiency. Amongst the mechanisms of resistance to third generation cephalosporins, production of ESBLs and AmpC- β -lactamases are the most common. AmpC- β -lactamases are clinically important because they confer resistance to narrow-, expanded-, and broad-spectrum cephalosporins, β -lactam- β -lactamase inhibitor combinations and aztreonam. Group1 AmpC- β -lactamases are poorly inhibited by clavulanic acid; however, they are inhibited by cloxacillin (Jarlier *et al.*, 1988; Fukuda and Hiramatsu, 1997; Gaillot *et al.*, 1997; Ananthan and Subha, 2005; Black *et al.*, 2005).

Acquired resistance, as opposed to intrinsic resistance, is the result of a change in the bacterial genome so that a drug that originally was effective *in vivo* is no longer active. General mechanisms of resistance fall into 1 of 4 categories: 1) decreased intracellular drug concentration, 2) drug inactivation, 3) target modification, or 4) target bypass. Intracellular drug concentration can be minimized by increased efflux

of an antibiotic from a bacterial cell, such as *Escherichia coli*'s tetracycline efflux system or *Staphylococcus aureus*' efflux system for fluoroquinolones (Frainow and Abrutyn, 1995). Decreased permeability of the bacterial outer membrane can result in reduced intracellular antibiotic concentration, such as the alterations in outer membrane porin proteins of *Pseudomonas aeruginosa* conferring resistance to Beta-lactam agents, imipenem and possibly fluoroquinolones and aminoglycosides. An additional mechanism contributing to diminished intracellular drug concentration is decreased uptake by the bacterial cytoplasmic membrane, as seen in some aminoglycoside-resistant staphylococcal species (Frainow and Abrutyn, 1995; Kaye *et al.*, 2000; Kaye *et al.*, 2004). Among gram-negative bacteria, the emergence of resistance to expanded-spectrum cephalosporins has been a major concern. It appeared initially in a limited number of bacterial species (*Enterobacter cloacae*, *Clostridium freundii*, *Serratia marcescens*, and *Pseudomonas aeruginosa*) that could mutate to hyperproduce their chromosomal class C β -lactamase. A few years later, resistance appeared in bacterial species not naturally producing AmpC enzymes (*K. pneumoniae*, *Salmonella* spp., *Proteus mirabilis*) due to the production of TEM- or SHV-type ESBLs (Philippon *et al.*, 2002). Characteristically, such resistance has included oxyimino- β -lactams (for example ceftizoxime, cefotaxime, ceftriaxone, and ceftazidime, as well as the oxyimino-monobactam aztreonam) but not 7-alpha-methoxy-cephalosporins (cephamycins). Plasmid-mediated AmpC β -lactamases represent a new threat since they confer resistance to 7-alpha-methoxy-cephalosporins (cephamycins) such as ceftaxime or cefotetan, are not affected by

commercially available β -lactamase inhibitors, and can, in strains with loss of outer membrane porins, provide resistance to carbapenems (Philippon *et al.*, 2002).

Table 1. Mechanisms of Antimicrobial Resistance among Enteric Gram-negative Bacilli

Microorganism	Antimicrobial Resistance	Usual Mechanisms	Other Mechanisms	Effective Antimicrobials
<i>Escherichia coli</i> and <i>Klebsiella</i> species	<u>Common</u> Some β -lactams: penicillins cephalosporin I*	Penicillinases or β -lactamases	Poor diffusion or altered porins	Cephalosporins II, III Aminoglycosides Fluoroquinolones
	<u>Emerging</u> Most β -lactams: cephalosporins aztreonam	Extended- spectrum β - lactamases (ESBL)	Amp C constitutive β -lactamases	Carbapenems Fluoroquinolones
<i>Enterobacter</i> , <i>Serratia</i> , <i>Citrobacter</i> , <i>Acinetobacter</i> , <i>Providencia</i> , and <i>Morganella</i> species	<u>Common</u> Many β -lactams	β -lactamases (broad spectrum)	Poor diffusion or altered porins	Aminoglycosides Carbapenems Fluoroquinolones
	<u>Emerging</u> Most β -lactams	Amp C constitutive β -lactamases	ESBL Plasmid-mediated (extremely rare)	Carbapenems Fluoroquinolones

* Cephalosporin I, II, III, and IV are sometimes referred to as 1st, 2nd, 3rd, and 4th generation cephalosporins, respectively

2.3.1 Plasmids

A plasmid is an independent, circular, self-replicating DNA molecule that carries only a few genes. Plasmids are autonomous molecules and exist in cells as extrachromosomal genomes, although some can be inserted into a bacterial chromosome, where they become a permanent part of the bacterial genome (<http://askabiologist.asu.edu/expstuff/mamajis/plasmids/plasmids.html>). Resistance and virulence genes have common features, both being located in the bacterial chromosome, as well as on plasmids, associated in gene clusters to form resistance or pathogenicity islands, transferred by mobile elements or phages (Villa and Carattoli,

2005). Many resistance genes found in clinical isolates of Gram-negative bacteria are parts of gene cassettes (Collis and Hall, 1992). Gene cassettes are discrete genetic elements that may exist as free, circular, non-replicating DNA molecules when moving from one genetic site to another, but which are normally found as linear sequences that constitute part of a larger DNA molecule, such as a plasmid or bacterial chromosome (Hall *et al.*, 1991). Hence, plasmids carry integrons which may contain gene cassettes encoding antibiotic resistance genes such as beta lactamases, amlyer class C beta-lactamases and aminoglycoside modifying enzymes (Bennett, 1999; Tran *et al.*, 2005; Villa and Carattoli, 2005; Ambrozic *et al.*, 2007).

2.3.2 Integrons

Integrons are mobile DNA elements with the ability to capture genes, notably those encoding antibiotic resistance, by site-specific recombination. Integrons have an intergase gene (*int*), a nearby recombination site (*attI*), and a promoter. Integrons are divided into two major groups: the resistance integrons (RI) and the super-integrons (SI). RI carries mostly gene cassettes that encodes resistance against antibiotics and disinfectants, and can be located either on the chromosome or on plasmids. The large chromosomally-located integrons, which contain gene cassettes with a variety of functions, belong to the SI group. At least four classes of chromosomal and plasmid-borne RI in Gram-negative bacteria have been described (Arakawa *et al.*, 1995; Hall and Collis, 1995). The four classes of RI are distinguished by their respective integrase (*int*) genes (Mazel *et al.*, 1998).

There are several classes of integrons based upon the type of integrase gene they possess. Class 1 integrons have been examined the most extensively. They consist of a variable region bordered by 5' and 3' conserved regions. The 5' region is made up of the *int* gene, *attI*, and the promoter part which drives transcription of genes within the variable region. The 3' region consists of an ethidium bromide resistance locus (*qacED1*), a sulfonamide resistance gene (*sulI*), and an open reading frame containing a gene of unknown function. Class 1 integrons are the most ubiquitous type among resistant clinical isolates of Gram negative bacteria, and are associated with the *Tn21* transposon family (Nesvera *et al.*, 1998; Sun *et al.*, 2002). Class 1 integrons can capture gene cassettes, which contain an *attC* recombination site, via a site-specific recombination event between *attI* and *attC*. The 3' conserved segment (3'CS) of integrons contains the *qacED1* and *sulI* genes, encoding resistance to quaternary ammonium salts and sulphonamides, respectively (Recchia and Hall, 1997). Since class 1 integrons were first described by Stokes and Hall in 1989 (Stokes and Hall, 1989). Integron-mediated resistance to antibiotics has been reported in clinical isolates of various Gram-negative bacteria (Sallen *et al.*, 1995; Hall and Collis, 1998; Houndt and Ochman, 2000). The integrase of Class 2 integrons is located within the 3' conserved region. Class 2 integrons are associated with the *Tn7* transposon family and have been identified in *Salmonella* so far only in the serovars *Typhimurium* and *Paratyphi B* (Miko *et al.*, 2003). They consist of an integrase gene followed by gene cassettes. Class 3 integrons have yet to be thoroughly studied (Stokes and Hall, 1989). The structure of class 3 integrons is comparable to that of class 2 integrons but contains the *blaIMP* gene cassette

conferring resistance to carbapenems (Arakawa *et al.*, 1995). Class 4 is a distinctive class of integrons located in the *Vibrio cholerae* genome and is not known to be associated with antibiotic resistance (Rowe-Magnus *et al.*, 1999).

2.3.2.1 Integrons as a source of resistance genes

Integrons have been identified as a primary source of resistance genes, and are suspected to form reservoirs of antimicrobial resistance genes within microbial populations (Stokes and Hall, 1989). However, with few exceptions, most of these studies have been performed with Gram negative bacteria (Nandi *et al.*, 2004). *Escherichia coli* (*E.coli*) isolates from clinical specimens may be resistant to multiple antimicrobial agents (Yu *et al.*, 2004; Bradford, 2001; Winokur *et al.*, 2001) and a substantial proportion of multiresistant *E. coli* isolates carry integrons (Goldstein *et al.*, 2001). Since many gene cassettes of integrons contain antimicrobial resistance genes in Gram-negative bacteria, the horizontal transfer of integrons through plasmids and transposons has been found to play an important role in the dissemination of antimicrobial resistance genes and the development of multiresistance (Recchia and Hall, 1997). Integrons are not self-transferred elements, and are commonly associated with various transposons. An IS26 insertion into the 5' conserved segment of an In4-type integron and an IS26-mediated recruitment of resistance genes of diverse origin have been suggested as a mechanism for the evolution of various multiresistant integrons, including those that harbour the bla_{VIM-1} genes (Miriagou *et al.*, 2005). However, further work on the exact mechanism of their development and dissemination is needed. Commensal *E. coli* isolates from

humans and animals can cause extra-intestinal diseases, including urinary tract infection, pneumonia, meningitis and bacteraemia (Guerra *et al.*, 2003). These bacterial strains are a potential reservoir for antimicrobial resistance genes and play an important role in the ecology of antimicrobial resistance of bacterial populations.

2.3.2.2 Prevalence of Integrons

Class 1 integrons are most commonly found in clinical isolates of Gram-negative bacteria and more than 60 distinct gene cassettes have been identified (Hall and Collis, 1998). In a previous study class 1 integrons detected in 54% of urinary *E. coli* isolates were shown to strongly associate with multiresistance (Yu *et al.*, 2003). Apart from being carried by clinical pathogens, few studies have reported the prevalence of integrons and gene cassettes in the enteric faecal flora of humans and animals (Hall and Collis, 1995; Leverstein *et al.*, 2001).

A type I integron carrying the bla_{VIM-1} gene and a 6'-N-aminoglycoside acetyltransferase (aac(6')-Ib) gene cassette was described in an *Enterobacter cloacae* clinical isolate (Galani *et al.*, 2005). Moreover, a different integron structure suggesting a different evolution process rather than a transfer, and the spread of the mobile element among the Greek hospitals was described in a cluster of four *E. coli* isolates in Crete (Scoulica *et al.*, 2004).

Similarly, a novel class 1 integron carrying a carbapenemase gene (bla_{VIM-1}) associated with a trimethoprim (dfrA1), a streptothricin (sat1) and two aminoglycoside resistance genes (aacA7 and aadA1) has been detected in a

Morganella morganii clinical isolate (Tsakris *et al.*, 2007). A class I integron carrying only the bla_{VIM-1}, and the dhfrI and aadA genes has been found in a plasmid isolated from three different bacterial genera (Galani *et al.*, 2005). An integron solely carrying the bla_{VIM-1} gene has also been described in an *E. coli* isolate (Galani *et al.*, 2007).

2.3.3 Extended Spectrum β -Lactamases (ESBLs) Production

Extended Spectrum β -Lactamases (ESBLs) are capable of conferring bacterial resistance to the penicillins, first- generation, second-, and third-generation cephalosporins, and aztreonam (but not the cephamycins e.g, ceftiofur or carbapenems such e.g. meropenem). ESBLs confer resistance to these antibiotics through their ability to hydrolyse the β -lactam ring (Bush *et al.*, 1995).

ESBLs-producing strains of *Enterobacteriaceae* have emerged as a major problem in hospitalized as well as community based patients. These strains have been isolated from abscesses, blood, catheter tips, lungs, peritoneal fluid, sputum, and throat culture (Naumovski *et al.*, 1992; Emery and Weymouth, 1997). Infections due to ESBL-producers range from uncomplicated urinary tract infection to life threatening sepsis (Bhattacharya, 2006). Beta-lactamases of Gram-negative bacteria are the most important mechanism of resistance against β - lactam drugs. These enzymes destroy the β -lactam ring of the β -lactam antibiotics. They bind to and prevent the action of penicillin binding proteins (PBPs), which are responsible for building and maintenance of peptidoglycan layer (Livermore, 1995).

ESBLs are especially problematic because they are plasmid-associated, and some of them are located within various transposable elements, which strongly facilitate their spread among bacterial strains, even those of different species (Tumbarello *et al.*, 2006). Partly because of the difficulty to differentiate infection from colonization, a number of studies have elucidated inconsistent results concerning the risk factors and clinical significance of infections due to ESBL-producing organisms (Schiappa *et al.*, 1996; Lautenbach *et al.*, 2001).

ESBL production is generally the result of point mutations in the *bla*_{TEM} and *bla*_{SHV} genes which alter the primary amino sequences of the respective β -lactamase enzymes (Livermore, 1995; Podschun and Ullmann, 1998; Bradford, 2001). The SHV-type ESBLs may be more frequently found in clinical isolates than any other type of ESBLs (Jacoby, 1997).

The TEM-type ESBLs are derivatives of TEM-1 and TEM-2. TEM-1 was first reported in 1965 from an *Escherichia coli* isolate from a patient in Athens, Greece, (Datta and Kontomichalou, 1965). TEM-1 is able to hydrolyze ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and has negligible activity against extended-spectrum cephalosporins. It is inhibited by clavulanic acid (Paterson and Bonomo, 2005). *Klebsiella oxytoca*, harboring a plasmid carrying a gene encoding ceftazidime resistance, was first isolated in Liverpool, England, in 1982 (Du Bois *et al.*, 1995). The responsible β -lactamase was what is now called TEM-12. Interestingly, the strain came from a neonatal unit which had been stricken by an outbreak of *Klebsiella oxytoca* producing TEM-1. Ceftazidime was used to treat infected patients, but subsequent isolates of *Klebsiella oxytoca* from the same unit

harbored the TEM-type ESBL (Du Bois *et al.*, 1995). This is a good example of the emergence of ESBLs as a response to the selective pressure induced by extended-spectrum cephalosporins (Paterson and Bonomo, 2005).

Other types of enzymes in particular, the cefotaximase (CTX-M) ESBLs have also been identified, and are being reported with increasing frequency throughout the world (Bradford, 2001; Bush, 2001; Edelstein *et al.*, 2003). The name CTX reflects the potent hydrolytic activity of these β -lactamases against cefotaxime. The OXA-type β -lactamases are so named because of their oxacillin-hydrolyzing abilities (Paterson and Bonomo, 2005). These β -lactamases (group 2d) are characterized by hydrolysis rates for cloxacillin and oxacillin greater than 50% that of benzylpenicillin (Bush *et al.*, 1995). Most OXA-type β -lactamases do not hydrolyze the extended-spectrum cephalosporins to a significant degree and are not regarded as ESBLs. However, OXA-10 weakly hydrolyzes cefotaxime, ceftriaxone, and aztreonam, giving most organisms reduced susceptibility to these antibiotics. Other OXA ESBLs include: OXA-11, -14, -16, -17, -19, -15, -18, -28, -31, -32, -35, and -45 (Toleman *et al.*, 2003). Novel chromosomally encoded ESBLs have also been described. VEB-1 has greatest homology with PER-1 and PER-2 (38%). It confers high-level resistance to ceftazidime, cefotaxime, and aztreonam, which is reversed by clavulanic acid. The gene encoding VEB-1 was found to be plasmid mediated; such plasmids also confer resistance to non- β -lactam antibiotics. GES, TLA and IBC are other examples of non-TEM, non-SHV ESBLs and have been found in a wide range of geographic locations (Paterson and Bonomo, 2005).

Many of these ESBLs have evolved from the TEM-1, TEM-2, and SHV-1 β -lactamases that are widely distributed among the *Enterobacteriaceae* (Sirot, 1995; Thomson *et al.*, 1996; Jacoby, 1997). Since the ESBL genes are usually found in large plasmids that also contain other antimicrobial resistance genes, ESBL-producing organisms may also be resistant to aminoglycosides, tetracyclines, chloramphenicol, and/or sulfonamides (Podschun and Ullmann, 1998; Bradford, 2001) and ESBL-producing *K. pneumoniae* strains are more likely to be resistant to fluoroquinolones than their non-ESBL-producing counterparts (Tumbarello *et al.*, 2006).

ESBLs may emerge as a result of excessive cephalosporin use, and this indicates that interventions designed to restrict cephalosporin use in order to reduce the level of antibiotic resistance merits further investigation (Du *et al.*, 2002). Outbreaks of *Klebsiella* infections with strains resistant to third-generation cephalosporins have also been reported in Kenya for ESBL production (Musoke and Revathi, 2000) implying a need for continuous surveillance.

Prevalence and Control of ESBL producing strains

ESBLs have been detected in many gram-negative species; *Klebsiella pneumoniae* is still the most frequently reported producer of these enzymes (Knothe *et al.*, 1983; Mena *et al.*, 2006). The first documented *K. pneumoniae* strain which produced an extended spectrum beta-lactamase (ESBL) was reported from Germany in 1983 (Knothe *et al.*, 1983) and during the last two decades in hospital settings there have

been several outbreaks of *K. pneumoniae* strains producing ESBLs of different types (Brun-Buisson *et al.*, 1987; Johnson *et al.*, 1992; Peña *et al.*, 1998; Rahal *et al.*, 1998; Mena *et al.*, 2006). In a different study the prevalence of ESBL was found to be 17.9% in *E. coli* and as high as 52.9% in *K. pneumoniae* isolated from blood cultures (Kim *et al.*, 2002). In some regions of Eastern Europe and Latin America, the prevalence of ESBL-producing *K. pneumoniae* in blood cultures has now approached 50% (Winokur *et al.*, 2001; Goossens and Grabein, 2005).

European surveys in 1994 and 1997–98 found ESBLs in a quarter of all *Klebsiella* spp. isolated from ICU patients (Livermore *et al.*, 2007). First reports of ESBL-producing organisms in the United States occurred in 1988 (Jacoby *et al.*, 1988) and in 1989, significant infections with TEM-10-producing *Klebsiella pneumoniae* were noted in Chicago by Quinn and colleagues (Quinn *et al.*, 1989). In Kenya CTX-M enzyme (CTX-M-12) has also been described among *K. pneumoniae* isolates causing sepsis in newborns (Kariuki *et al.*, 2001).

In an attempt to reduce the prevalence of ESBL producers, restricted use of extended-spectrum cephalosporins and encouraged use of β -lactam/ β -lactamase inhibitor combinations (piperacillin/tazobactam or ampicillin/sulbactam) has been tried for empirical therapy and/or specific therapy (Rice *et al.*, 1996; Piroth *et al.*, 1998). That attempt was followed by a successful reduction in ESBL-producing *K. pneumoniae* and *E. coli* isolates in an institute for children where ESBL-producing *E.*

coli and *K. pneumoniae* were endemic. The impact of the change in antibiotic policy was more evident in *K. pneumoniae* than in *E. coli* (Lee *et al.*, 2007).

2.3.4 Ambler Class C β -Lactamases Production

The production of Ambler class C beta-lactamases (AmpC β -lactamases) is one of the prevalent mechanisms of β -lactam resistance (Verdet *et al.*, 2006). Various types of AmpC enzymes have been found, particularly in *Escherichia coli* and *Klebsiella pneumoniae* which are the most commonly isolated species of the Enterobacteriaceae family in the clinical laboratory (Hanson, 2003). Several members of the family *Enterobacteriaceae* are naturally resistant to cephalosporins due to the production of an inducible, chromosomally encoded cephalosporinase. In the uninduced state, transcription of the structural β -lactamase gene, *ampC*, is repressed by the product of the linked *ampR* gene (Lindberg *et al.*, 1985; Honoré *et al.*, 1986; Poirel *et al.*, 1999). Plasmid-mediated AmpC enzymes, such as CMY-2, DHA, ACT-1, and CMY-2, were first reported in Germany and Taiwan (Bauernfeind *et al.*, 1990) while DHA-2 was first identified in *K. pneumoniae* in France (Fortineau *et al.*, 2001). Plasmid mediated AmpC β -lactamases confer transmissible cephalosporin resistances to pathogens (Paterson, 2006), which may pose an important problem to public health. In China DHA-type AmpC β -lactamases in *E. coli* and *K. pneumoniae* isolates have also been reported (Xiong *et al.*, 2002). In particular, *K. pneumoniae* strains have also acquired plasmid-mediated AmpC enzymes. Unlike ESBLs, AmpCs are poorly inhibited by β -lactamase inhibitors and are less active against cefepime and

cefpirome than ESBLs. AmpCs confer resistance to oxyimino- and 7- α -methoxy-cephalosporins (Philippon *et al.*, 2002).

Prevalence of AmpC β -lactamase Producing bacteria

More than 20 different plasmid-borne *ampC* genes have been identified (Pérez-Pérez and Hanson, 2002). One of the encoded enzymes, DHA, was first described for *Salmonella enterica* serovar *enteritidis* in Saudi Arabia in 1997 (Gaillot *et al.*, 1997). A detailed study demonstrated that *blaDHA-1* was located on an integron that originated from *Morganella morganii* (Verdet *et al.*, 2000). The increasing prevalence of plasmid-mediated AmpC β -lactamases in *E. coli* and *K. pneumoniae* is becoming a serious problem worldwide. In addition, many laboratories are having difficulties in detecting these enzymes using unified criteria in clinical isolates since there are no Clinical Laboratory Standards Institute (CLSI) guidelines regarding the methodology for detection of this type of resistance (Song *et al.*, 2007; Woodford *et al.*, 2007). A report has shown that, in the United States, among 752 *K. pneumoniae* and *E. coli* strains from 70 sites in 25 states, 7 to 8.5% of the *K. pneumoniae* and 4% of the *E. coli* strains contain plasmid-mediated AmpC-type enzymes (Alvarez *et al.*, 2004).

The prevalence of AmpC-type β -lactamases among resistant *K. pneumoniae* isolates has been shown to vary by region, from 8.5% in the USA (Alvarez *et al.*, 2004) to 55% in the UK. As the prevalence of *E. coli*- and *K. pneumoniae*-producing plasmid-mediated AmpC β -lactamases arise in tandem with the pervasive use of the broad-

spectrum cephalosporins (Moland *et al.*, 2007) it is getting difficult to select antibiotics to treat patients, especially pediatric patients (Woodford *et al.*, 2007). The prevalence of plasmid-mediated AmpC β -lactamases in *K. pneumoniae* has been rising in Korea. Of the ESBLs and the acquired AmpC enzymes, SHV-12 and DHA-1 are the most prevalent and widespread in Korea, respectively (Lee *et al.*, 2006; Ryoo *et al.*, 2005). Occurrence of AmpC β -lactamase co-producing *K. pneumoniae* has been reported (Moland *et al.*, 2007; Yan *et al.*, 2004) but outbreaks of the isolates are extremely rare.

2.3.5 Aminoglycoside Modifying Enzymes

In bacteria, resistance to aminoglycosides which include ; amikacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, rhodostreptomycin, streptomycin, tobramycin, and apramycin, is often due to enzymatic inactivation by Aminoglycoside-modifying enzymes (Benveniste and Davies, 1973). These enzymes are grouped into three families, aminoglycoside phosphotransferases (APHs), aminoglycoside acetyltransferases (AACs), and aminoglycoside nucleotidyltransferases (ANTs). The turnover products of these reactions lack antibacterial activity and each class performs a specific reaction. Furthermore, several subtypes of each class have been identified with distinct regioselectivities in substrate modification (Kim *et al.*, 2007). For instant the *aac(6')*-Ib gene (synonymously called *aacA4*) encodes many variants of an aminoglycoside-acetyltransferase enzyme that is responsible for amikacin resistance but not gentamicin resistance (van Nhieu and Collatz, 1987). Bifunctional enzymes are

generally rare in bacteria. Four genes encoding bifunctional aminoglycoside-resistance enzymes have now been discovered within the past few years. These genes encode enzymes that have been designated as AAC(6')/APH(2''), ANT(3'')-Ii/AAC(6')-IId, AAC(3)-Ib/AAC(6')-Ib', and AAC(6')-30/AAC(6')-Ib' (Kim *et al.*, 2007).

Aminoglycoside-resistant strains often emerge as a result of acquiring plasmid-borne genes encoding aminoglycoside-modifying enzymes (Courvalin and Carlier, 1981). Furthermore, many of these genes are associated with transposons, which aid in the rapid dissemination of drug resistance across species boundaries.

Bifunctional aminoglycoside-modifying enzymes have been shown to influence resistance to fluoroquinolones (Kim *et al.*, 2007). Fluoroquinolones are broad-spectrum antimicrobial agents widely used in clinical medicine. Resistance to this class of antibacterials is usually caused by mutations in the chromosomal genes that code for DNA gyrase and/or DNA topoisomerase IV, the target enzymes, and/or mutations resulting in alterations in drug accumulation (Ruiz, 2003). Recently, plasmid mediated quinolone resistance (PMQR) mechanisms have also been described. The first comprises *qnr* genes that encode target protection proteins of the pentapeptide repeat family (Tran *et al.*, 2005; Martínez-Martínez *et al.*, 1998). The second mechanism involves the *aac (6')-Ib-cr* gene, which encodes a new variant of the common aminoglycoside acetyltransferase. Two single-nucleotide substitutions at codons 102 and 179 in the wild-type allele *aac (6')-Ib* enable the gene product to

be capable of acetylating and thus reducing the activity of some fluoroquinolones, including norfloxacin and ciprofloxacin. Over the past decade, the prevalence of fluoroquinolones-resistant Enterobacteriaceae has been increasing (Neuhauser *et al.*, 2003) and is independently associated with higher mortality in adults. Fluoroquinolones resistance in Enterobacteriaceae has been associated most consistently with prior fluoroquinolones use. The emergence of bifunctional aminoglycoside-resistance enzymes is yet another level of sophistication. All three examples that have been studied AAC(6')/APH-(2''), ANT(3)-Ii/AAC(6')-IId, and AAC(3)-Ib/AAC-(6')-Ib' reveal that the merger of the two genes results in bifunctional enzymes whose substrate profile is broadened. The cases of AAC(6')/APH(2'') and ANT-(3'')-Ii/AAC(6')-IId are somewhat intuitive, as two domains that catalyze distinct reactions merged to result in the large enzymes (Kim *et al.*, 2007). Monitoring of this emergence is thus essential. Resistance development against the aminoglycosides as well as their relative toxicity has spurred medicinal chemistry approaches to develop improved aminoglycoside derivatives and mimetics (Vakulenko and Mobashery, 2003).

Prevalence of Aminoglycoside Modifying Enzymes

In a study by Park *et al.*, (2006) *aac (6')-Ib-cr* was detected in 15 (32%) of 47 *E. coli* isolates, 17 (16%) of 106 *K. pneumoniae* isolates, and 12 (7.5%) of 160 *Enterobacter* isolates collected during the study period. The analysis of the relationship between *aac(6')-Ib-cr* and susceptibility to ciprofloxacin, gentamicin, and trimethoprim-sulfamethoxazole among all isolates had the *aac(6')-Ib-cr* allele present in almost

equal proportions of ciprofloxacin-susceptible (MIC, 0.25 to 1.0 µg/ml) and -resistant (MIC > 2.0 µg/ml) isolates, and neither ciprofloxacin nor trimethoprim-sulfamethoxazole resistance was associated with *aac(6′)-Ib-cr* prevalence (Lee *et al.*, 2006).

In the study, there was no statistically significant change in the overall prevalence of *aac(6′)-Ib-cr* over time, although *aac(6′)-Ib-cr* was detected in a slightly smaller proportion of isolates in 2004 than in the prior years studied (Park *et al.*, 2006).

In terms of geographical distribution the Middle Atlantic region provided the largest number of *aac(6′)-Ib-cr*-positive *K. pneumoniae* and *E. coli* isolates and the Pacific region had the highest prevalence of *aac(6′)-Ib-cr* (22%) overall, but *aac(6′)-Ib-cr*-positive isolates of *K. pneumoniae* were found in all but three census regions in (Park *et al.*, 2006) study. Overall, there was no geographic clustering of *aac(6′)-Ib-cr* and there was no relationship between *aac(6′)-Ib-cr* prevalence and patient age, patient gender, or inpatient status. There was also no relationship between the presence of *qnrA*, *-B*, or *-S* genes and *aac(6′)-Ib-cr* (Park *et al.*, 2006).

The contributions of the *qnr* and *aac(6′)-Ib-cr* genes to the increasing quinolone resistance worldwide and the association between quinolone resistance and extended-spectrum-β-lactamase (ESBL)-producing strains remain largely unknown (Ambrozic *et al.*, 2007).

2.4 Clinical importance of antibiotic resistant pathogens

Many ESBL-producing outbreak strains of *K. pneumonia* and *E. coli* are isolated most frequently from critically ill patients, within specialist units. These strains carrying ESBL are clinically important because they cause nosocomial infections that commonly appear in outbreaks and are associated with treatment failure, prolonged hospital stay, increased health expenditure as well as a possible increase in mortality (Meyer *et al.*, 1993). In addition strains of *K. pneumoniae* and *E. coli* that are resistant to multiple antibiotics, including the newer cephalosporins, have developed. The infections caused by them are frequently epidemic in nature and have complicated chemotherapy significantly (Bryan *et al.*, 1983; Meyer *et al.*, 1993).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study site and Specimen source

The study was carried out at the Centre for Microbiology Research (CMR) at the Kenya Medical Research Institute (KEMRI). Environmental bacterial strains were isolated from water collected from sewage dams, streams/waste effluents, rivers and bore holes in Nairobi and its environs (Table 2).

Table 2. Sources of water samples and locations collected

Water source	Locations
Sewage	Juja sewage, Ruai sewage plant.
Streams/waste effluents	Kenyatta National Hospital stream, Ruiru, Juja, Mbagathi way, Makadara, Kawangware.
Rivers	Nairobi river, Ruiru river.
Boreholes	Langata, South B, Kahawa area.

3.2 Sampling and study design

A cross sectional design was used in this study. Environmental samples were obtained by convenient sampling whereby water and sediments were collected from sewage systems and waste effluents within Nairobi. From each site 500ml of water was collected in sterile narrow-mouth plastic bottles. All samples were transported

to the laboratory in an insulated cool box and processed within 24 hours of collection.

3.3 Sample Size

Information from an earlier study where the prevalence of *Klebsiella* species and *E. coli* were 26% and 29%, respectively was used (Pathak and Gopal, 2008). The higher prevalence of 29% was used to estimate the number of isolates required so as to provide a statistically significant sample size. A single prevalence was used since from a single sample it was expected that both *E. coli* and *Klebsiella* species would be present. Sample size was therefore estimated using the formula in Lwanga (1975) biostatistics for medical students as shown below:

$$Z_{\alpha} = \frac{\delta}{\sqrt{P(1-P)/n}}$$

Where:

$$Z_{\alpha} = Z_{0.05} = 1.96 \text{ (95\% Confidence limit)}$$

$$\delta = 0.07 \text{ (7\% True proportion estimate)}$$

$$P = 0.29 \text{ (29\% Probability of } \textit{Klebsiella} \text{ or } \textit{E. coli} \text{ isolation) (Pathak and Gopal, 2008)}$$

n = Minimum sample size

$$n = (1.96)^2 * (0.29 * 0.71) / (0.07)^2 = 0.7910 / (0.07)^2 = 161$$

A minimum of 161 none replica bacterial isolates were required to recover a statistically significant number of *Klebsiella* species and *E. coli* isolates.

3.4 Laboratory Procedures

3.4.1 Isolation of bacteria

Culture media used were quality controlled using standard strains; *Escherichia coli* ATCC® 25922 and *Staphylococcus aureus* ATCC® 25923. The protocol below was used in bacterial isolation:

The water and sediment samples were transported to the laboratory in a cool box then immediately 3ml of the sample was inoculated into 5ml of Peptone water (Oxoid Ltd.) in a test tube and incubated at 37°C for 24 hours. A duplicate was made of the same batch of water sample and inoculated in Peptone water (Oxoid Ltd.) and incubated at 44°C for 24 hours. After the 24 hours incubation a loopful of the broth culture from both 37°C and 44°C were then sub-cultured on Macconkey (Oxoid Ltd.), *Salmonella*, *Shigella* (Oxoid Ltd.), and Xylose lysine deoxycholate (XLD) (Oxoid Ltd.) media and incubated at 37°C for 24 hours.

3.4.2 Identification of bacteria

Colony morphology and sugar fermentation reactions were used for presumptive identification of the isolates. Biochemical typing was used for further identification of the isolates. The biochemical metabolic characteristics were tested on triple sugar iron agar (Oxoid Ltd.), lysine indole motility agar (Oxoid Ltd.), Simmon's citrate agar (Oxoid Ltd.), Methyl Red Voges- Proskauer (MRVP) broth(Oxoid Ltd.) and

urea agar (Oxoid Ltd.). Organisms once identified were stocked in 1ml capacity vials containing tryptic soy broth (Oxoid Ltd.), containing 15% (v/v) glycerol and stored at -70°C until analysed.

3.4.3 Antibiotic susceptibility testing

Antibiotic disks viability was quality controlled using *Escherichia coli* ATCC® 25922. Each identified bacteria was revived from -70°C on Mueller-Hinton agar (Oxoid Ltd.). Kirby-Bauer disk diffusion method was used for susceptibility testing. Briefly, a single pure bacterial colony was picked and suspended in normal saline to give an opacity equivalent to that of 0.5 McFarland standards. Using a sterile cotton swab a uniformly thin lawn of the suspension was carefully spread on Mueller-Hinton agar (Oxoid Ltd.) (MH) plates. A maximum of six antibiotic disks were placed radially on inoculated plates. The classification and specific antibiotic disks (all obtained from Oxoid Ltd.) used were:

The **beta-lactams**: Ampicillin/Sulbactam (AMS 20µg), Ampicillin (AMP 10µg) (moderate spectrum), ampicillin/clavulanate (augmentin) (AUG 30 µg) (combination), piperacillin–tazobactam (TZP 110µg) (combination), aztreonam (ATM, 30 µg) (monobactam), cefepime (FEP, 30µg), (4th generation cephem), cefotaxime (CTX 30 µg) (broad spectrum 3rd generation cephem), cefixime (CFM, 5µg) (broad spectrum 3rd generation), Ceftriaxone (CRO 30µg) (broad spectrum 3rd generation cephem), cefoxitin (FOX, 30 µg) (2nd generation cephem) and ceftazidime - CAZ (30 µg) (3rd generation cephem).

The **aminoglycosides**: amikacin (Ak 30 µg), neomycin (N 30 µg), tobramycin (TOB10 µg), kanamycin (K 30µg), tetracycline (Te 30µg), minocycline (MN 30µg), gentamicin (Cn, 10µg) and streptomycin (S 30µg) all target the 30S subunit. Also included was chloramphenicol (C 30µg) an amphenicol targeting the 50S subunit.

Nucleic acids inhibitors: Nitrofurantoin (F 300µg) and Sulfamethoxazole (SMX, 50µg).

Fluoroquinolones: Ciprofloxacin (Cip, 5µg) and norfloxacin (NOR 10 µg).

The Mueller Hinton (Oxoid Ltd.) agar plates with the antibiotic disks placed on were incubated at 37°C for 24 hours aerobically and diameters of zones of inhibition measured and classified as resistant or sensitive according to the clinical and laboratory standards institute standards (CLSI) (2008) recommended breakpoints (Appendix E).

3.4.4 Determination of minimum inhibitory concentrations

Minimal inhibitory concentrations (MICs) were determined by the agar micro dilution method according to the CLSI guidelines. The antimicrobial agents used were ampicillin, amikacin, tetracycline, sulphamethoxazole, ceftazidime, cefotaxime and gentamicin (Adatabs; Mast Laboratories, Liverpool, United Kingdom). Serial dilutions of antimicrobials were done in Mueller-Hinton agar. The range of dilutions of antibiotics used was: ampicillin 128, 64, 32, 16, 8, 4, 2, 1 and 0.5 mg/L, amikacin 64, 32, 16, 8, 4, 2, 1 and 0.5 mg/L, tetracycline 128, 64, 32, 16, 8, 4, 2, 1 and 0.5 mg/L, sulphamethoxazole 128, 64, 32, 16, 8, 4, 2, 1 and 0.5 mg/L, ceftazidime 128, 64, 32, 16, 8, 4, 2, 1 and 0.5 mg/L, cefotaxime 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125

mg/L and gentamicin 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/L. One discrete bacterial colony from an overnight incubation was resuspended in normal saline to give an opacity equivalent to that of the 0.5 McFarland standards. Using a sterile Pasteur pipette each bacterial suspension was dispensed into a single well of a multiple-well template. Quality control was performed by including, *Escherichia coli* ATCC® 25922 and *Staphylococcus aureus* ATCC® 25923 during each experiment. The bacterial suspension was inoculated from the template wells to the antibiotic containing agar plates using a multiple inoculator with rods sterilised with 70% alcohol and dried by flaming. Inoculation sequence was done from the least potent to the most potent antibiotic and from the lowest to the highest dilution with sterilisation between changes of antibiotics. A culture plate containing Mueller Hinton agar (Oxoid Ltd.) with no antibiotic was inoculated before and after inoculation of antibiotic to control for bacterial viability.

3.4.5 Identification of Extended Spectrum β -Lactamases (ESBLs)

Synergy between ceftazidime and clavulanate was determined by placing a disk of Augmentin (20 μ g of amoxicillin plus 10 μ g of clavulanic acid) and a disk of ceftazidime 30 mm apart (center to center). A clear-cut extension of the edge of the ceftazidime inhibition zone toward the disk containing clavulanic acid was to be interpreted as synergy, which suggests the production of ESBL. The double-disk synergy test was considered positive when decreased susceptibility to ceftazidime was combined with synergy between ceftazidime and augmentin (Jarlier *et al.*, 1988). Repeat experiments were done whereby in each repeat, the cephalosporin

was replaced with a representative drug from 1, 2nd, 3rd and 4th generation cephalosporin plus a monobactam (aztreonam).

3.4.6 Isolation of DNA by boiling method

A single colony of each isolate was suspended in 0.5 ml of sterile water and boiled for 15 min. After boiling, centrifugation was done at 13,000 xg for 5 min at 4°C. The DNA-containing supernatant was stored in -20°C until later used as the source of DNA template for further PCR amplification experiments (Solberg *et al.*, 2006).

3.4.7 Conjugation experiments

Fourteen isolates that were multidrug resistant and had plasmids detected were selected to be donors for conjugation experiments. Those selected isolates were sensitive to ciprofloxacin and resistant to ampicillin and rifampicin plus any other aminoglycoside. *E. coli* C600 which is resistant to rifampicin was used as the recipient. Resistant organisms (donor) and *E. coli* C600 (recipient) were cultured onto Mueller-Hinton agar and incubated at 37°C overnight. The cultured recipient and resistant donors were inoculated in normal saline to make 0.5 Macfarland solution. Donor and recipient strains were diluted to 1:3 by adding 0.5ml of donor Macfarland solution into 1.5ml recipient macfarland solution into freshly prepared Mueller hinton broth and incubated at 37 degrees for 24 hours. After the 24 hours using a sterile inoculating wire loop each of the mixtures was sub-cultured onto: MacConkey agar containing 30µg/ml ampicillin and 30µg/ml rifampicin (for *E. coli*

C600) to select for transconjugants. Antibiotic susceptibility was repeated using the disk diffusion method to confirm transferred resistance phenotypes.

3.4.8 Polymerase chain reaction (PCR) amplification of resistance genes

PCR amplification was carried out in 25 μ L reaction volumes containing 2.5 μ l 10x concentrated PCR buffer [100 mM Tris/HCl (pH 8.3), 500 mM KCl, 15 mM MgCl₂], 2.5 μ l (10 pmol μ l⁻¹) each of appropriate primers, 2 μ l dNTP mix (2.5 mM each dNTP), 0.2 μ l (5 U μ l⁻¹) *Taq* DNA polymerase, 5 μ l of template DNA and 12.8 μ l sterilized distilled water. The thermo-cycler PCR conditions were primer specific.

3.4.8.1 Detection of aminoglycosides modifying enzymes

Organisms resistant to quinolones and aminoglycosides were screened for the presence of bifunctional aminoglycosides modifying enzymes (aac) using the following set of primers and PCR conditions:

aac(6')-II Forward 5' – CGCTTGTTGATTTGCTGCTGTTCGC – 3'
Reverse 5' - TTGAAACGACCT TG ACCTTCCG – 3'

PCR Conditions: 94⁰C 5min, [94⁰C 45s, 55⁰C 30s, 72⁰C 1min] 30 times, 72⁰C 10min, 10⁰C hold

aac(6')-1b-cr Forward 5' – TTGCGATGCTCTATGAGTGGCTA – 3'
Reverse 5' - CTCGAATGCCTGGCGTGTTT – 3'

PCR Conditions: 94⁰C 5min, [94⁰C 45s, 55⁰C 45s, 72⁰C 1min] 35 times, 72⁰C 10min, 10⁰C hold

aac(3)-I Forward 5' – AGCCCGCATGGATTTGA – 3'
Reverse 5' - GGCATACGGGAAGAAGT – 3'

PCR Conditions: 94⁰C 5min, [94⁰C 1min, 55⁰C 1min, 72⁰C 1min] 35 times, 72⁰C 10min, 10⁰C hold

aac(3)-IIa Forward 5' – GCTAAACTCCGTTACC – 3'
Reverse 5' - TAGCACTGAGCAAAGCC – 3'

PCR Conditions: 94⁰C 5min, [94⁰C 45s, 60⁰C 30s, 72⁰C 90s] 30 times, 72⁰C 10min, 10⁰C hold

3.4.8.2 Detection of Integrons class 1 and 2

All isolates resistant to two or more antibiotics were screened for presence of integron class 1 and Integron class 2 genes. The following set of primers and PCR conditions were used:

Int 1 Forward 5' – GTTCGGTCAAGGTTCTG – 3'
Reverse 5' - GCCAACTTTCAGCACATG– 3'

PCR Conditions: 94⁰C 5min, [94⁰C 30s, 55⁰C 30s, 72⁰C 90s] 35 times, 72⁰C 10min, 10⁰C hold

Int 2 Forward 5' – ATGTCTAACAGTCCATTTT– 3'
Reverse 5' - AAATCTTTAACCCGCAAAC – 3'

PCR Conditions: 94⁰C 5min, [94⁰C 30s, 55⁰C 30s, 72⁰C 1min] 35 times, 72⁰C 10min, 10⁰C hold

3.4.8.3 Electrophoresis

Agarose gel (1% agar wt/v) was prepared containing 0.05% ethidium bromide and once set it was mounted in the electrophoresis tank and 1X electrophoresis buffer Tris Borate EDTA (TBE) (sigma) (Appendix B) added to cover the gel to a depth of about 1mm. The PCR products (10 µl) were mixed with gel-loading buffer and loaded into the wells using a disposable micropipette. Voltage was applied at 115 volts DC for 30 minutes.

3.4.8.4 Photography

Visualization of the bands was done on an UV transilluminator (UVP Inc., San Gabriel, Calif.) and photography of the gel was done using a transmitted illumination camera fitted with a Polaroid film.

3.4.9 Plasmid studies

3.4.9.1 Harvesting and Extraction.

Bacteria found resistant to two or more antibiotics were selected for plasmid analysis using the protocol by Sambrook *et al.* (1989) described below. Bacteria stored at -70°C were revived by subculturing on Mueller Hinton agar (Oxoid Ltd.) and incubated at 37°C for 24 hours aerobically. A single bacterial colony was transferred into 2 ml of LB medium in a loosely capped 15 ml tube. The culture was incubated at 37°C with vigorous shaking overnight. A volume of 1.5 ml of the culture was transferred into a microfuge tube and centrifuged at 13,000g for 30 seconds at 4°C in a microfuge. Supernatant was removed by aspiration and the dry pellet was resuspended in 100 µl of ice cold solution I (Appendix A) by gentle agitation. Bacterial cell wall was digested by addition of 200 µl of freshly prepared solution II (Appendix A) and on gentle shaking 150 µl of ice cold solution III was used to precipitate cell debris plus protein material (Appendix A). Centrifuged in a microfuge at 13,000g for 5 minutes at 4°C and The supernatant was transferred to a fresh tube. The double stranded DNA was precipitated using 2 volumes of ethanol at room temperature, vortexed and allowed to stand for 2 minutes at room temperature. Centrifugation was then done at 13,000g at 4°C in a microfuge for 5 minutes and the

supernatant was removed by gentle aspiration. The tube was then placed in an inverted position and all fluid allowed to drain. The pellet was rinsed using 1 ml of 70% ethanol at 4°C, the supernatant removed and the pellet allowed to dry in air for 10 minutes. Plasmid DNA was then redissolved in 50 µl of TE (pH 8.0) containing DNase free pancreatic RNase (20 µg/ml) and stored at -20°C until use. Then DNA was resolved by electrophoresis and images taken as described earlier in sections 2.4.8.3 and 2.4.8.4.

3.5 Data management

Laboratory procedures and results were recorded in the laboratory note book. Later (same day) all data was transferred to Microsoft excel[®] software and saved in the hard disk drive and flash disks. The notebook and flash disks were kept in a securely locked cabinet when not in use and a password used for data in the computer hard disk drive. Data presentation was done by use of bar graphs, charts and tables. Chi-square or Fisher's exact test was used for test of association between proportions as appropriate. Stat View[®] and INSTAT[®] statistical application software were used for analysis of the data. Analysed data and results will be availed by publication in journals, seminars presentation and thesis.

3.6 Ethical considerations

Laboratory procedures were performed in accordance to the standard operating procedures. No human subjects were involved in the current study hence ethical requirements were exempted by the Kenya Medical Research Institute (KEMRI) National Ethical Review Committee. Permission to carry out the study was granted

by the Scientific Steering Committee (SSC), KEMRI and Jomo Kenyatta University of Agriculture Technology.

CHAPTER FOUR

4.0 RESULTS

4.1 Samples

Total of seventy nine (79) water samples were collected from boreholes (9 samples), sewages (20 samples), streams (34 samples) and rivers (16 samples) within Nairobi, Kenya and its environs.

4.2 Isolation and Identification of bacteria.

Bacteria were isolated on MacConkey agar after enrichment in broth media at 37°C and 44°C overnight. Discrete colonies with characteristic pink (lactose fermenters – characteristic of *Escherichia coli*) (Figure 1) and pink raised mucoid (characteristic of *Klebsiella*) (Figure 2) were observed on Mac Conkey agar plates that had been inoculated with cultures enriched at 44°C overnight.

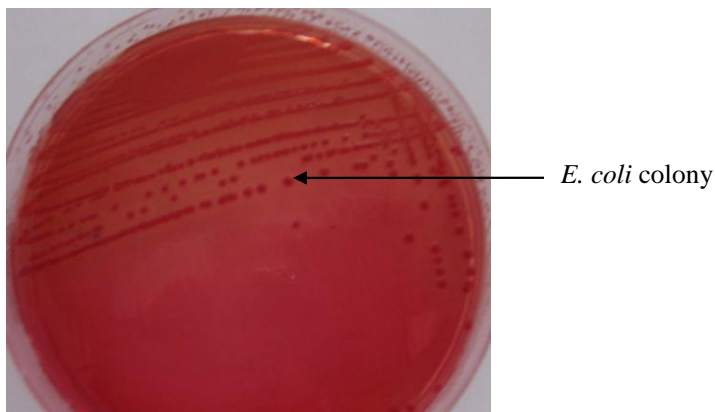


Figure 1. Colony morphology of *Escherichia coli* (*E.Coli*)

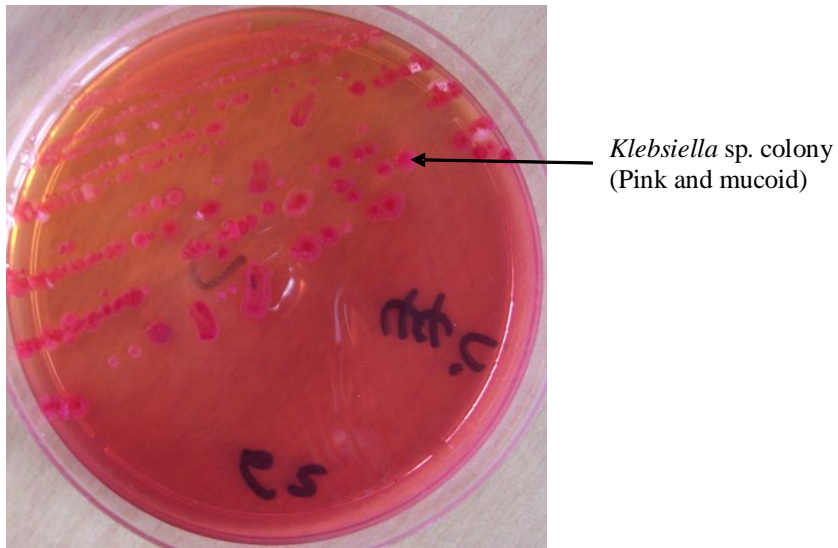


Figure 2. Colony morphology of *Klebsiella* species

Growth on Mac Conkey agar inoculated with samples from 37°C overnight enrichments was characterized by an overgrowth of pale colonies (none lactose fermenters) and thus no follow up of the samples was done.

4.3 Identification of isolates by biochemical typing

A total of 180 isolates were presumptively identified as *E. coli* and *Klebsiella* based on their growth characteristic on Mac Conkey agar and were further identified by biochemical typing. Of the 180 isolates biotyped *E. coli* (Figure 3) were 73 (40.6%) and *Klebsiella* sp. (Figure 4) were 53 (29.4%).



TSI: Gas +Ve, Slant +Ve, Butt +Ve. SIM: Sulphur –Ve, Indole +Ve, Motility +Ve. SC: –Ve. U: -Ve. MR:+Ve
TSI- Triple sugar iron, SIM- Sulphur Indole motility, SC- Simmons Citrate, U-Urea, MR-VP- Methyl red-Vogues Proskeur.

Figure 3. Biochemical typing characteristics of *Escherichia coli*



TSI: Gas +Ve, Slant -Ve, Butt +Ve. SIM: Sulphur –Ve, Indole +Ve, Motility +Ve. SC: +Ve. U: -Ve. MR:-Ve
TSI- Triple sugar iron, SIM- Sulphur Indole motility, SC- Simmons Citrate, U-Urea, MR-VP- Methyl red-Vogues Proskeur.

Figure 4. Biochemical typing characteristics of *Klebsiella* species.

Biochemical typing of colony morphology identified isolates gave identities of *Klebsiella* and *E. coli* as well as other organisms including *Enterobacter*, *Citrobacter*. Others were not identifiable with the biochemical tests used (Table 3).

Table 3. Isolates identified by biochemical typing

Identity	Number	Percentage
<i>Escherichia coli</i>	73	40.6%
<i>Klebsiella species</i>	53	29.4%
<i>Enterobacter</i>	3	1.7%
<i>Citrobacter</i>	2	1.1%
Unidentified	49	27.2%
Total	180	100%

Escherichia coli and *Klebsiella* species were more prevalent in sewage samples (56%, 66%), followed by streams/rivers (34%, 28%) and least isolated from boreholes (10%, 6%), respectively (Table 4)

Table 4. Isolates distribution by source

Source	<i>E. coli</i>	<i>Klebsiella sp.</i>	Total
Bore holes	7 (10%)	3 (6%)	10
Streams/Rivers	25 (34%)	15 (28%)	40
Sewage	41 (56%)	35 (66%)	76
Total	73	53	126

4.4 Antibiotics susceptibility testing

Susceptibility testing was done against four broad categories of antibiotics namely beta – lactams, aminoglycosides (protein synthesis inhibitors), fluoroquinolones and nucleic acid inhibitors.

The diameter of the inhibition zones (zone without growth) was measured and interpreted to resistant (R) or intermediate (I) or sensitive (S). Representative measured zone diameters and their interpretations are shown in Tables 5, 6, 7 and 8. Table 5 represents zones and diameters for beta lactams. Table 6 represents zones and diameters for aminoglycosides. Table 7 represents zones and diameters for nucleic acid inhibitors. Table 8 represents zones and diameters for fluoroquinolones.

The inhibition zones appeared as shown in Figure 5 below:

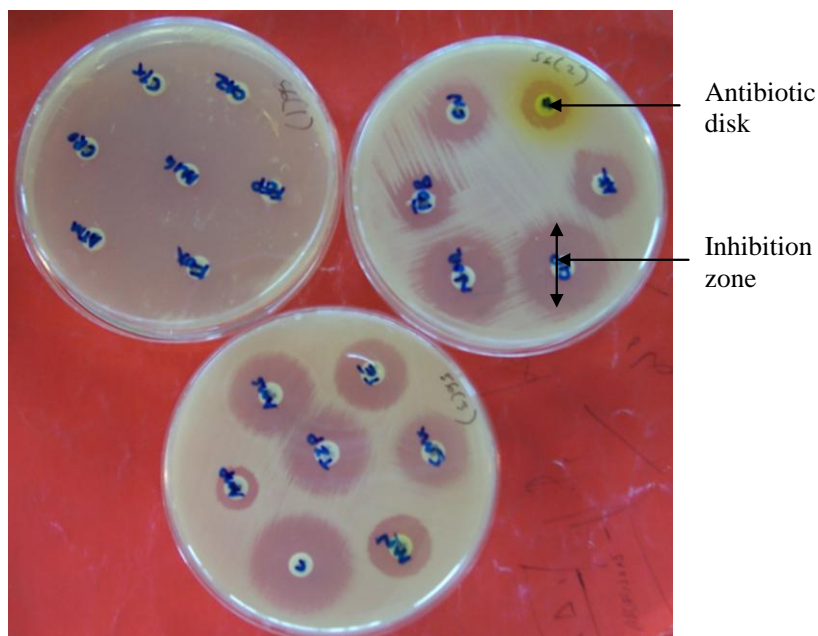


Figure 5. Antibiotics susceptibility testing by Disk diffusion

Table 5. Representative zones of inhibition diameters and their interpretations for beta lactam antibiotics

Box No.	Bacteria Identity	CAZ 30µg		FOX 30µg		CRO 30µg		CTX 30µg		FEP 30µg		ATM 30µg		AUG 30µg		AMP 10µg		AMS 20µg		TZP 110µg		CFM 5µg	
4	<i>Escherichia coli</i>	28	S	18	S	30	S	30	S	34	S	36	S	10	R	10	R	18	S	36	S	22	S
35	<i>Escherichia coli</i>	26	S	15	I	28	S	30	S	30	S	28	S	11	R	7	R	17	S	16	R	24	S
70	<i>Escherichia coli</i>	11	R	19	S	12	r	14	R	34	S	13	R	20	S	10	R	10	R	22	S	18	I
71	<i>Escherichia coli</i>	16	I	18	S	21	S	21	I	32	S	16	I	20	S	10	R	10	R	19	I	26	S
72	<i>Escherichia coli</i>	10	R	20	S	16	I	18	I	30	S	10	R	16	I	10	R	10	R	24	S	17	I
79	<i>Klebsiella</i>	15	I	19	S	20	I	18	I	32	S	18	I	16	I	12	R	12	I	32	S	22	S
80	<i>Klebsiella</i>	18	S	20	S	25	S	26	S	28	S	30	S	20	S	13	R	17	S	29	S	26	S
82	<i>Klebsiella</i>	16	I	19	S	25	S	20	I	34	S	21	I	15	I	10	R	14	I	27	S	25	S

Key: S – Sensitive; I – Intermediate; R – Resistant

CAZ 30µg - ceftazidime, FOX 30µg – cefoxitin, CRO 30µg – Ceftriaxone, CTX 30µg – cefotaxime, FEP 30µg – cefepime, ATM 30µg – aztreonam, AUG 30µg - ampicillin/clavulanate (augmentin) AMP 10µg – Ampicillin, AMS 20 µg - Ampicillin/Sulbactam, TZP 110µg - piperacillin–tazobactam, CFM 5µg - cefixime.

Each drug’s zone diameters were interpreted to R or I or S independently according to the NLSI breakpoints for beta lactams antibiotics

Table 6. Representative zones of inhibition diameters and their interpretations for aminoglycosides antibiotics

Box No.	Bacteria Identity	S 30µg		N 30µg		AK 30µg		CN 10µg		K 30µg		TOB 10µg		MN 30µg		C 30µg		TE 30µg	
4	<i>Escherichia coli</i>	16	S	20	S	21	S	22	S	22	S	20	S	18	I	26	S	24	S
35	<i>Escherichia coli</i>	14	I	17	S	15	I	15	S	14	I	12	R	15	I	16	R	14	R
70	<i>Escherichia coli</i>	14	I	19	S	15	I	13	I	14	I	22	S	10	R	10	R	22	S
71	<i>Escherichia coli</i>	10	R	20	S	15	I	15	S	15	I	23	S	11	R	16	R	10	R
75	<i>Escherichia coli</i>	14	I	24	S	18	S	20	S	18	S	23	S	19	S	13	R	16	I
79	<i>Klebsiella</i>	12	I	15	I	16	I	17	s	16	I	20	S	10	R	15	R	12	R
80	<i>Klebsiella</i>	13	I	13	I	16	I	16	S	16	I	24	S	11	R	20	I	15	I
83	<i>Klebsiella</i>	17	S	23	S	15	I	15	S	15	I	28	S	12	R	16	R	15	I

Key: S – Sensitive; I – Intermediate; R – Resistant

S 30µg – streptomycin, N 30 µg - neomycin, Ak 30 µg - amikacin, Cn 10µg – gentamicin, K 30µg – kanamycin, TOB10 µg - tobramycin, MN 30µg - minocycline, C 30µg – chloramphenicol, Te 30µg - tetracycline.

Each drug’s zone diameters were interpreted to R or I or S independently according to the NLSI breakpoints for aminoglycosides.

Table 7. Representative zones of inhibition diameters and their interpretations for nucleic acid inhibitor antibiotics

Box No.	Bacteria Identity	F 300µg		SMX 50µg	
4	<i>Escherichia coli</i>	13	R	11	R
35	<i>Escherichia coli</i>	14	R	8	R
70	<i>Escherichia coli</i>	12	R	10	R
71	<i>Escherichia coli</i>	13	R	10	R
72	<i>Escherichia coli</i>	10	R	10	R
79	<i>Klebsiella</i>	15	R	10	R
80	<i>Klebsiella</i>	13	R	10	R
82	<i>Klebsiella</i>	15	R	10	R
83	<i>Klebsiella</i>	15	R	10	R

Key: S – Sensitive; I – Intermediate; R – Resistance

F 300µg – Nitrofurantoin, SMX 50µg – Sulfamethoxazole.

Each drug's zone diameters were interpreted to R or I or S independently according to the NLSI breakpoints for nucleic acid inhibitor antibiotics

Table 8. Representative zones of inhibition diameters and their interpretations for fluoroquinolones antibiotics

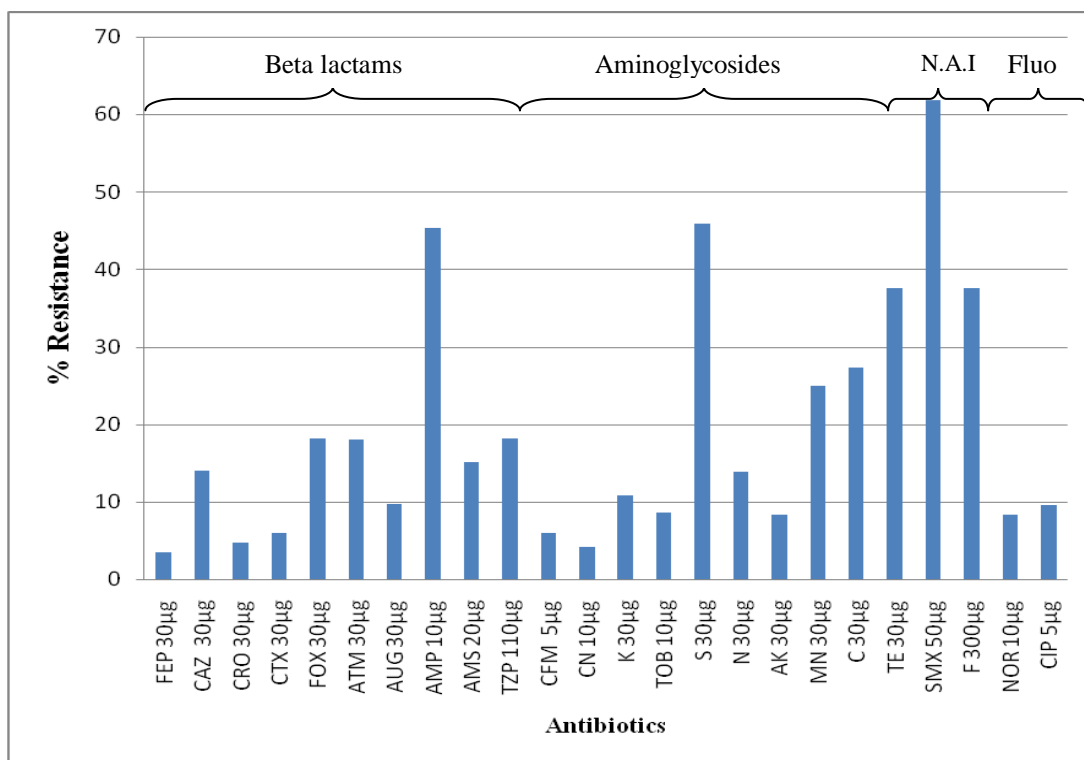
Box No.	Bacteria Identity	NOR 10µg		CIP 5µg	
5	<i>Escherichia coli</i>	38	S	22	S
1	<i>Escherichia coli</i>	21	S	26	S
2	<i>Escherichia coli</i>	15	I	17	I
3	<i>Escherichia coli</i>	16	I	20	I
15	<i>Klebsiella</i>	20	S	28	S
16	<i>Klebsiella</i>	20	S	23	S
17	<i>Klebsiella</i>	11	R	10	R

Key: S – Sensitive; I – Intermediate; R – Resistant

NOR 10 µg – norfloxacin, Cip 5µg – ciprofloxacin.

Each drug's zone diameters were interpreted to R or I or S independently according to the NLSI breakpoints for fluoroquinolones antibiotics.

No isolate was identified as an Extended Spectrum β -Lactamase (ESBL) producer. Among the *E. coli* isolates 62% were resistance to sulfamethoxazole with only 3% being resistance to cefepime (FEP) and 4% to gentamicin (CN) (Figure 6). The different antibiotic groups differed significantly in terms of resistance levels ($p < 0.05$; chi-squared for independence) with, nucleic acid inhibitors having the highest average resistance level (57%) followed by aminoglycosides (27%), beta lactams (17%) and lastly fluoroquinolones (10%).



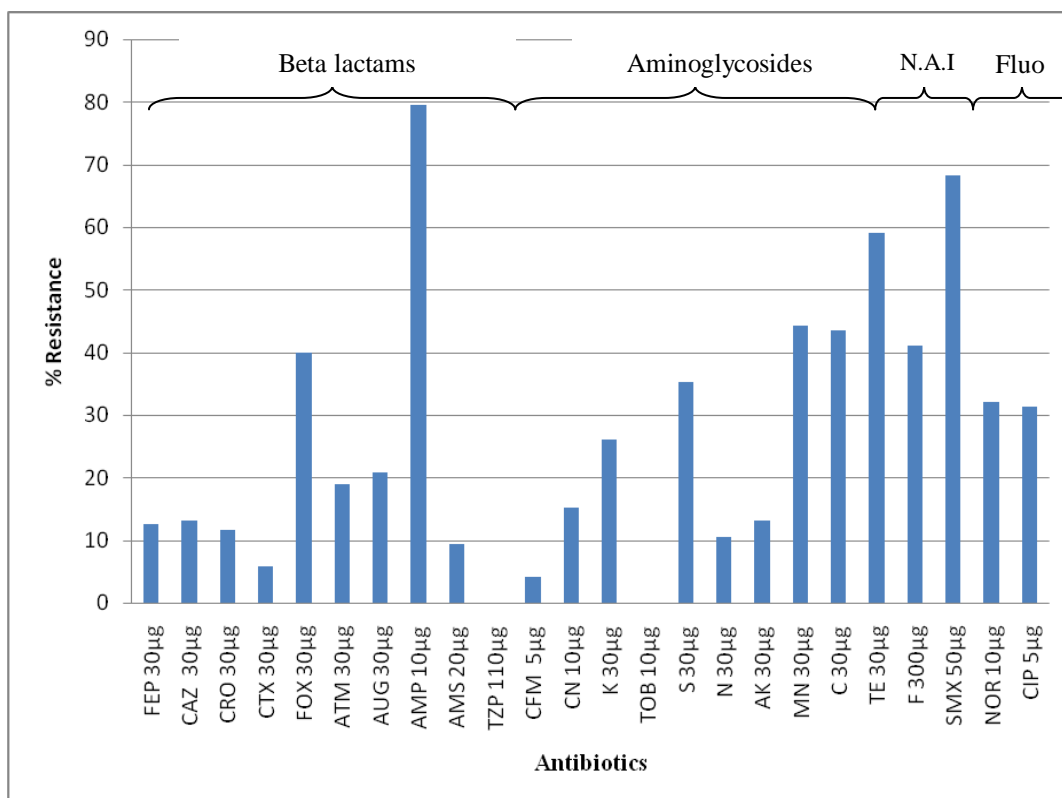
Key: Fluo- fluoroquinolones; N.A.I –nucleic acids inhibitors

FEP 30µg – cefepime, CAZ 30µg - ceftazidime, CRO 30µg - Ceftriaxone, CTX 30µg - cefotaxime, FOX 30µg - cefoxitin, ATM 30µg - aztreonam, AUG 30µg - ampicillin/clavulanate (augmentin) AMP 10µg - Ampicillin, AMS 20 µg - Ampicillin/Sulbactam, TZP 110µg – piperacillin-tazobactam, CFM 5µg - cefixime, Cn 10µg - gentamicin, K 30µg - kanamycin, TOB 10 µg - tobramycin, S 30µg - streptomycin, N 30 µg - neomycin, Ak 30 µg - amikacin, MN 30µg - minocycline, C 30µg - chloramphenicol, Te 30µg - tetracycline, SMX 50µg – Sulfamethoxazole, F 300µg – Nitrofurantoin, NOR 10 µg - norfloxacin, Cip 5µg – ciprofloxacin.

Figure 6. Antibiotics susceptibility profile of *Escherichia coli*

Klebsiella species showed highest resistance to Ampicillin (Amp) (80%) and no resistance to tobramycin (TOB) (0%) and piperacillin-tazobactam (TZP) (0%) (Figure 7). For *Klebsiella* isolates the different antibiotic groups tested differed significantly in terms of resistance levels ($p < 0.05$; chi-squared for independence).

Nucleic acid inhibitors had the highest average resistance level (65%) followed by aminoglycosides (36%), fluoroquinolones (33%), and lastly beta lactams (25%).

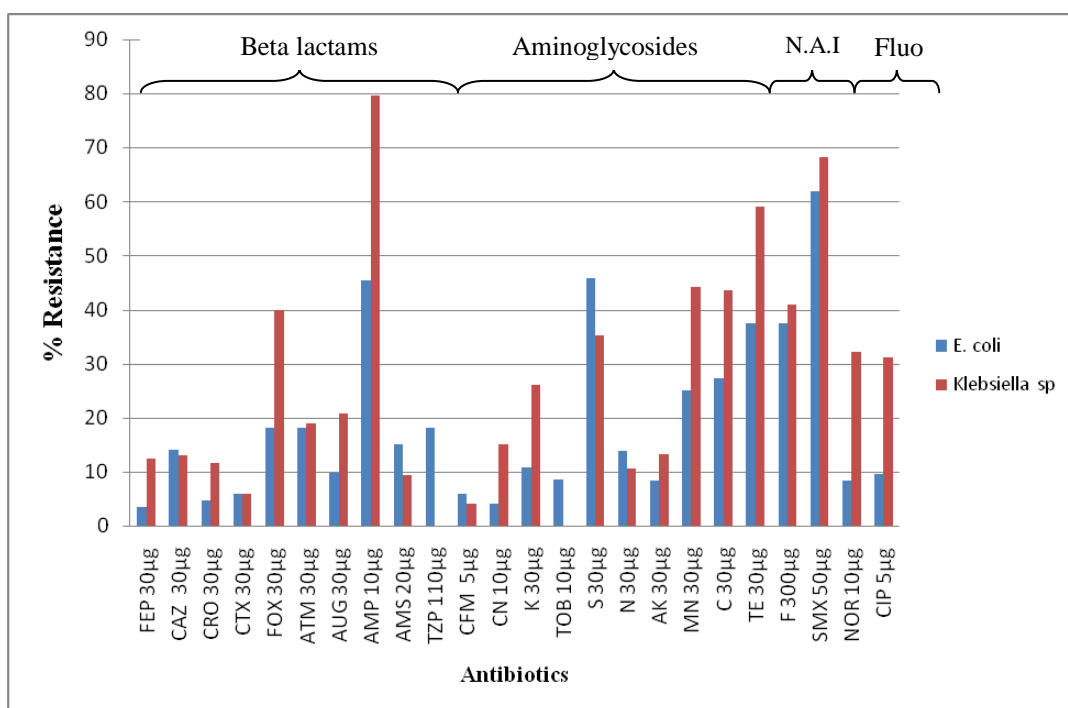


Key: Fluo- fluoroquinolones; N.A.I –nucleic acids inhibitors

FEP 30µg – cefepime, CAZ 30µg - ceftazidime, CRO 30µg - Ceftriaxone, CTX 30µg - cefotaxime, FOX 30µg - cefoxitin, ATM 30µg - aztreonam, AUG 30µg - ampicillin/clavulanate (augmentin) AMP 10µg - Ampicillin, AMS 20 µg - Ampicillin/Sulbactam, TZP 110µg – piperacillin-tazobactam, CFM 5µg - cefixime, Cn 10µg - gentamicin, K 30µg - kanamycin, TOB10 µg - tobramycin, S 30µg - streptomycin, N 30 µg - neomycin, Ak 30 µg - amikacin, MN 30µg - minocycline, C 30µg - chloramphenicol, Te 30µg - tetracycline, SMX 50µg – Sulfamethoxazole, F 300µg – Nitrofurantoin, NOR 10 µg - norfloxacin, Cip 5µg – ciprofloxacin.

Figure 7. Antibiotics susceptibility profile of *Klebsiella* species

A higher percentage of *Klebsiella* isolates were resistant to most drugs compared to *E. coli* (Figure 8). The difference though was only significant in ciprofloxacin, norfloxacin and ampicillin ($p < 0.05$; Fisher's exact test).

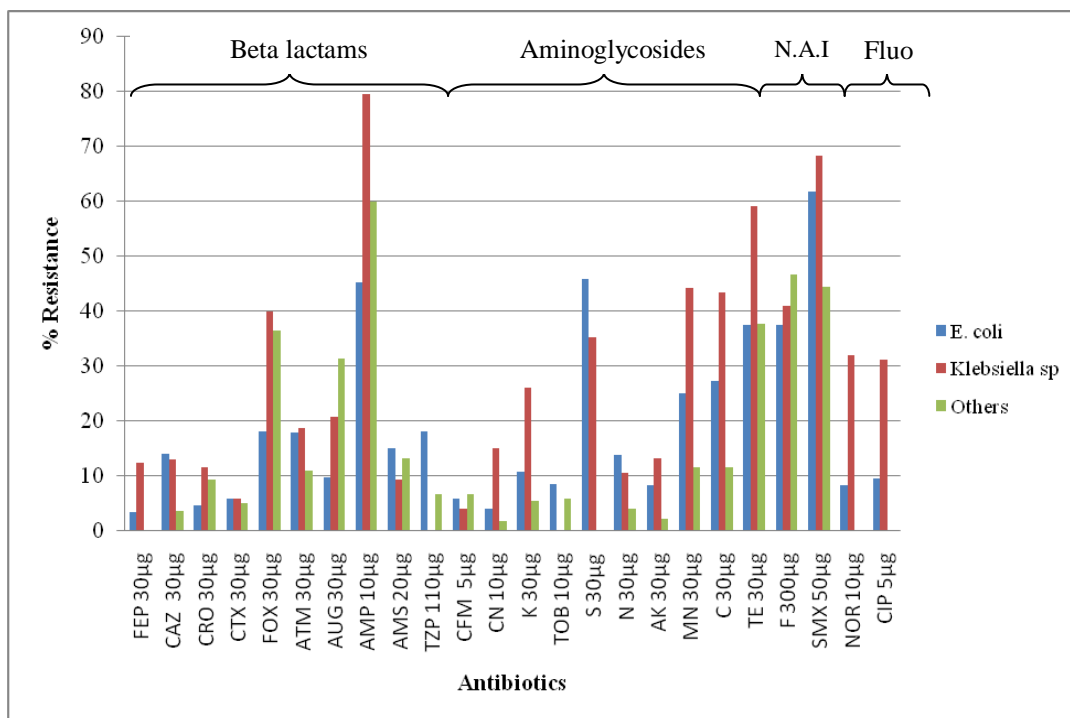


Key: Fluo- fluoroquinolones; N.A.I –nucleic acids inhibitors

FEP 30µg – cefepime, CAZ 30µg - ceftazidime, CRO 30µg - Ceftriaxone, CTX 30µg - cefotaxime, FOX 30µg - cefoxitin, ATM 30µg - aztreonam, AUG 30µg - ampicillin/clavulanate (augmentin) AMP 10µg - Ampicillin, AMS 20 µg - Ampicillin/Sulbactam, TZP 110µg – piperacillin-tazobactam, CFM 5µg - cefixime, Cn 10µg - gentamicin, K 30µg - kanamycin, TOB10 µg - tobramycin, S 30µg - streptomycin, N 30 µg - neomycin, Ak 30 µg - amikacin, MN 30µg - minocycline, C 30µg - chloramphenicol, Te 30µg - tetracycline, SMX 50µg – Sulfamethoxazole, F 300µg – Nitrofurantoin, NOR 10 µg - norfloxacin, Cip 5µg – ciprofloxacin.

Figure 8. Comparison of susceptibility pattern of *Escherichia coli* and *Klebsiella* species

Unidentified isolates and other isolates comprising of Citrobacter and Enterobacter showed highest resistance observed in ampicillin (Figure 9).



Key: Fluo- fluoroquinolones; N.A.I –nucleic acids inhibitors

FEP 30µg – cefepime, CAZ 30µg - ceftazidime, CRO 30µg - Ceftriaxone, CTX 30µg - cefotaxime, FOX 30µg - cefoxitin, ATM 30µg - aztreonam, AUG 30µg - ampicillin/clavulanate (augmentin) AMP 10µg - Ampicillin, AMS 20 µg - Ampicillin/Sulbactam, TZP 110µg – piperacillin-tazobactam, CFM 5µg - cefixime, Cn 10µg - gentamicin, K 30µg - kanamycin, TOB10 µg - tobramycin, S 30µg - streptomycin, N 30 µg - neomycin, Ak 30 µg - amikacin, MN 30µg - minocycline, C 30µg - chloramphenicol, Te 30µg - tetracycline, SMX 50µg – Sulfamethoxazole, F 300µg – Nitrofurantoin, NOR 10 µg - norfloxacin, Cip 5µg – ciprofloxacin.

Figure 9. Comparison of susceptibility profiles of all the groups

4.5 Minimum inhibitory concentrations (MIC)

Minimum inhibitory concentrations (MICs) of twenty eight multidrug resistant isolates were determined. The isolates selected were all resistant to ampicillin, tetracycline, sulphamethoxazole and ceftazidime by disk susceptibility testing. The isolates resistant by disk diffusion method were also all resistance (100%) by MICs cut off points of the respective drugs Table 9.

Table 9: MICs of multidrug resistant *Klebsiella* sp. and *E. coli* isolates

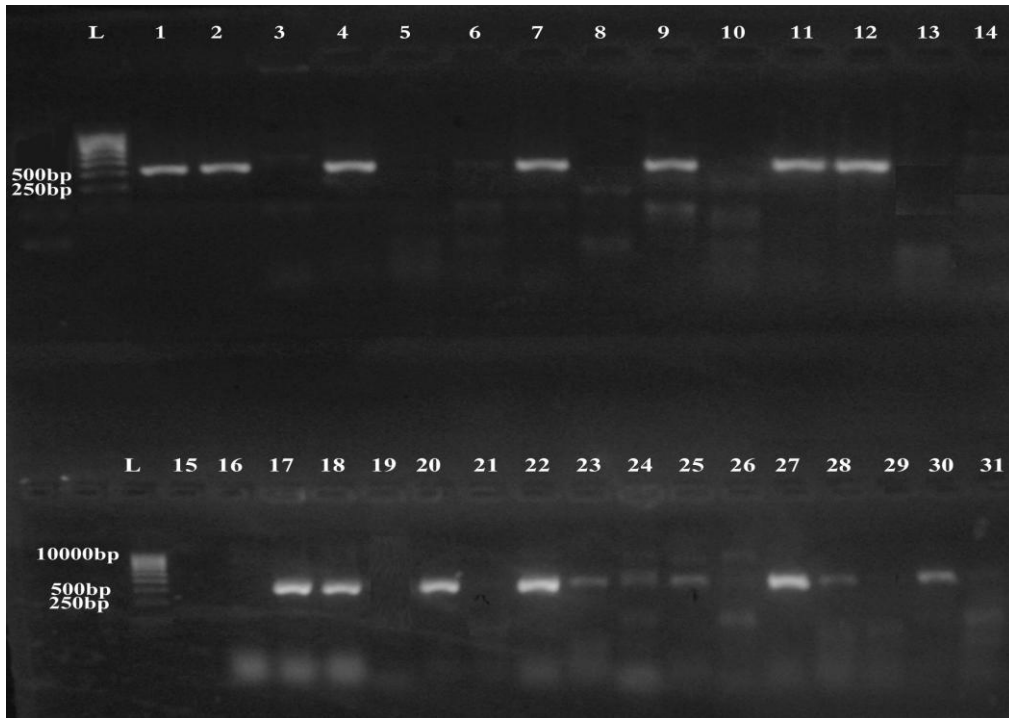
Antibiotic	Range (mg/l)	Minimum Inhibitory Concentrations			N=28
		Mode (mg/l)	MIC50 (mg/l)	MIC90 (mg/l)	% Resistant
Ampicillin	0.5 - 128	>128	128	>128	100
Amikacin	0.5 - 64	32	16	32	0
Tetracycline	0.5 - 128	128	128	128	100
Sulphamethoxazole	0.5 - 128	128	128	128	100
Ceftazidime	0.5 - 128	128	128	128	100
Cefotaxime	0.125 - 32	32	16	32	89
Gentamicin	0.0313 - 32	32	16	32	68

4.6 Detection of resistance genes.

Isolates found to be resistant to more than two antibiotics were tested for presence of Integrase 1, Integrase 2 and Ame genes.

4.6.1 Detection of *Int 1* and *Int 2* genes

Among the isolates screened for resistance genes by PCR, the presence of *Int 1* was significantly lower in *E. coli* (47.4%; [18/38]) than in *Klebsiella* species (73.0% [27/37]), ($p < 0.05$ Chi-square). The presence of *Int 1* gene was demonstrated by the presence of 523bp amplicon (Figure 10, 11).



1 to 14: *E. coli* isolates, 15 to 29: *Klebsiella* isolates, 30: positive control, 31: negative control, L – 1Kb Ladder (Promega)

Figure 10. Gel photo of representative isolates with and without *Int 1* gene



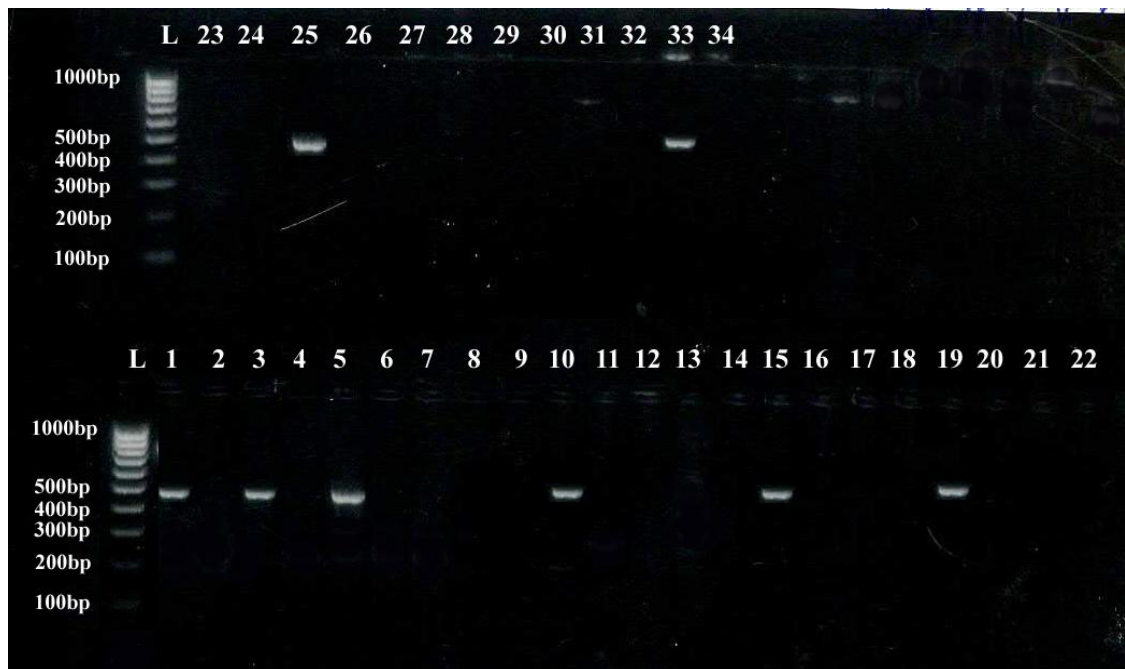
Key: L- 1Kb ladder (Promega), 1 to 8 – *Klebsiella* isolates, 9 to 15 – *E. coli* isolates

Figure 11: Representative pooled positive PCR products of *Int 1* gene amplification

All the resistant isolates including those positive for *Int 1* gene were negative for *Int 2* gene.

4.6.2 Detection of AME genes

The distribution of genes encoding four AMEs (*aac*(6')-1b-cr, *aac*(3)-1, *aac*(6')-II and *aac*(3)-IIa) was investigated by simplex PCR and *aac*(6')-1b-cr was detected in 18.2% (2/11) of *E. coli* and 25% (5/20) of *Klebsiella* species all giving an amplicon of 482bp (Figure 12). Though detected in a higher percentage in *Klebsiella* species than in *E. coli* the difference is not significant ($p > 0.05$; Fisher's exact test). The genes *aac*3-1, *aac*(6')-II and *aac*(3)-IIa were not detected in any of the isolates.



Key: L:100bp ladder (Promega), 1: Positive control, 2: Negative control; 3 to 22: *Klebsiella* isolates, 23 to 33: *E. coli* isolates, 34: Negative control

Figure 12. PCR products of *aac*(6')-1b-cr AME genes

4.7 Plasmid studies

Among the 34 multidrug resistant isolates used for plasmid studies 41% (14/34) had plasmids detected (Figure 13).



Key: M1 – *E.coli* V517, 1-5- *E.coli* isolates, 6-11 – *Klebsiella* species, M2 – *E.coli* 39R861

Figure 13. Representative plasmid DNA isolated from multidrug resistant isolates

4.8 Conjugation experiments

Conjugation experiments were done for the 14 isolates found to have plasmids so as to determine their ability to transfer resistance to *E. coli* C600. Transconjugants were identified by the ability to grow in the presence of ampicillin and rifampicin (Figure 14). The donor and recipient had no growth observed.

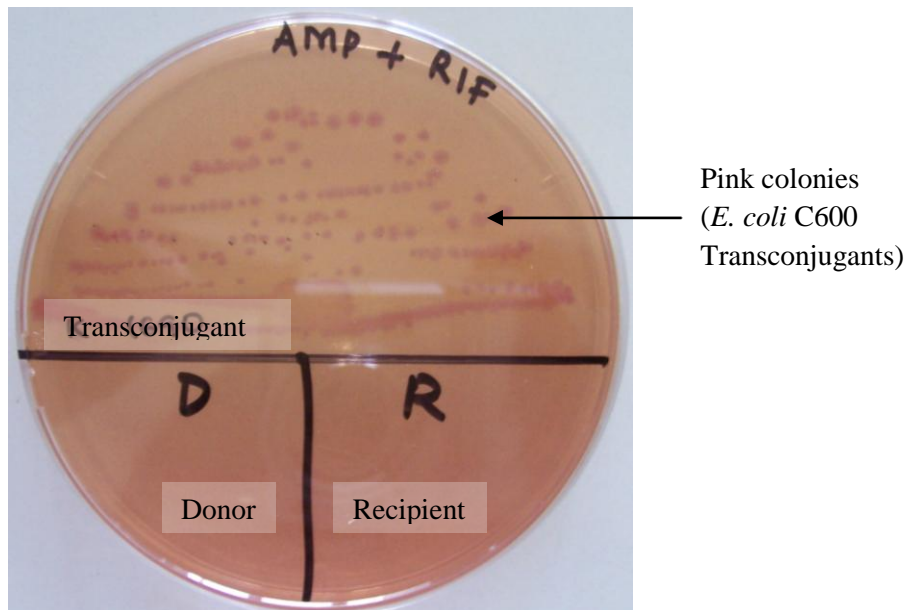


Figure 14. Transconjugant selection

Two *E. coli* and three *Klebsiella* spp transferred resistance to *E. coli* C600. Confirmation by disk susceptibility test on the transconjugants indicated presence of ampicillin resistance in all the five isolates. In addition, two *Klebsiella* transconjugants and one *E. coli* transconjugant were additionally resistant to nitrofurantoin. No plasmids were detected from the transconjugants.

CHAPTER FIVE

5.0 DISCUSSION

In the present study water samples enriched by incubation at 44°C before culturing on agar plates at 37°C showed better recovery rates for *Klebsiella* spp and *Escherichia coli*. Different studies have shown variation in the optimal growth temperatures for different bacteria. Increasing the incubation temperature to 44°C, the process used in standard methodology to distinguish *E. coli* from coliforms (Environmental Protection Agency, 1989) has also been shown to inhibit growth of heterotrophs (Packer *et al.*, 1995). The better recovery after enrichment at 44°C observed in the present study is therefore, likely due to the inhibition of heterotrophs thus providing a less competitive ground for the growth of the two bacterial types.

Klebsiella species comprised of 29% (Table 3) of the total organisms isolated. Most (66% of the *Klebsiella* isolates, Table 4) were isolated from sewage systems which are within normal expectations considering that *Klebsiella* species are considered to be among the fecal coliform organisms (Calva *et al.*, 1996; Munoz *et al.*, 2006). Their high presence in sewage is thus likely to be from fecal sources. Previous studies have also shown occurrence of *Klebsiella* species in hospital foods and kitchen which were identified as the source of organisms in the bowels of patients (Cooke *et al.*, 1980). In the hospital kitchen study; food was sampled as it entered the kitchen and of 136 salads constituents sampled before preparation 10.3% had *Klebsiella* species while 46% of raw meat samples contained *Klebsiella* species. Different serotypes were identified and they were similar to the serotypes isolated

from the hospitalized patients (Cooke *et al.*, 1980). With increasing reports of *Klebsiella* species being involved in opportunistic infections and developing new mechanisms of antibiotic resistance such as extended spectrum beta-lactamase production (Bergogne-Bérézin, 1995), its presence in bore holes and streams/rivers is of significant importance. The water sources may easily be a source of *Klebsiella* species contaminating foods in households and possibly in hospitals.

Prevalence of *E. coli* was highest in sewage (56%) followed by streams/rivers (34%) and drastically reduced in bore holes (10%) (Table 4). Similar to *Klebsiella* species, *E. coli* is also in the category of fecal coliforms (Calva *et al.*, 1996; Munoz *et al.*, 2006), hence its high presence especially in sewage samples is expected. Some of the streams were contaminated with chemical effluents which may explain the reason for reduction in *E. coli* and *Klebsiella* species isolation. Other studies have shown the impacts of chemical wastes on microbial ecology of different ecosystems. For instance, differences in bacterial community structure have been shown to be largely driven by pH and conductivity. In support of this, the bacterial communities in pristine streams with naturally low pHs (pH 5.5) have been shown to be more similar to the bacterial communities in streams impacted by Acid Marine Drainage than to the bacterial communities in other pristine sites with near-neutral stream water (pH 6.5) (Lear *et al.*, 2009). Studies on *Vibrio cholerae* have revealed evidence that *V. cholerae* is an autochthonous inhabitant of brackish water and estuarine systems (Colwell *et al.*, 1977; Islam *et al.*, 1990). These studies also showed that a close association between plankton and *V. cholera* influences the survival of these bacteria.

The survival of this organism between cholera epidemics and its isolation from such waters, even in non-epidemic regions, can be explained on the basis of its persistence in a viable, though often nonculturable, state. Several factors, therefore, render the aquatic environment ideal for the survival and growth of *V. cholerae*, which, in their turn, are important factors in the epidemiology and pathogenesis of cholera (Patel and Isaacson, 1999). Likewise, *E. coli* and *Klebsiella* species may also be affected by the pH and vegetation present in the streams and rivers which are greatly affected by the chemical effluents.

Most bore holes were also treated though the treatment methods could not be established. This may have had an impact on the reduction of *E. coli* and *Klebsiella* species isolates obtained from bore holes. Other studies have shown the effects of decontaminants on growth of microorganisms. For instance, when mung bean seeds are treated with hot water at 85°C for 40 sec followed by dipping in cold water for 30 sec and lastly soaked into chlorine water (2000 ppm) for 2 hours, no viable *Escherichia coli* O157:H7 and *Salmonella enteritidis* are found in the enrichment medium and during the sprouting process (Bari *et al.*, 2010). Disinfection of wastewater by chlorination has also been found to be 100% effective in removal of fecal coliforms (Pant and Mittal, 2007), although, other studies have also shown that pH affects the effectiveness of organic chloramines. This is supported by a study where no evidence of inactivation was observed at pH 8.1 for any of the tested organic N-chloramines (Amiri *et al.*, 2009). This may explain why some bore holes

may have been treated but water collected from the bore holes had *E. coli* and *Klebsiella* species isolated.

Klebsiella species (68%) and *E. coli* (62%) had high rates of resistance to nucleic acid inhibitor, sulfamethoxazole. High rates of resistance to both ampicillin and trimethoprim/sulfamethoxazole has been reported in many countries in Europe prompting a close review of treatment success rates in settings in which they are commonly used in empiric therapy (Kahlmeter, 2000; Hooton *et al.*, 2004). In addition, it is assumed that commensal and environmental bacteria have comparatively lower antibiotic resistance prevalence than their counterparts isolated from clinical samples, but many studies have shown that the resistance prevalence for sulfamethoxazole/ trimethoprim, ciprofloxacin and amoxicillin are of the same order of magnitude in clinical and environmental isolates (Stelling *et al.*, 2005). It is therefore; not surprising that in Kenya the resistance to sulphamethoxazole is very high among environmental isolates of *Klebsiella* and *E. coli* and including the unidentified isolates. Similarly *E. coli* had high resistance to ampicillin though *Klebsiella* had a significantly higher resistance level than *E.coli* (Figure 8). The high resistance level is similar to what was previously observed in Europe (Kahlmeter, 2000; Hooton *et al.*, 2004). The recommendation in Europe to reconsider the use of ampicillin and sulphamethoxazole in empiric therapy would be useful in Kenya as well.

Resistance to aminoglycosides was observed where, 38% of *E. coli* and 58% of *Klebsiella* were resistant. Resistance to tetracycline was the highest among the aminoglycosides (Figure 8). *Klebsiella* (42%) and *E. coli* (28%) had lower resistance levels to chloramphenicol when compared to tetracycline. This observation of chloramphenicol being more effective than tetracycline is similar to findings of a study that attempted to evaluate the use of old antibiotic compounds in treatment of biofilm forming multidrug resistant organisms (Liaqat *et al.*, 2009). The study observed a highly significant decrease ($P < 0.01$) in biofilm formation by treatment with chloramphenicol compared to tetracycline (Liaqat *et al.*, 2009). Tobramycin would be the best drug of choice based on the findings of the present study especially for use against *Klebsiella* species (0% resistance; Figure 7).

Resistance to β – lactam antibiotics by *Klebsiella* and *E. coli* was below 20% for most antibiotics tested. Exceptions were observed in *Klebsiella* where 40% were resistant to cefoxitin and in *Klebsiella* and *E. coli* where 80% and 45%, respectively, were resistant to ampicillin (Figure 8). With environmental *Klebsiella* isolates having a high resistance to cefoxitin, it indicates a concern based on the findings of the current study. Other studies have also shown reduced susceptibility to cefoxitin in the *Enterobacteriaceae* which may be an indicator of AmpC activity, but cefoxitin resistance may also be mediated by alterations to outer membrane permeability (Hernández-Allés *et al.*, 2000). The capability to detect AmpC is important to improve the clinical management of infections and provide sound epidemiological data, but at present, there are no standardized phenotypic screening methods that are

readily available to microbiology laboratories (Tan *et al.*, 2009). Though, no standardized method is available for detection of AmpC, in the present study double-disk synergy method was used for detection of ESBLs (Jarlier *et al.*, 1988) and there was no organism found to be positive. Bearing in mind that AmpC and ESBLs are both β -lactamase enzymes the resistance to beta lactams in the present study may likely be mediated by alterations to the outer membrane permeability as earlier suggested (Hernández-Allés *et al.*, 2000).

The prevalence of Integron class 1 genes was significantly higher in *Klebsiella* species over *E. coli*. Several studies have reported the presence of class 1 integrons in gram-negative bacteria from patients with hospital-acquired infections (i.e., *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *E. coli*, and *Citrobacter freundii* (Jones *et al.*, 1997) and in *Klebsiella oxytoca* strains responsible for nosocomial outbreaks (Preston *et al.*, 1997). Resistance to streptomycin, sulphonamides and trimethoprim has been identified in gene cassettes of the class 1 integron (Jones *et al.*, 1997). The findings agree with those of another study where the content of class 1 integrons in *V. cholerae* strains from Thailand and in members of the family *Enterobacteriaceae* and *Pseudomonas* spp., had gene cassettes encoding for resistance to aminoglycosides, trimethoprim, and β -lactamases (Dalsgaard *et al.*, 2000). In the current study the samples tested for presence of Int 1 gene were preselected from a list of multidrug resistant isolates which were resistance to aminoglycosides, β -lactamases and nucleic acids inhibitors. Integron 1 is hence likely to be playing a role in resistance of the isolates in the present study. No Integron class 2 was observed

from any of the isolates positive for Int 1 gene. This observation is highly possible considering that class 1 integrons are the most abundant and are found in a diverse range of other mobile elements such as transposons and plasmids. However, class 2 integrons are also found in 4 to 20% of uropathogenic *Escherichia coli* strains as well as in other human pathogens, other animal pathogens and various commensal bacteria (Solberg *et al.*, 2006; Ranjbar *et al.*, 2007). In all of these cases, though, where examined, the *intI2* gene is inactive by virtue of possessing a premature in-frame stop codon (Márquez *et al.*, 2008). However, two *E. coli* strains, designated 3843 and 8157, from separate individuals have generated a sequence implying a functional *intI2* gene presence (Márquez *et al.*, 2008).

All the 7 isolates positive for aminoglycosides modifying enzyme *aac(6')-Ib-cr* gene were also positive for Int 1 gene. Surprisingly they were all resistant to sulphamethoxazole, several aminoglycosides, and either intermediate or resistant to norfloxacin and ciprofloxacin. The *cr* variant of *aac(6')-Ib* encodes an aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin by *N*-acetylation of its piperazinyl amine (Robicsek *et al.*, 2006). The gene may also be the course of resistance to fluoroquinolones in the present study.

In addition, it was previously considered that plasmid-mediated resistance to quinolones could not be easily developed *in vivo* due to recessivity of a mutant gyrase gene compared with dominance of a wild-type chromosome encoded allele and to the plasmid curing effect of quinolones (Courvalin, 1990; Gómez-Gómez *et al.*, 1997). The first plasmid-mediated quinolone resistance protein Qnr (later termed

QnrA) was identified from urine in a *Klebsiella pneumoniae* isolate in Birmingham, AL, USA, in 1994 (Martínez-Martínez *et al.*, 1998). Though not determined in the present study, the QnrA gene may also be present in the isolates resistant to fluoroquinolones in the present study.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

- The findings in this study show the prevalence of *Escherichia coli* and *Klebsiella* spp. is 40.6% and 29.4% respectively, in environmental water sources.
- Plasmids, Integron 1 genes and aminoglycoside modifying enzyme genes are present in both environmental isolates of *E. coli* and *Klebsiella* species and they may be responsible for resistance to quinolones, aminoglycosides and beta lactam antibiotics tested.
- The genetic mechanism of resistance to ampicillin by *Klebsiella* species and *E. coli* was transferrable to *E. coli* C600 in a few instances as evidenced by positive conjugation experiments. Hence, the two organisms may transfer resistance to other organisms including those that are pathogenic.

6.2 Recommendations

- The prevalence of *Escherichia coli* and *Klebsiella* spp. being 40.6% and 29.4% respectively, in environmental water sources and both showing presence of resistance genes there is need for continuous monitoring of resistance patterns. There also a need of studies comparing clinical isolates with the environment isolates to identify the source of the resistant genes.
- The isolates *E.coli* and *klebsiella* spp. were able to transfer resistance to *E.coli* C600. Therefore, it would be important to carry out a study on resistance patterns of other opportunistic bacteria from environmental sources. This would identify the risk they pose in the event of infecting immuno-compromised patients.

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APPENDICES

Appendix A. Plasmid extraction reagents

Solution I

50 mMol glucose

25 mMol Tris Cl (pH 8.0)

10 mMol EDTA (pH 8.0)

Solution I can be prepared in batches of approximately 100 ml, autoclaved for 15 min at 10 lb/sq. in. on liquid cycle and stored at 4°C.

Solution II

0.2 N NaOH (freshly diluted from a 2 N stock).

1% SDS

Solution III

5 M potassium acetate 60 ml

glacial acetic acid 11.5 ml

H₂O 28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetic acid.

Appendix B. Tris-borate Electrophoresis buffer (TBE)

Buffer	Working Solution	Concentrated stock solution (per litre).
Tris-borate (TBE)	0.5 X: 0.045 M Tris-borate 0.001 M EDTA	5 X: 54g Tris base 27.5g boric acid 20ml 0.5 M EDTA (pH 8.0)
		Filter sterilize, store at RT

Appendix C. Bacteria media preparation

MACCONKEY AGAR

Formula

Gelatin peptone 17.0

Bile salts No: 3 1.5g

Lactose 10.0g

Neutral red 0.03g

Sodium chloride 5.0g

Peptone mixture 3.0g

Bacteriological agar 13.5g

Preparation

Suspend 50 g in 1 litre of distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 45-50 °C and pour in 15 – 20 ml amounts into Petri dishes.

Use: For selection of enteric bacteria.

MUELLER HINTON AGAR.

Formula

Beef infusion 300.0g

Acid hydrolysate of casein 17.5g

Starch 1.5g

Agar 17.0g

Preparation

Dissolve 42 g in 1 litre of distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15 minutes

Use: For sensitivity testing.

TRYPTIC SOY BROTH

Formula

Pancreatic digest of casein 17.0g

Papaic digest of soy meal 3.0g

Sodium chloride 5.0g

Dipotassium phosphate 2.5g

Dextrose 2.5g

Preparation

Dissolve 30g in 1 litre of distilled water. Mix and distribute into final containers. Autoclave at 121°C for 15 minutes.

Use: General purpose broth media.

BRAIN HEART INFUSION BROTH

Formula

Calf brain infusion solids 12.5g

Beef heart infusion solids 5.0g

Sodium chloride 5.0g

Proteose peptone 10.0g

Glucose 2.0g

Disodium phosphate 2.5g

Preparation

Dissolve 37g in 1 litre of distilled water. Mix and distribute into final containers. Autoclave at 121°C for 15 minutes.

Use: General purpose broth media.

Appendix D. Biochemical tests

1. Urease Test.

The urease test helps in the identification of certain species of Enterobacteraceae. Urease enzyme hydrolyses substrate urea into ammonia, water, and carbon dioxide. The presence of the enzyme is determined by inoculating an organism to broth or agar that contains urea as the primary carbon source and detecting the production of ammonia. Ammonia increases the pH of the medium so its presence is readily detected using a common indicator of metabolic process and, because pH indicators change color which increases (alkalinity) or decrease (acidity) in the medium's Ph, they are commonly used in many identification tests schemes. to pink red. The test is used to determine the ability of an organism to produce the enzyme urease, which hydrolyses urea. Hydrolysis of urea produces ammonia and carbon dioxide. The formation of ammonia alkalinizes the medium, and the Ph shift is detected by the color change of phenol red indicator from light orange at pH 6.8 to magenta pH 8.1.

Procedure

- 1) Streak the surface of a urea agar slant with a portion of a well isolated colony or inoculate slant with 1 to 2 drops from an overnight brain heart infusion broth culture.
- 2) Leave the cap on loosely and incubate tube at 35-37°C in ambient air for 48 hours to 7 days.

Expected results

Positive: Change in color of slant from light orange o magenta

Negative: No color change in media.

Quality control:

Known urea positive, *Proteus vulgaris* and urea negative *Escherichia coli* should always be included when doing the test.

Both *S.typhimurium* and *S.enteritidis* are urease negative.

2. Citrate utilization test

This test is used o determine the ability of an organism to utilize sodium citrate as its only carbon source and inorganic ammonium salts as its nitrogen source. Bacteria that can grow on this medium turn bromothymol blue indicator from green to blue.

Method

Inoculate Simmons citrate agar lightly on the slant by touching the tip of a needle to a colon that is 18-24 hours old. Broth culture is not recommended as the inoculum will be too heavy.

Incubate at 35-37°C for upto 7 days.

Observe for development of blue color, denoting alkalization.

Expected Results

Positive: Growth on the medium, with or without change in color of the indicator

The color change of the indicator is due to acid or alkali production by the test organism as it grows on the medium. Growth usually results in the bromothymol blue indicator, turning from green to blue.

Quality control:

Known citrate positive; *Klebsiella pneumonia* and citrate negative; *Escherichia coli*.

Both *S. enteritidis* and *S. typhimurium* do not utilize citrate as their source of carbon hence they do not grow in this medium.

3. Indole test

Bacteria that produce the enzyme tryptophanase are able to degrade the amino acid tryptophan into pyruvic, ammonia and indole. Indole is detected by combining with an indicator, aldehyde (1 % paradimethylaminoaldehyde), that results in a blue color formation. This test is used in many identification schemes, especially to presumptively *Escherichia coli*, the many gram negative bacilli most commonly encountered in diagnostic bacteriology.

This test is used to determine the ability of an organism to split tryptophan to form the compound indole.

Method

Inoculate tryptophane broth with a drop from a 24 hour brain heart infusion broth culture.

Incubate at 35°C in ambient air for 24- 48 hours.

Add 0.5 ml. of Kovacs reagent

Expected results

Positive: Pink to wine colored red ring after addition of appropriate reagent

Negative: No color change after addition of the appropriate reagent.

S. Typhimurium and *S. enteritidis* are indole negative

4. Triple Sugar Iron (TSI)

Principle

TSI is used to determine whether a gram negative bacilli utilizes glucose and lactose or sucrose fermentatively and forms hydrogen sulfide (H₂S). TSI contains 10 parts lactose: 10 parts sucrose: 1 part glucose and peptone. Phenol red and ferrous sulfate serve as indicators of acidification and (H₂S) formation respectively. When glucose is utilized by a fermentative organism, the entire media becomes acidic (yellow) in 18 to 24 hours. The butt remains acidic after the recommended 18 to 24 hours incubation period because of the presence of organic acids resulting from the fermentation of glucose under anaerobic conditions in the butt of the tube. The slant however reverts to the alkaline (red) state because of oxidation of the fermentation products under aerobic conditions in the slant. This change is a result of the formation of carbon dioxide and water and the oxidation of peptones in the medium to alkaline amines. When in addition to glucose, lactose and/or sucrose are fermented, the large amount of fermentation products formed on the slant will more than neutralize the alkaline amines and render the slant acidic (yellow), provided the

reaction is read in 18 to 24 hours. Reactions in TSI should not be read beyond 24 hours of incubation, because aerobic oxidation of the fermentation products from lactose and/or sucrose does not proceed and the slant will eventually revert to the alkaline state. The formation of carbon dioxide and water (hydrogen gas) is indicated by the presence of bubbles or cracks in the agar or by separation in the tube. The production of H₂S requires an acidic environment and is manifested by blackening of the butt of the medium.

Method

- 1) With a straight inoculation needle, touch the top of a well isolated colony.
- 2) Inoculate TSI by first stabbing through the center of the medium the bottom of the tube and then streaking the surface of the agar slant.
- 3) Leave the cap on loosely and incubate the tube at 35° - 37°C in ambient air for 18 -24 hours.

Expected Results

Alkaline slant/no change in the butt (K/NC = glucose, lactose, and sucrose non utilizer: this may also be recorded as K/K (alkaline slant /alkaline butt).

Alkaline slant/acid butt (K/A) = glucose fermentation only.

Acid slant/acid butt (A/A) = glucose, sucrose, and /or lactose fermenter.

Note: A black precipitate in the butt indicates production of ferrous sulfide and H₂S gas (H₂S +)

Bubbles or cracks in the tube indicate the production of carbon dioxide or hydrogen. Drawing the circle around the A for acid butt, this is A/A; usually this means the organism ferments glucose and sucrose, glucose and lactose, or sucrose and lactose, with the production of gas.

Quality control

A: *Escherichia coli*

K/A H₂S+: *Salmonella typhi*, *S.typhimurium*

K/NC: *Pseudomonas aeruginosa*

S.typhimurium produces H₂S while *S.enteritidis* does not

5. TE (Tris hydromethyl amino methane EDTA (ethylene diamine tetraacetic acid)

For 100 ml TE

10 mM Tris-HCl pH 8.0

1 mL 1 M Tris-HCl, pH 8.0

1 mM EDTA pH 8.0

0.2 ml 0.5M EDTA pH 8.0

Q.S to 100 ml with H₂O

Filter sterilize, store at RT

Appendix E. Clinical and laboratory standards institute standards (CLSI)

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Table 2A
Enterobacteriaceae
M2-Disk Diffusion

Table 2A. Zone Diameter Interpretive Standards and Equivalent Minimal Inhibitory Concentration (MIC) Breakpoints for Enterobacteriaceae

Testing Conditions	
Medium:	Mueller-Hinton agar
Inoculum:	Growth method or direct colony suspension, equivalent to a 0.5 McFarland standard
Incubation:	35 ± 2 °C; ambient air; 16 to 18 hours

Minimal QC Recommendations (See Table 3 for acceptable QC ranges.)

Escherichia coli ATCC® 25922
Escherichia coli ATCC® 35218 (for β-lactam/β-lactamase inhibitor combinations)

General Comments

(1) When fecal isolates of *Salmonella* and *Shigella* spp. are tested, only ampicillin, a quinolone, and trimethoprim-sulfamethoxazole should be reported routinely. In addition, chloramphenicol and a third-generation cephalosporin should be tested and reported for extraintestinal isolates of *Salmonella* spp.

NOTE: Information in boldface type is considered tentative for one year.

Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter, Nearest Whole mm			Equivalent MIC Breakpoints (µg/mL)		Comments
			R	I	S	R	S	
PENICILLINS								
A	Ampicillin	10 µg	≤ 13	14-16	≥ 17	≥ 32	≤ 8	(2) Class representative for ampicillin and amoxicillin.
B	Mezlocillin or piperacillin	75 µg	≤ 17	18-20	≥ 21	≥ 128	≤ 16	
B	Ticarcillin	100 µg	≤ 17	18-20	≥ 21	≥ 128	≤ 16	
B	Ticarcillin	75 µg	≤ 14	15-19	≥ 20	≥ 128	≤ 16	
U	Carbenicillin	100 µg	≤ 19	20-22	≥ 23	≥ 64	≤ 16	
U	Mecillinam	10 µg	≤ 11	12-14	≥ 15	≥ 32	≤ 8	(3) For use against <i>E. coli</i> urinary tract isolates only.
β-LACTAM/β-LACTAMASE INHIBITOR COMBINATIONS								
B	Amoxicillin-clavulanic acid or ampicillin-sulbactam	20/10 µg	≤ 13	14-17	≥ 18	≥ 32/16	≤ 8/4	
B	ampicillin-sulbactam	10/10 µg	≤ 11	12-14	≥ 15	≥ 32/16	≤ 8/4	
B	Piperacillin-tazobactam	100/10 µg	≤ 17	18-20	≥ 21	≥ 128/4	≤ 16/4	
B	Ticarcillin-clavulanic acid	75/10 µg	≤ 14	15-19	≥ 20	≥ 128/2	≤ 16/2	

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Table 2A. (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter, Nearest Whole mm			Equivalent MIC Breakpoints (µg/mL)		Comments	
			R	I	S	R	S		
CEPHEMS (PARENTERAL) (Continued)									
B	Cefamandole or cefonicid or cefuroxime sodium (parenteral)	30 µg	≤ 14	15-17	≥ 18	≥ 32	≤ 8		
B		30 µg	≤ 14	15-17	≥ 18	≥ 32	≤ 8		
B		30 µg	≤ 14	15-17	≥ 18	≥ 32	≤ 8		
B	Cefepime	30 µg	≤ 14	15-17	≥ 18	≥ 32	≤ 8		
B	Cefmetazole	30 µg	≤ 12	13-15	≥ 16	≥ 64	≤ 16		
B		75 µg	≤ 15	16-20	≥ 21	≥ 64	≤ 16		
B		Cefotetan	30 µg	≤ 12	13-15	≥ 16	≥ 64	≤ 16	
B		Cefoxitin	30 µg	≤ 14	15-17	≥ 18	≥ 32	≤ 8	
B	Cefotaxime or ceftizoxime or ceftriaxone	30 µg	≤ 14	15-22	≥ 23	≥ 64	≤ 8	(9) Cefotaxime and ceftriaxone should be tested and reported on isolates from CSF in place of cephalothin and cefazolin. See comment (5).	
B		30 µg	≤ 14	15-19	≥ 20	≥ 32	≤ 8		
B		30 µg	≤ 13	14-20	≥ 21	≥ 64	≤ 8		
C	Ceftazidime	30 µg	≤ 14	15-17	≥ 18	≥ 32	≤ 8	See comment (5).	
O	Moxalactam	30 µg	≤ 14	15-22	≥ 23	≥ 64	≤ 8		
CEPHEMS (ORAL)									
B	Cefuroxime axetil (oral)	30 µg	≤ 14	15-22	≥ 23	≥ 32	≤ 4		
U	Loracarbef	30 µg	≤ 14	15-17	≥ 18	≥ 32	≤ 8	(10) Because certain strains of <i>Citrobacter</i> , <i>Providencia</i> , and <i>Enterobacter</i> spp. have been reported to give false-susceptible results with cefdinir and loracarbef disks, strains of these genera should not be tested and reported with these disks.	
O	Cefaclor	30 µg	≤ 14	15-17	≥ 18	≥ 32	≤ 8		
O	Cefdinir	5 µg	≤ 16	17-19	≥ 20	≥ 4	≤ 1	See comment (10).	
O	Cefixime	5 µg	≤ 15	16-18	≥ 19	≥ 4	≤ 1	(11) Not applicable for testing <i>Morganella</i> spp.	
O	Cefpodoxime	10 µg	≤ 17	18-20	≥ 21	≥ 8	≤ 2	See comments (5) and (11).	
O	Cefprozil	30 µg	≤ 14	15-17	≥ 18	≥ 32	≤ 8	(12) Because certain strains of <i>Providencia</i> spp. have been reported to give false-susceptible results with cefprozil disks, strains of this genus should not be tested and reported with this disk.	
Inv.	Cefetamet	10 µg	≤ 14	15-17	≥ 18	≥ 16	≤ 4	See comment (11).	
Inv.	Ceftibuten	30 µg	≤ 17	18-20	≥ 21	≥ 32	≤ 8	(13) Indicated for urine isolates only.	
CARBAPENEMS									
B	Ertapenem	10 µg	≤ 15	16-18	≥ 19	≥ 8	≤ 2		
B	Imipenem or meropenem	10 µg	≤ 13	14-15	≥ 16	≥ 16	≤ 4		
B		10 µg	≤ 13	14-15	≥ 16	≥ 16	≤ 4		
MONOBACTAMS									
C	Aztreonam	30 µg	≤ 15	16-21	≥ 22	≥ 32	≤ 8	See comment (5).	

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Table 2A. (Continued)

Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter, Nearest Whole mm			Equivalent MIC Breakpoints (µg/mL)		Comments
			R	I	S	R	S	
AMINOGLYCOSIDES								
(14) WARNING: For <i>Salmonella</i> spp. and <i>Shigella</i> spp., aminoglycosides may appear active <i>in vitro</i> , but are not effective clinically and should not be reported as susceptible.								
A	Gentamicin	10 µg	≤ 12	13-14	≥ 15	≥ 8	≤ 4	
B	Amikacin	30 µg	≤ 14	15-16	≥ 17	≥ 32	≤ 16	
C	Kanamycin	30 µg	≤ 13	14-17	≥ 18	≥ 25	≤ 6	
C	Netilmicin	30 µg	≤ 12	13-14	≥ 15	≥ 32	≤ 12	
C	Tobramycin	10 µg	≤ 12	13-14	≥ 15	≥ 8	≤ 4	
O	Streptomycin	10 µg	≤ 11	12-14	≥ 15	-	-	
TETRACYCLINES								
C	Tetracycline	30 µg	≤ 14	15-18	≥ 19	≥ 16	≤ 4	(15) Organisms that are susceptible to tetracycline are also considered susceptible to doxycycline and minocycline. However, some organisms that are intermediate or resistant to tetracycline may be susceptible to doxycycline or minocycline or both.
O	Doxycycline	30 µg	≤ 12	13-15	≥ 16	≥ 16	≤ 4	
O	Minocycline	30 µg	≤ 14	15-18	≥ 19	≥ 16	≤ 4	
FLUOROQUINOLONES								
(16) Fluoroquinolone-susceptible strains of <i>Salmonella</i> that test resistant to nalidixic acid may be associated with clinical failure or delayed response in fluoroquinolone-treated patients with extraintestinal <i>salmonellosis</i> . Extraintestinal isolates of <i>Salmonella</i> should also be tested for resistance to nalidixic acid. For isolates that test susceptible to fluoroquinolones and resistant to nalidixic acid, the physician should be informed that the isolate may not be eradicated by fluoroquinolone treatment. A consultation with an infectious disease practitioner is recommended.								
B	Ciprofloxacin or levofloxacin	5 µg	≤ 15	16-20	≥ 21	≥ 4	≤ 1	
B		5 µg	≤ 13	14-16	≥ 17	≥ 8	≤ 2	
U	Gatifloxacin	5 µg	≤ 14	15-17	≥ 18	≥ 8	≤ 2	
B	Gemifloxacin	5 µg	≤ 15	16-19	≥ 20	≥ 1	≤ 0.25	(17) FDA-approved for <i>Klebsiella pneumoniae</i> .
U	Lomefloxacin or norfloxacin or ofloxacin	10 µg	≤ 18	19-21	≥ 22	≥ 8	≤ 2	
U		10 µg	≤ 12	13-16	≥ 17	≥ 16	≤ 4	
U		5 µg	≤ 12	13-15	≥ 16	≥ 8	≤ 2	
O	Enoxacin	10 µg	≤ 14	15-17	≥ 18	≥ 8	≤ 2	
O	Grepafloxacin	5 µg	≤ 14	15-17	≥ 18	≥ 4	≤ 1	
Inv.	Fleroxacin	5 µg	≤ 15	16-18	≥ 19	≥ 8	≤ 2	

Table 2A
Enterobacteriaceae
M2-Disk Diffusion