PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF UROPATHOGENIC *ESCHERICHIA COLI* ISOLATES FROM PATIENTS IN SELECTED HOSPITALS IN KENYA

BY

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DECLARATION

I declare that this thesis is my original work and has not been presented to any other university or institution for a degree or any other award.

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ABSTRACT

Uropathogenic *Escherichia coli* (UPEC) is the major cause of community and hospital-acquired UTI cases.However,there is increasing resistance to antibiotics used in the treatment of UPEC infections. *Escherichia coli* can be classified into eight phylo-groups, which differ in antimicrobial resistance. Production of extended-spectrumβ-lactamases (ESBLs) and plasmid-mediated AmpC (pAmpC) β-lactamases are important mechanisms of resistance toβ-lactams antibiotics. In Kenya, β-lactams are used increasingly in the treatment of UTIs, most without a sensitivity test, a practice that may result in increased resistance due to misuse of the drugs. In 2010,the occurrence of ESBL in UPEC isolates was 27%. Since then,few studies on genetic determinants of resistance to β-lactams have been carrying out in Kenya. Moreover, the phylo-groups of UPEC in Kenya have not been determined due to lack of continuous surveillance studies. This study, therefore, undertook to determine the occurrence of Extended-Spectrum β-lactamases (ESBLs) and plasmid-mediated β-lactamases (pAmpC), the genetic determinants of resistance to β-lactams, and phylo-grouping of UPEC isolates in Kenya. This retrospective, laboratory-based survey study is nested in a parent protocol (KEMRI# 2767/WRAIR#2089) that has been running from 2015 to date. In this study,95 archived UPEC isolates obtained from patients in different hospitals in Kenya were purposely selected from the database at the start of the study. Data were analyzed in the statistical package for social sciences (SPSS) version 20, where the frequencies and percentages for occurrence of ESBLs and pAmpC β-lactamases plus phylo-groups for UPEC isolates were obtained through cross-tabulation in descriptive statistics. In this study, Nairobi, Kisumu, Kericho, Kilifi, and Kisii had 39/95;(41.1)%, 28/95;(29.5)%, 14/95;(14.7)%, 10/95;(10.5)% and 4/95;(4.2)% UPEC isolates respectively. The ESBLs were characterized by phenotype and genotype using a combined disk diffusion test and real-time PCR for identification of the bla_{TEM}, $bla_{\text{CTX-M,}}$ and bla_{SHV}β-lactamase genes. Twenty three out of 95 UPEC isolates were positive for ESBL production.Nairobi, Kisumu, Kilifi, Kericho, and Kisii Counties had 12/23;(52.2)%, 5/23;(21.7)%, 4/23;(17.4)%, 2/23;(8.7)% and zero ESBL producers respectively.AmpC screening was done using the cefoxitin disk diffusion test and screen positives phenotypically confirmed by the disk approximation test. Five out of 23 ESBL producers were cefoxitin resistant. However, all five isolates were negative for AmpC production by phenotypic and genotypic methods. The predominant ESBL genes were $bla_{\text{CTX-M,}}$ and bla_{TEM} each with 95.6% followed by bla_{SHV}21.7%. Sixteen isolates (69.6%) had both $bla_{\text{CTX-M}}$andbla_{TEM} genes, whereas five isolates (21.7%) had all three genes (bla_{TEM}, $bla_{\text{CTX-M,}}$ and bla_{SHV}. A total of 93 out of 95 UPEC isolates were assigned to 5 of the eight phylo-groups by the quadruplex PCR methods of which thephylo-groups B231/95; (32.6%) and D 30/95; (31.6%) predominated.The results of this study could guide clinicians in the identification of the correct antibiotics forpatients with UTIs. Further,the results canbe used in mapping the phylo-groups associated with ESBL resistance genes to prevent there spread.This study recommendscontinuous surveillance studies to monitor the genes associated with ESBL resistance, futher tests to identify novel and emerging phylo-types such as the unassigned isolate

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LIST OF ABBREVIATIONS AND ACRONYMS

AFLP	:	Amplified Fragment Length Polymorphism
AST	:	Antibiotic Susceptibility Testing
CARB	:	Combating Antibiotic-Resistant Bacteria
CAZ	:	Ceftazidime
CTX	:	MCefotaximase-Munich
CDDT	:	Combination Disc Diffusion Test
CLA	:	Clavulanic acid
CLSI	:	Clinical Laboratory Standard Institute
CT	:	Cycle Threshold
DAEC	:	Diffusely Adherent *E. coli*
DNA	:	Deoxyribonucleic acid
DFMH	:	Defense Force Memorial Hospital
EAEC	:	Enteroaggregative *E. coli*
EHEC	:	Enterohemorrhagic *E. coli*
EIEC	:	Entero-invasive *E. coli*
ETEC	:	Enterotoxigenic *E. coli*
ESBL	:	Extended Spectrum β-lactamases
EXPEC	:	Extra-intestinal Pathogenic *E. coli*
GEIS	:	Global Emerging Infections Surveillance and Response System
HSPB	:	Human Subjects Protection Branch
HUS	:	Hemolytic Uremic Syndrome
IRB	:	Institutional Review Board
IPEC	:	Intestinal Pathogenic *E. coli*
IPR	:	Intellectual Property Rights
KCH	:	Kericho County Hospital
KCH	:	Kisumu County Hospital
KEMRI	:	Kenya Medical Research Institute
MIC	:	Minimum Inhibition Concentration
MLST	:	Multilocus Sequence Typing
MVLA	:	Multiple-locus variable tandem repeat number analysis

MCH	:	Malindi County Hospital
NMEC	:	Neonatal Meningitis-Associated *E. coli*
PAMPC	:	Plasmid-Mediated AmpC
PCR	:	Polymerase Chain Reaction
PI	:	Principle Investigator
PFGE	:	Pulsed-Field Gel Electrophoresis
RNA	:	Ribonucleic acid
RPM	:	Revolution Per Minute
SEPEC	:	Sepsis-causing *E. coli*
SERU	:	Scientific Ethics Review Unit
SDG	:	Sustainable development goal
SHV	:	Sulfhydryl Variable
SMART	:	Antimicrobial Resistance Trends
STEC	:	Shiga toxin–producing *E. coli*
TEM	:	Temoinera
Trna	:	Trasfer ribonucleic acid
UHC	:	Universal health coverage
USA	:	United States of America
UTIs	:	Urinary Tract Infections
UPEC	:	Uropathogenic *Escherichia coli*
VTEC	:	Verocytotoxigenic *E. coli*
WRAIR	:	Walter Reed Army Institute of Research

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CHAPTER ONE

INTRODUCTION

1.1 Background Information

Escherichia coli (*E. coli*) is a Gram-negative bacillus from the family *Enterobacteriaceae*(Sanches Trevizol *et al.,* 2018). Clinically,*Escherichia coli* is classified into three broad groups, namely: commensal, intestinal pathogenic, and extraintestinal pathogenic *E.coli*,based on their interaction with their host and site of infection(Iliyasu *et al.,* 2018). The commensal *E. coli* is part of the normal flora of the intestine(Salehzadeh & Ghauor, 2017). Intestinal pathogenic *E. coli* strains cause intestinal diseases such as diarrhea. In contrast, the extra-intestinal pathogenic *E. coli* (EXPEC)strains cause diseases such as bacteremia, neonatal meningitis, and urinary tract infections (UTIs) (Iliyasu *et al.,*2018). *Escherichia coli* strains isolated from the urinary tract are known as uropathogenic *E. coli* (UPEC).

Hospital and community-acquired urinary tract infections (UTIs) are a common cause of morbidity in persons of all age categories, worldwide(Alqasim *et al.,* 2018). Globally, it is estimated that about 150 million people suffer from UTIs per year(Koshesh *et al.,* 2017). Uropathogenic *Escherichia coli* (UPEC) is the primary etiological agent of UTIs accounting for about 70-95% of the out-patient and 30-50% in-patient cases(Al-Jamei *et al.,* 2019).The continuing emergence and spread of bacterial resistance to commonly available antibiotics used in the treatment of UTIs are of clinical concern(Onanuga *et al.,*2019).β-lactam antibiotics are the main class of drugs used to treat hospital and community-acquired UPEC infections(Dasgupta *et al.,* 2018). β-lactamases are a family of enzymes that hydrolyze the amide bond of the β-lactam ring and therefore disrupt their antimicrobial action(Dasgupta *et al.,* 2018; Bajpai *et al.,*2017). In *E.coli,*theproduction of extended-spectrum β-lactamases (ESBLs) and plasmid-mediated β-

lactamases (pAmpC) are the key mechanisms of resistance to β-lactams that results in treatment failure(Ghonaim and Moaety, 2018; Gupta *et al*., 2013). The susceptibility patterns for ESBL and AmpC β-lactamases producing organisms differ for different drugs in the β-lactamase class; therefore, detection of the mechanism of resistance in clinical isolates of UPEC is essential for guiding clinicians toward appropriate therapy.

Extended-spectrum β-lactamases (ESBL) are acquired enzymes that hydrolyze penicillins, oxy-imino-cephalosporins(e.g., ceftazidime, cefotaxime, ceftriaxone, cefepime, cefuroxime) and monobactams (e.g., aztreonam), but not cephamycins(e.g., cefoxitin, cefotetan, and carbapenems)(F. J. Giwa *et al.,* 2018). They are, however, inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam(Gupta *et al.,* 2013). The genes encoding ESBLs frequently co-exist on the same plasmid with genes conferring resistance to other antibiotics such as fluoroquinolones, cotrimoxazole, tetracycline, and aminoglycosides(Gupta *et al.,* 2013). These plasmids facilitate the horizontal transmission of resistance to multiple antimicrobials among bacteria of the same or different species in both clinical and community settings(Ruppé *et al.,*2015). Therefore, ESBL production by UPEC is a significant hindrance to the effective management of both nosocomial and community-acquired infections caused by *E.coli* and other *Enterobacteriaceae*(Van Hoek *et al.*, 2011), as it limits therapeutic options which may lead to increased morbidity and cost of health care(Desai*et al.,*2013; Giwa *et al.,* 2018).

The AmpC β-lactamases are clinically vital because they can confer resistance to penicillins, cephems (cephalosporins and cephamycins), and monobactams but not to carbapenems(Gupta *etal.*, 2013). These enzymes are not inhibited by β-lactamase inhibitors such as clavulanate, tazobactam, and sulbactam(Gupta *et al.,* 2013),but are inhibited by boronic acid and

cloxacillin(Ghonaim & Moaety, 2018).In *E. coli*, the AmpC β-lactamases are encoded by CMY-type genes located either on the chromosome or plasmids(Helmy & Wasfi, 2014). Plasmid-mediated AmpCβ-lactamases (pAmpC) have risen through the transfer of chromosomal genes for the inducible AmpC β-lactamases of other members of *Enterobacteriaceae*onto plasmids, and they display structural and functional similarity to their chromosomal origin(Khari *et al.*, 2016). The most commonly detected plasmid-mediated AmpC is CMY-type (Kiiru *et al.,* 2012), of which there are 64 plasmid-mediated variants (Helmy & Wasfi, 2014). The CMY-2 type is the most frequently recovered pAmpC β-lactamase, worldwide, from patients in hospitals and in the community(Kiiru *et al.,* 2012).Other pAmpC resistance genes include DHA variants, ACC variants, MOX variants, FOX variants, LAT variants, BIL-1, MIR-1, and ACT-1(Helmy & Wasfi, 2014).Plasmid-mediated AmpC β-lactamases production in bacteria is also a medical concern as it is associated with multidrug resistance due to the co-existence of these genes with other horizontally transferable resistance determinants for other antibiotic classes on the same plasmids (Kiiru *et al.*, 2012).

The occurrence of ESBL and AmpC β-lactamases producing bacteria such as *E. coli* has been reported to be on the rise globally. However, there is considerable variance in prevalence data from facility to facility and differences in the methodology and the designs of the study (Ghonaim and Moaety, 2018).Therefore, continuous surveillance to provide knowledge of genetic determinants of resistance to commonly prescribed antibiotics in different regions is essential to update or guide the selection of effective antibiotic therapy and to implement infection control strategies to minimize the spread of resistant bacteria. β-lactams antibiotics are often used for the empirical treatment of UTIs. Despite the changing epidemiology of β-lactamases genes, there is insufficient data on the occurrence ofthese genes in *E. coli* isolates

from UTI patients in Kenya to inform treatment practices. For instance, a study analyzed β-lactamase genes among *E.coli* strains isolated from different clinical samples in 18 years (from 1992-2010), found that the diversity of *bla* genes encountered in isolates from hospitalized and non-hospitalized patients was high and very similar(Kiiru *et al.*, 2012). In that study, the ESBL phenotype was detected in 27% of 912 isolates of which29% were carriers of $bla_{\text{CTX-M-14}}$,24% carried $bla_{\text{CTX-M-15}}$,2% $bla_{\text{CTX-M-9}}$,4%$bla_{\text{CTX-3}}$, 11%$bla_{\text{CTX-M-1}}$, 6% $bla_{\text{SHV-5}}$,3% $bla_{\text{SHV-12}}$, and 5%$bla_{\text{TEM-52}}$ genes. The pAmpC phenotype was observed in 10% of the isolates of which,82% carried $bla_{\text{CMY-2}}$, while 18% contained $bla_{\text{CMY-1}}$(Kiiru *et al.*, 2012). Overall, isolates from urine accounted for 53% and 72% of strains exhibiting ESBL and plasmid-mediated AmpC phenotypes, respectively. However, the study did not look at the diversity of *bla* genes isolated from samples collected in various geographical regions of Kenya. Moreover, in Kenya since 2010,there have been no additional studies published monitoring the trends in ESBL and AmpC producing UPEC over time in different regions in Kenya.

Clermont *et al.* (2000) developed a triplex PCR assay for phylogenetic analysis that classified *E.coli* isolates into four phylo-groups:A, B1,B2, and D, based on a combination of the three genetic markers *chuA*,*yjaA*, and the DNA fragment TspE4.C2. It was demonstrated that 80-85% of phylogenetic groups were correctly assigned (Gordon *et al.*, 2008), and therefore, this method was widely used (Doumith *et al.*, 2012).However, some *E. coli* strains were incorrectly assigned using the Clermont method(Doumith *et al.*, 2012). Consequently, a modified quadruplex PCR assay was developed by (Clermont *et al.*, 2013), which had an additional gene target, *arpA*. This new scheme classified*E.coli* isolates into one of eight different phylogenetic groups: A, B1, B2, C, D, E, F and *Escherichia* Clade I.It has been suggested that phylogenetic groups B2, D, E, and F are more likely to be involved in extra-intestinal infections compared to A, B1 or C, and Clade

isolates are thought to reside outside of the gut (Walk *et al.,* 2009). Uropathogenic *E.coli*isolates in Phylogenetic groups B2 and D are frequenty associated with antimicrobial resistance compared to those of group A or B1 (López-banda *et al.,* 2014).

The only study so far on phylogenetic analysis of *E. coli* strains from a patient with urinary tract infections, based on the quadruplex PCR method was done in Iran (Iranpour *et al.,* 2015). The study found that 25% of the isolates belonged to groups C, E, F, and clade I (Iranpour *et al.,* 2015). More studies on phylogenetic analysis using the quadruplex method are required to provide a better understanding of the*E.coli* phylo-groups. This study aims to determine the phylo-groups of *E. coli* isolates from patients with UTI based on the quadruplex PCR method, and also compare the distribution of various phylo-groups of UPEC within the study areas distributed across Kenya.

1.2 Statement of the Problem

Urinary tract infections (UTIs) is a major public health problem in both out-patient and in-patient department in health care facilities in Kenya. Uropathogenic *Escherichia coli*are the main etiological agent causing UTIs among all age groups in Kenya. Most patients with symptoms of UTIs in Kenyan hospitals are treated with antibiotics without laboratory results. This empirical approach to treatment has created massive antibiotic pressure due to drug overuse leading to the emergence and spread of multi-drug resistant bacterial strains among the patients. Production of ESBL and pAmpC β-lactamases are an important mechanism of resistance to β-lactams used in UTI treatment. Information on the occurrence of ESBLand PampC β-lactamases is limited in most developing countries in Africa, including Kenya.

Escherichia coli can be grouped into eight phylogenetic groups using the Clermont modified quadruplex PCR method. These phylogenetic groups differ in characteristics such as

antimicrobial resistance traits. In Kenya, few studies on phylo-grouping of UPEC are published. More so, there is no data to date to show the association of virulent phylo-groups and antimicrobial resistance trains in UPEC isolates.This could be due to a lack of continuous surveillance studies.More studies on phylo-grouping of UPEC isolates using the modified quadruplex method are required to provide a better understanding of the *E. coli* phylogenetic groups and their characteristics in different geographical regions in Kenya.

1.3 Objectives of the Study

1.3.1 General Objective

i. To determine the phenotypic and genotypic characterization of uropathogenic *Escherichia coli* isolates from patients in selected hospitals in Kenya.

1.3.2 Specific Objectives

i. To determine the occurrence of ESBL and AmpC resistance in UPEC isolates from patients in selected hospitals in Kenya.

ii. Todetermine the genes involved in ESBL and AmpC β-lactamases resistance in uropathogenic *E. coli.*

iii. To determine the phylo-groups of uropathogenic *E. coli* isolates obtained from patients in hospitals from different regions in Kenya.

1.4 Research Questions

i. Is there ESBL and AmpC resistance in uropathogenic *E. coli* isolates from patients in hospitals from different geographical regions in Kenya?

ii. Which genes are responsible for ESBL and AmpC resistance in uropathogenic *E. coli*?

iii. What are the phylogenetic groups of uropathogenic *E. coli* isolates obtained from patients in hospitals from different geographical regions in Kenya?

1.5 Study Significance

The results obtained from this study haveprovided information on the occurrence of ESBL and AmpC β-lactamase producers and the genes associated with their resistance to β-lactams.Thatinformation is useful in guiding the health care workers towards the selection of effective antibiotic therapy, for treating patients with UTIs after culture and sensitivity testsaccording to Clinical Laboratory Standard Institute guidelines (Gupta *et al.,* 2013). The data on phylo-grouping of UPEC isolates in Kenya canguide inthe mapping ofthe phylo-groups associated with ESBL resistance.Where these high-risk phylo-groups exist, enhanced infection control strategies could be applied to minimize the spread of resistant bacteria. Further,the report createsan avenue for amore significant study whose findingscan guide in fulfilment of better heathy for all;Sustainable Development Goal (SDG#3) of the Universal Health Care (UHC)(International Health Partnership, 2018).

CHAPTER TWO

LITERATURE REVIEW

2.1 Definition and morphology of *Escherichia coli*

*Escherichia coli*is a gram-negative non-spore forming bacillus from the family *Enterobacteriaceae*(Delmus & Dalmasso, 2015). The bacterial is a facultative anaerobe andmay or may not be mobile. Some of it's rods are flagellated; others are not. *Escherichia coli* exists in one vegetative life stage regardless of the site or host infected. The optimal conditions for growth are a temperature of 25-35°C. The colonies of *E.coli* are approximately 0.3-1.0× 1.0-6.0 µm and usually pink in colour on MacConkey media(Al-Baer & Hussein, 2017).In the presence of Kovac's reagent, *E.coli* slant cultures form a characteristic pink colour ring.Gaseous bubbles are presentin the bacteria'scolony in the presence of hydrogen peroxide(Delmus & Dalmasso, 2015).

2.1.1 Classification of *E. coli*

Clinically,*E.coli* can be classified as part of the normal flora of the gastrointestinal tract of humans and animals(Sarowska *et al.,* 2019). A majority of the *E. coli* strains are harmless, but some strains have acquired virulence genes that have enabled them to be pathogenic to human beings and animals (Khairy *et al.,* 2019). These pathogenic strains are categorized into two groups, which are in accordance with the site of infection. *E*scherichia*coli*strainsthat infect and cause disease syndromes in the gastrointestinal tract are known as the intestinal pathogenic *E. coli* (IPEC). Those that cause disease outside the gastrointestinal tract are called extra-intestinal *E. coli* (EXPEC) (Iliyasu *et al.*, 2018).

The gastrointestinal pathogenic *E. coli* strains includes:Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Diffusely Adherent *E. coli*(DAEC), and Verocytotoxigenic *E. coli* (VTEC).The

Extraintestinal pathogenic *E. coli* includes uropathogenic *E. coli* (UPEC), Neonatal Meningitis-Associated *E. coli* (NMEC), and Sepsis-causing *E. coli* (SEPEC)(Toval *et al.,* 2014).

2.1.2 Escherichia coli strains

2.1.2.1 Enteropathogenic E. coli

Enteropathogenic *E. coli* (EPEC) encode*eae* genesjust as some strains of VTEC. Due to that, there able to attach and efface lesion causing diarrhea. Classical EPEC possessthe *bfpA* gene, while atypical EPEC is a more prevalent cause of diarrhea(Lupindu, 2012). Human beings are infected by EPEC throughthe fecal-oral route. Isolation of EPEC can be done from different sources such as water, food, animal, and the environment. However, emphasise should be put on distinguishing EPEC from VTEC by the presence of the *eae* gene and the absence of *vtx* genes. Also, classical EPEC and atypical EPEC should be differentiated by assessing the presence of the *bfpA* gene that encodes for bundle-forming pili.Pulsed-field Gel Electrophoresis (PFGE)typing can be applied to compare strains during outbreaks(Lupindu, 2012)

2.1.2.2 Enterohemorrhagic *E. coli*.

Enterohemorrhagic *E. coli* (EHEC) strains are part ofa pathogenic subgroup of Shiga toxin-producing *E. coli* (STEC).They cause severe diseases, such ashemolytic uremic syndrome (HUS) and bloody diarrhea in humans. The ability to cause severe disease differentiates EHEC from other STEC found in the environment that are less harmful. *Escherichia coli* O157:H7 is the most common EHEC that cause HUS, but non–O157:H7 EHEC are inconstantly present as the only pathogens in stools from HUS patients(Lupindu, 2012).

2.1.2.3 Enteroaggregative *E. coli*

Enteroaggregative *E. coli*(EAEC) cause watery Diarrhea with the mucus, with or without blood and abdominal pain with vomiting and low fever. Acute self-limiting diarrhea is the usual pathology, but some patients may develop chronic diarrhea. Prolonged diarrhea occurs

depending on the host's immunity, nutritional status, and genetic susceptibility(Gomes *et al.*, 2016). The EAEC strains can be detected by conventional biochemical PCR methods(Lupindu, 2012).

2.1.2.4 Enterotoxigenic *E. coli*

Enterotoxigenic *E. coli* (ETEC) strains are responsible for watery diarrhea in children and travellers in developing nations (Gomes *et al.*, 2016). These are due to poor absorption of sodium ions and enhanced chloride secretion caused by enterotoxins. The transmission of ETEC is through the consumption of contaminated food and water.Enterotoxigenic *E. coli* (ETEC) produce heat-stable and heat-labile enterotoxins that are responsible for the cause of diarrhea. The toxins can be detected by serological assays (Lupindu, 2012).

2.1.2.5 Entero-invasive *E. coli*

Enteroinvasive *E. coli* (EIEC) is a causative agent of profuse diarrhea in humans living in developing Nations. They cause infections through damage to host epithelial cells by the use of adhesin protein to bind and enter intestinal cells. Enteroinvasive *E. coli* resembles Shigella species, both genetically and biochemically. Enteroinvasive *E. coli* are often detected by invasion plasmid antigens (*ipaH*) gene-targeted PCR (Lupindu, 2012). The invasiveness of EIEC can be assessed by guinea pig conjunctivitis assays(Gomes *et al.*, 2016).

2.1.2.6 Verocytotoxigenic *E. coli*

Verocytotoxigenic *E. coli* (VTEC) produces verocytotoxins, also known as Shiga toxins. The most common VTEC is O157:H7 strain. The main reservoirs for VTEC are the animals, and the route of transmission is fecal-oral. In human beings, VTEC causes abdominal cramps associated with diarrhea, especially in children and the elderly. Complicated cases of VTEC infection may lead to HUS. Characterization of isolates for VTEC detection may include immunological methods by using specific antibodies against target VTEC strain or PCR by targeting specific

genes. Verocytotoxigenic *E. coli*isolates typing can be done by serology, PFGE, DNA hybridization,and sequencing(Lupindu, 2012).

2.1.2.7 Diffusely adherent *E. coli*

Diffusely adherent *E. coli* (DAEC) are responsible for acute diarrhea in humans. Diffuselyadherent*E. coli*are distinguished by the ability to adhere to Hep-2 cells in a diffuse fashion. Diffusely adherent *E. coli* can bedetected by the PCR method(Lupindu, 2012).

2.2 Strain typing of *Escherichia coli*

*Escherichia coli*straintypinginvolves serological and molecular methods such as Multilocus Sequence Typing(MLST),Pulsed-Field Gel Electrophoresis (PFGE),Amplified Fragment Length Polymorphism (AFLP) analysis, other PCR fingerprinting methods and Multiple-locus variable tandem repeat number analysis (MVLA)(Woodford *et al.*, 2011).

2.2.1 Serological typing

Serological typing is used to detect the presence of antigenic components of a specific *E. coli* strain. The antigen can be detected using specific antibodies. For example,the presence of somatic antigen O, capsular antigen K, and flagella antigen H can be detected by agglutination tests and using specific antisera. The somatic and flagellar antigens are tested against each specific antiserum, or against pools of antisera first and then tested against each of the specific antisera from the positive results. The number of positive antisera is used in O and H antigen nomenclature(Lupindu, 2012).

2.2.2 Molecular methods

2.2.2.1 Multilocus sequence typing

Multilocus sequence typing (MLST)is great for evolutionary studies and for comparing isolates. However, it lacks the discrimination required for outbreak analysis.The MLST has guided in the

definition of sequence typesand clonal complexes in species such as *K. pneumonia*e ST258 and community-acquired *K. pneumoniae*(Woodford *et al.,* 2011).

2.2.2.2 Pulsed-Field Gel Electrophoresis

Pulsed-Field Gel Electrophoresis (PFGE) is a molecular technique that is used broadly for the typing of a range of bacterial species. This technique has been used to provide excellent discrimination of bacterial species. However, the method is not as portable as MLST and MVLA techniques that describe isolates numerically(Woodford *et al.,* 2011).

2.2.2.3 PCR fingerprinting method

Polymerase chain reaction fingerprinting methods are common typing methods too, but without AFLP, there considered unreproducible in various laboratories.An automated PCR method, however, is increasingly being used and may provide discrimination similar to that of PFGE(Woodford *et al.,* 2011).

2.2.2.4 Multiple-locus variable tandem repeat number analysis

Multiple-locus variable tandem repeat number analysis is a PCR method that determines the number of repeat units at multiple loci with short sequence repeats. This method is quickly gaining popularity for epidemiological investigations, has it's portable. The discriminatory power of the technique varies with the loci chosen, providing the potential to tailor the typing at an appropriate resolution. The MVLA technique provides higher resolution than PFGE(Woodford *et al.*, 2011).

2.2.2.5 Whole-genome sequencing

The DNA analysis has progressed from the analysis of a few DNA fragments into whole-genome sequencing. Next-generation sequencing analyses the entire genome in a short time of a single sequencing run. Has a result, analysis, and comparison of the whole-genome of isolates lead to a correct diagnostic inference. The major advantage of this technique is that millions of reactions

take place in parallel, and many samples can be analyzed at once. Sequencing is superior to other methods in the characterization of genetic material. For instance, whole-genome sequencing has the ability to detect the false positive and false negative clonal relationship of isolates from PFGE fingerprinting (Lupindu, 2012). However, this technique can exaggerate minor differences and fail to recognize that the isolates are largely similar(Woodford *et al.*, 2011).

2.3 Uropathogenic *Escherichia coli* infections

The presence of bacteria in urine is known as bacteriuria(Leung *et al.,*2019). Bacteriuria may beindicative of an infection in the urinary tract.When bacteriuria occurs with symptoms, it is called symptomatic bacteriuria, but without symptoms, it is known as asymptomatic bacteriuria(Leung *et al.,* 2019). The infections of the lower urinary tract are characterizedby frequency, urgency, and dysuria, while upper urinary tract infections are characterized by fever,chills, and flank pain(Leung *et al.,* 2019).Urinary tract infections are divided into two main categories complicated and uncomplicated diseases.The uncomplicated are defined as those occurring in a healthy, non-pregnant woman with a structurally and neurologically normal urinary tract. Complicated infections include those caused by a foreign body such as catheters or patients with urinary obstruction,pregnancy, or retention due to neurologic impairment. The organs commonly infected with UTIs are the urethra, bladder, ureters, and the kidneys, which comprise the urinary system (Mashwal *et al.,* 2017).

2.3.1 Epidemiology of uropathogenic E.coli

Globally, UTIs are the most common infections after respiratory tract infections affecting an estimated 150 million people annually(Koshesh *et al.,* 2016).Among the infants with febrile illnessand sick children with symptoms of urinary infections,6%-8% of them will have the UTIs(Kaufman *et al.,* 2019).Urinary tract infections are more common among women, with 50%-60% of adult women having at least one UTI in their lifetime(Medina *et al.,* 2019).About

25% of women will have a recurrent UTI within six months after initial infection, and about 12% will experience at least one UTI per year (Matulay *et al.,* 2016). Women are more prone to UTIs than males due to there shorter urethral distance and proximity of the urethra tract to the anus (Scribano *et al.,* 2020). The foreskin surface area in uncircumcised males makes them prone to UTIs (Kaufman *et al.*, 2019). In children,during the toddler years,toilet training can lead to volitional holding and bladder stasis,promoting UTIs(Leung *et al.,* 2019).Young adolescent girls will tend to experience UTIs when sexual activities disrupt bacteria near the urethral orifice(Kaufman *et al.,* 2019). It is estimated that about15% of the children with UTI will develop permanent kidney damage,leading to renal failure (Kaufman *et al.,* 2019).The chances of UTI to recur often a few weeks after an initial acute infection is a problem in UTImanagement.

2.3.2 Pathogenesis of uropathogenic Escherichia coli.

In most cases, UTIs result from the ascent of the bacteria from the periurethral areato the urethra to the bladder, and finally to the upper urinary tract(Leung *et al.,* 2019).The colonization of the periurethral area with uropathogenic bacteria is considered an important factorin causing UTIs (Leung *et al.,* 2019).Factors that contribute to the high colonization of the perineum include raised vaginal PH,increased adhesiveness of bacteria to vaginal cells, and reduced cervicovaginal antibodies(Matulay *et al.,* 2016). In uncircumcised males, the foreskin is a potential reservoir of bacterial pathogens to cause UTIs (Kaufman *et al.,* 2019).The use of instruments like catheters may also introduce bacteria into the urinary tract(Matulay *et al.,* 2016).Worthnotingis that a majority of the UTIs occur in the lower urinary tract and only a few results in pyelonephritis.Invasion of the kidney by pathogens leads to an intense inflammatory response that can result in renal failure (Leung *et al.,* 2019).

Clinical manifestations of uropathogenic *E.coli* infections include vaginitis, which is an infection of the vagina, cystitis (infection of the bladder), pyelonephritis (infection of the kidneys) and urethritis (infection of the urethra). The common signs and symptoms of UTIs include dysuria, fever>38°C,suprapubic tenderness,haematuria, and polyuria(Amiri *et al.,* 2017).

2.3.3 Diagnosis of uropathogenic E.coli infections

Uropathogenic *E. coli* infections are diagnosed using a combination of presenting symptoms and a positive laboratory test by microscopy or dipstick.Information such as the history of UTIs and last sexual activity is used to determine the likelihood of UTIs(Matulay *et al.,* 2016).The most common symptoms of UTIs includedysuria,frequency,urgency, suprapubic pain,and haematuria(Kaufman *et al.,* 2019).Symptoms of pyelonephritis, which is the infection of the upper tract infections, including fever,chills, and flank pain(Kaufman *et al.,* 2019). Most women with one or three symptoms of dysuria,urgency, or frequency of urination without vaginal discharge will have a 90% chance of UTIs(Kaufman *et al.,* 2019). Hence attentionto clinical history and physical exanimation is sufficient for diagnosis of uncomplicated UTI in most cases.A positive laboratory test without symptoms is considered asymptomatic bacterium and does not require antimicrobial treatment (Leung *et al.,* 2019).Unfortunately, pregnant women with a positive culture and sensitivity result will be put on the antibiotics whether symptomatic or not,so that they do not progress to a more serious infection of the bladder and kidneys(Matulay *et al.,* 2016).

2.3.3.1 Laboratory tests for UTIs

The laboratory tests for the diagnosis of UTIs include urine dipstick testing,microscopic urinalysis,and urine culture and sensitivity test(Matulay *et al.,* 2016).A culture and sensitivity

test is done in case of recurrent or complicated UTIs before antibacterial treatment is commenced (Matulay *et al.,* 2016).

2.3.4 Treatment of UTIs

The β-lactams antibiotics are the most common and readily available treatment for UTIs widespread among *Enterobacteriaceae* species, particularly among *E. coli* isolated from UTIs worldwide (Onanuga *et al.,* 2019). Symptomatic UTIs are treated with the antibiotics based on the clinical history and positive urinary tests while waiting for culture and sensitivity results to clear the infections(Leung *et al.,* 2019).On the other hand, asymptomatic UTIs do not need to be treated. The right antibiotic chosen should be easy to administer, have little or no effecton the fecal or vaginal flora,have minimal or no toxicity, and be of low cost (Leung *et al.,* 2019). The least broad-spectrum antibiotic should be used. The commonly used antibiotics to treat UTIs include; cefixime (Suprax), cefdinir (such as ciprofloxacin (Cipro,Ciloxan, Neofloxin), nitrofurantoin (Macrobid, Omnicef), ceftibuten (Cedax), cefpodoxime (Vantin), cefuroxime (Ceftin), and cefprozil (Cefzil), fluoroquinolones Macrodantin), trimethoprim-sulfamethoxazole (TMP-SMX), ampicillin (Ampi, Omnipen, Principen, Penglobe), amoxicillin (Amoxil, Moxatag, Trimox, Wymox), and amoxicillin-clavulanate(Clavulin, Augmentin) (Leung *et al.,* 2019).

2.4 Modes of antibiotic mechanisms of activity towards the bacterial isolates

There are five modes of antibiotic mechanisms of activity towards the bacteria. Namely:

2.4.1 Inhibition of cell wall synthesis

The β-lactam antibiotics, such as the penicillin and cephalosporins, will impede enzymes that prevent the synthesis of the bacteria cell walls that are responsible for the formation of the peptidoglycan layer (Kapoor *et al.*,2017).

2.4.2 Inhibition of nucleic acid (nucleotide) synthesis

The antimicrobial agents such as the Rifampicin interferes with a DNA-directed Ribonucleic acid (RNA) polymerase preventing the RNA synthesis. Quinolones such as the ciprofloxacin and nalidixic acid inhibit DNA synthesis by interfering with type II topoisomerase, DNA gyrase, and type IV topoisomerase during replication cycle causing double-strand break(Ali *et al.*, 2017).

2.4.3 Inhibition of protein synthesis and the arrest of bacterial growth

For instance, the tetracyclines interfere with protein synthesis by binding to the 30S subunit of the ribosome, thereby weakening the ribosome tRNA interaction. The macrolides such as Erythromycin binds to the 50S ribosomal subunit and inhibit the elongation of nascent polypeptide chains. Chloramphenicol binds to the 50S ribosomal subunit blocking peptidyltransferase reaction(Kapoor *et al.*, 2017).

2.4.4 Inhibition of a metabolic pathway

The metabolic pathway can be inhibited by sulfonamides such as the sulfamethoxazole and trimethoprim that block the critical steps in the folate synthesis. Folate is a co-factor in the biosynthesis of nucleotidesthat form the building blocks of DNA and RNA (Kapoor *et al.*, 2017).

2.4.5 Damaging of the cell membrane leading to loss of contents and then death

The primary site of action of the antimicrobial agent is the cytoplasmic membrane of Gram-positive bacteria or the inner membrane of Gram-negative bacteria. For instance, the polymyxins exert their inhibitory effects by increasing bacterial membrane permeability, causing leakage of bacterial content and daptomycin displays rapid bactericidal activity by binding to the cytoplasmic membrane in a calcium-dependent manner and oligomerizing in the membrane, leading to efflux of potassium from the bacterial cell and cell death (Ali *et al.*, 2017).

2.5 Antimicrobial resistance

Antimicrobial resistance is the abilityofanorganism to resist the action of an antimicrobial agent to which it was previously susceptible(Sheikh *et al.,* 2019).The antimicrobial resistance can either be intrinsic or acquired (Munita *et al.,* 2016).

2.5.1 Intrinsic antimicrobial resistance

The intrinsic antimicrobial resistanceis conferred by naturally occurring genes in the bacterium's genome or bythe inherent characteristics of the bacterium, which allow tolerance to specific antimicrobials(Peterson & Kaur, 2018). This type of resistance is common for all members of a bacterial species and is independent of the selective pressure from antimicrobials.For instance,the Mycoplasma are resistant to penicillin because they lack peptidoglycan (Peterson & Kaur, 2018).

2.5.2 Acquired antimicrobial resistance

Acquired antimicrobial resistanceis a type of antimicrobial resistance that occurs when a naturally susceptible microorganism obtains the ability to resist a specific antimicrobial agent to which it was previously susceptible(Santajit & Indrawattana, 2016). Acquired antimicrobial resistance can be attained from a mutation of cellular genes, the acquisition of foreign resistance genes, or a combination of these two mechanisms(Munita *et al.,* 2016).There are two main mechanisms by which bacteria acquire resistance:These mechanisms are by spontaneous mutations in chromosomal genes or through horizontal gene transfer,which isthe acquisition of naturally occurring resistance genes from other bacteria(Santajit & Indrawattana, 2016). The main mechanism for the spread of antibiotic resistance is by horizontal transfer of genetic material.Antibiotic resistance genes may be transferred by conjugation, transformation or transduction(Munita *et al.,* 2016).

2.6 Bacterial resistance strategies

There are four major bacterial resistance strategies that the bacteria uses to protect itself from the antimicrobial agent. These strategies include; prevention of the antimicrobial from reaching its target by reducing its ability to penetrate the cell, the expulsion of the antimicrobial agents from the cell via general or specific efflux pumps, inactivation of antimicrobial agents via modification or degradation finally, by modification of the antimicrobial target within the bacteria.Important to note is that a single bacterial strain may possess several types of resistance mechanisms of which these mechanisms prevail depends on the nature of the antibiotic, it's target site, the bacterial species and whether it is mediated by a resistance plasmid or by a chromosomal mutation (Laws *et al.*, 2019).

2.6.1 Inactivation of antimicrobial agents via modification or degradation

Antibiotic inactivation is a strategy by which some bacteria preserve themselves by destroying the active component of the antimicrobial agent.For instance, the β-lactamases hydrolyticallycleave the β-lactam ring of penicillin and cephalosporin antibiotics.The inactivated penicilloic acid is then ineffective in binding to penicillin-binding proteins, thereby protecting the process of cell wall synthesis(Santajit & Indrawattana, 2016).

2.6.2 Antibiotic resistance via modification of target within bacteria

Target modification is a strategy that the bacteria uses to modify the antibiotic target site so that the antibiotic is unable to bind correctly. Due to the vital cellular functions of the target sites, organisms cannot evade antimicrobial action by dispensing with them entirely.However, mutational changes can occur in the target that reduces susceptibility to inhibition while retaining cellular function.The altered targets may include the DNA gyrase, RNA polymerase, and Prokaryotic ribosome, which are targets of quinolone, antimicrobials, rifampin, and tetracycline, and other protein synthesis inhibitors respectively(Santajit & Indrawattana, 2016).

2.6.3 Expulsion of the antimicrobial agents from the cell via general efflux pumps

The efflux pumps are membrane proteins that export the antibiotics out of the cell and keep their intracellular concentrations at low levels. For the antimicrobial agents tobe effective, they must be present at a sufficiently high concentration within the bacterial cell. Some bacteria possess membrane proteins that act as an export or efflux pump for certain antimicrobials, extruding the antibiotic out of the cell as fast as it can enter. This results in low intracellular concentrations that are insufficient to elicit an effect. Some efflux pumps selectively extrude specific antibiotics, for example, the macrolides and tetracyclines. Others possess multiple drug resistance pumps that are capable of expelling a broad spectrum of structurally unrelated drugs with different modes of action—for instance, the multidrug resistance (MDR)(Munita *et al.*, 2016).

2.6.4 Prevention of the antimicrobial from reaching its target

The antimicrobial compounds always require access into the bacterial cell to reach their target site where they can interfere with the normal function of the bacterial organism. Porin channels are the passageways by which these antibiotics would typically cross the bacterial outer membrane. Some bacteria protect themselves by prohibiting these antimicrobial compounds from entering past their cell walls. For example,the Gram-negative bacteria reduce the uptake of certain antibiotics, such as aminoglycosides and β-lactams, by modifying the cell membrane porin channel frequency, size, and selectivity. Prohibiting entry prevents antimicrobials from reaching their intended target. The target for aminoglycosides and β-lactams are the ribosomes and penicillin-binding proteins (PBPs), respectively (Santajit & Indrawattana, 2016).However, there are increased reports of drug resistance to β-lactams antibiotics (Giedraitiene *et al.*, 2011). The most common and important mode of resistance is the expression of β-lactamases such as the extended-spectrum β-lactamases (ESBL), plasmid-mediated AmpC enzymes, and carbapenem hydrolyzing β-lactamases (carbapenemases)(Moxon & Paulus, 2016).

2.7 Extended-spectrum β-lactamases

Extended-spectrum β-lactamases (ESBL) are acquired enzymes that hydrolyze penicillins, oxy-imino-cephalosporins, (ceftazidime, cefotaxime, ceftriaxone, cefepime, cefuroxime) and monobactams (aztreonam), but not cephamycins (cefoxitin and cefotetan) and carbapenems(Bajpai *et al.*, 2017; F. Giwa *et al.*, 2018).They are, however, inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam(Dasgupta *et al.*, 2018; F. Giwa *et al.*, 2018).The genes encoding ESBLs frequently co-exist on the same plasmid with genes conferring resistance to other antibiotics such as fluoroquinolones(Moxon & Paulus, 2016), thus facilitating the horizontal transmission of resistance to multiple antimicrobials among bacteria of the same or different species in both clinical and community settings (Ruppé *et al.*, 2015). The problems associated with ESBL production include multidrug resistance, difficulty in detection and treatment, and an increase in transmission of the resistance phenotype (Iliyasu *et al.*, 2018).

Globally, the occurrence of ESBL varies according to different geographic locations and is correlated with the overuse of antibiotics(F. Giwa *et al.*, 2018). Production of ESBL by pathogenic organisms has been reported in several African countries:Egypt,Morocco,Tunisia, Senegal, South Africa, and Nigeria, with prevalence rates ranging from 5% to 44.3%(F. Giwa et al., 2018).The current occurrence of ESBL production in Kenya is 27%(Kiiru *et al.*, 2012).Worldwidestrains of *E. coli* and *Klebsiella pneumoniae* that produce ESBL range from 10%-40%(F. Giwa *et al.*, 2018). A study on monitoring Antimicrobial Resistance Trends (SMART) in the Asia Pacific region in 2007 revealed that ESBL production by *Enterobacteriaceae*species was highest in India, with79% ESBL positives among *E.coli* isolates(F. Giwa *et al.*, 2018).

The most common ESBL types in *E. coli*are temoneira (TEM), sulfhydryl variable (SHV), and cefotaximase-Munich (CTX-M) families(Shaikh *et al.,* 2015).The TEM type ESBL is derived by mutations in their parental enzyme TEM-1 and TEM-2(Shaikh *et al.,* 2015),whereas the SHV type ESBL is derived from SHV-1 (Shaikh *et al.,* 2015). The plasmid-encoded TEM-1, TEM-2, and SHV-1 confer resistance to penicillins but not extended-spectrum cephalosporins(Moxon & Paulus, 2016).The CTX-M type ESBL originated by horizontal transfer of a chromosomal β-lactamase gene from the non-pathogenic *Kluyvera* species found in the soil and has potent hydrolytic activity against cefotaxime(Bourjilat *et al.,* 2011). Until the discovery of CTX-M in 1990 in *E. coli*, most ESBL producing pathogens in clinical specimens were primarily hospital acquired and belonged to TEM and SHV families (Bourjilat *et al.,* 2011). The CTX-M β-lactamases are becoming the most widespread among *Enterobacteriaceae*species, particularly among *E. coli* isolated from UTIboth in nosocomial and community settingsworldwide (Bourjilat *et al.,*2011; Giedraitiene *et al.,* 2011).

2.7.1 Microbiology techniques for the detection of ESBL

Most of the proposed ESBL detection methods are based on the Kirby-Baur disk diffusion test methodology.In clinical microbiology, test β-lactamase inhibitor usually clavulanate,in combination with an oxyimino-cephalosporin such as ceftazidime or cefotaxime, are employed.In this case, the clavulanate inhibits the ESBL,thereby reducing the level of resistance to the cephalosporin(Rahman *et al.,* 2015).

2.7.2 ESBL Screening

According to the CLSI, ESBL screeningcan be performed using selected antimicrobial agents. The In-vitro sensitivity testing is carried out with ceftazidime (30 µg), cefotaxime (30µg), ceftriaxone (30µg), aztreonam (30µg) and cefpodoxime (10 µg). Any zone diameter within the "grey zone" is considered as a probable ESBL producing strain requiring phenotypic

confirmatory testing. Each *Klebsiella pneumoniae* or *Escherichia coli* isolate should be regarded as a potential ESBL-producer(Rahman *et al.,* 2015).

2.7.3 Combination disc diffusion test - the confirmatory phenotypic method for ESBL production

The phenotypic confirmatory methods for ESBL production involves the use of Ceftazidime (30µg) versus ceftazidime/clavulanic (30/10µg) and cefotaxime (30µg) versus (cefotaxime/clavulanic acid (30/10µg) that are placedonto a Muller-Hinton agar plate inoculated with the test organism and incubated at 37°Cfor 24 hours.The results are determined regardless of the zone diameters, a ≥ 5mm increase in zone diameter for an antimicrobial agent tested in combination with clavulanic acid versus its zone size when tested alone, indicates probable of ESBL production(Dolinsky, 2017).

2.7.4 Molecular detection of ESBL genes

Up to now,thereis no gold standard test for the detection of ESBLs(Rahman *et al.,* 2015).Clinical Laboratory Standard Institutes recommended the CDDT method as a confirmatory test(Dolinsky, 2017).However, the traditional methods are labour intensive and time-consuming and require at least 24hr incubation after isolated colonies are available from primary culture.This process takes about 48 hours to get the ESBLs reports (Rahman *et al.,* 2015).Multiplex real-time PCR with oligonucleotide primers is the easiest and most common molecular method used to detect the presence of β-lactamase genes. In this test, the primers are usually chosen to anneal to regions where various point mutations are not known to occur (Rahman *et al.,* 2015). The DNA extracts are screened for the presence of bla_{TEM},$bla_{\text{SHV,}}$ and $bla_{\text{CTX-M,}}$ genes by fluorescent probe-based multiplex real-time PCR using a PCR instrument/equipment.

2.8 AmpC β-lactamases

According to the ambler structural classification of β-lactamases, the AmpC enzymes belong to class C, Madhumati, *et al.*, 2015; Helmy and Wasfi, 2014).In *E. coli*, the AmpC β-lactamases are encoded by genes located either on the chromosome or on plasmids (Khari *et al.,* 2016). Chromosomal genes are expressed constitutively at low levels, but various mutations in the promotor/attenuator region of the AmpC gene can result in increased AmpC expression and β-lactam resistance(El-Hady & Adel, 2015). Plasmid-mediated AmpCβ-lactamases have spread via the transfer of chromosomal genes for the inducible AmpC β-lactamases of other members of *Enterobacteriaceae*including *E. cloacae, Citrobacterfreundii,* and *Morganellamorganii* onto plasmids(Shagufta *et al.,* 2017). They display structural and functional similarity to their chromosomal origin (Helmy & Wasfi, 2014). The most common reported plasmid-mediated AmpCgenes encode the CMY-type enzymes(El-Hady & Adel, 2015), of which there are 64 plasmid-mediated variants(Reuland *et al.*, 2014). Other plasmid-mediated AmpC'sinclude DHA variants, ACC variants, MOX variants, FOX variants, CIT variants, BIL-1, MIR/ACT (associated with EBC family gene)(Khari *et al.*, 2016). Excess production of AmpC β-lactamases in *E.coli*confers resistance to the majority of β-lactam antibiotics,except for carbapenems and fourth-generation cephalosporins (cefepime)(Khari *et al.*, 2016). Plasmid-borne AmpC genes are considered to be significant because they may serve as a reservoir for the emergence of antibiotic resistance (Madhumati *et al.,* 2015). Both excess production of AmpC and porin mutations affecting the bacterial outer membrane can reduce susceptibility to carbapenems, especially in plasmid-mediated AmpC microorganisms (Madhumati *et al.,*2015). Porins are chemically selective and allow one molecule at a time to pass through (Madhumati *et al.,* 2015). Fluoroquinolones and β-lactam antibiotics must pass through porins to reach their targets in gram-negative bacteria (Madhumati *et al.*, 2015). Bacteria confer resistance to these antibiotics

by mutating the gene that encodes the porins making it difficult for the antibiotic to pass through the outer membrane(Madhumati *et al.,* 2015). Despite many reported cases of plasmid-mediated AmpC β-lactamases among *Klebsiella pneumoniae,*the rate of detection in *E.coli* remains unknown(Madhumati *et al.,*2015; El-hady and Adel, 2015). These organisms typically show multidrug resistance but are often not flagged as AmpC positive isolates in routine antimicrobial susceptibility tests as there are no Clinical Laboratory Standard Institute (CLSI) standards for AmpC detection(Madhumati *et al.,*2015; El-hady and Adel, 2015). This leads to therapeutic failure and the spread of resistant genes(Cugati & Lyra, 2019).

2.9 Phylo-typing of UPEC isolates

The*E.coli* phylo-groups differ in characteristics such as ecological niches, pathogenicity, and antimicrobial resistance (Gordon *et al.,* 2008; Clermont *et al.,* 2000). These traits can be tracked using the phylo-groups as proxies; therefore, assessing their geographical distribution is a convenient method of mapping the traits spatially. Phylo-typing analysis using the triplex PCR method has shown that extraintestinalpathogenic *E.coli* strains belong mostly to phylo-group group B2 followed by group D(Iranpour *et al.,* 2015; Abdi and Rashki, 2014; Girardini *et al.,* 2012). Most commensal *E. coli* strains are associated with phylogenetic group A and B1 *E. coli* (Giray *et al.,* 2012; Abdi and Rashki, 2014; Peerayeh *et al.*, 2018). Isolates of groups B2 and D have been reported to have decreased rates of antimicrobial resistance compared to those of group A or B1 (López-banda *et al.,* 2014). However, there are also contradicting reports of higher antimicrobial resistance of uropathogenic *E. coli* strains belonging to phylogenetic group B2 (Kawamura-Sato *et al.,* 2010). Such differences may be attributed to different bacterial characteristics in different geographic areas influenced by regional differences in antibiotic usage (Lee & Lee, 2010).

CHAPTER THREE

MATERIALS AND METHODS

3.1Study Area

This study was conducted at the Center for Microbiology Research in Kenya Medical Research Institute (KEMRI), Nairobi. The Department of Emerging Infectious Diseases (DEID) of the United States Army Medical Directorate-Africa (USAMRD-A) has been conducting antimicrobial resistance surveillance in Kenya since April 2015 (KEMRI SERU#2767/WRAIR IRB#2089). This study utilized archived uropathogenic *E. coli* (UPEC) isolates that were collected in the parent study between 2015 and 2018. The isolates were identified from urine samples collected from patients who visited health care facilities with symptoms of UTIs.The health care facilities from which the samples wereobtained are located in five Counties: Kisumu,Kericho, Malindi,Kisii,and Nairobi (Figure 3.1). These health care facilities are part of a surveillance network of hospitals utilized in the parent protocol and established historically by the United States Army Medical Directorate-Africa USAMRD-A in Kenya as they represent diverse geographic locations in Kenya, which provide better nationally relevant data than single-site studies.

Kisumu County lies within Longitudes 33^{0}20E and $35^{0'}$20'E and Latitude 0^{0}20'south and 0^{0}50'.TheCounty covers a total land area of 2085.9$km^{2.}$ The total population is 1,155,574persons, of which 560,942 are males, 594,609 female, and 23 intersexes this is according to Kenya National Census 2019(Kenya National Bureau of Statistics, 2019). The administrative and political unit of Kisumu County is in Kisumu City.The main economic activities of this County include fishing and agriculture.

Kericho County lies between longitude $35^0$02'and $35^0$40'East and between the equator and latitude 023' south and covers an area of 2454.5km^2 (947.7sq m)(County Government of Kericho, 2014).The administrative and political unit of Kericho County is in Kericho town. The total population as per the 2019 census is 901,777 persons of which,450,741 are males,451,008 are females, and 28 intersexes(Kenya National Bureau of Statistics, 2019).The main economic activities in this County are tea and dairy farming.

Kilifi County lies at $3^0$56' and $4^0$10 'south of the equator and between 39^0 34'39^0 46' east of Green wish meridian. The county covers an area of 12.245.9km^2 (4.728.2sqmi),with a population of 1,453,787 people. Males 704,089, females 749,673, and 25 intersexes as per the Kenya National Bureau of statistics 2019(Kenya National Bureau of Statistics, 2019).The administrative and political units of Kilifi County are in Malindi town headquarter. The major economic activities in Kilifi County are agriculture,tourism, and fishing due to proximity to the Indian Ocean.

Kisii Countylies between latitude 00 30' and 100 South and longitude 340 38' and 350 East. The County covers an area of 1,317.9km^2(5.08.8sq mi), with a population of 1.266860 persons. Males are 605,784,female 661,038 and intersex 38 persons(Kenya National Bureau of Statistics, 2019). The administrative and political unit of Kisii County is in Kisii town headquarter. The main economic activities in Kisii county include tea and coffee farming plus small scale daily farming(Kisii County Government, 2020).

Nairobi County covers a total area of 696.1km^2 and is located between longitude 36^0 45'East and latitude 1^0 18'south. The County lies at an altitude of 1.798 meters above sea level. The total population is 2,192,452persons, of which 2,192,452 are males, 2,204,376 are female, and 245

intersexes(Kenya National Bureau of Statistics, 2019). The administrative and political unit of Nairobi County is in Nairobi City. In terms of economic activities, Nairobi County is a commercial businesshub.

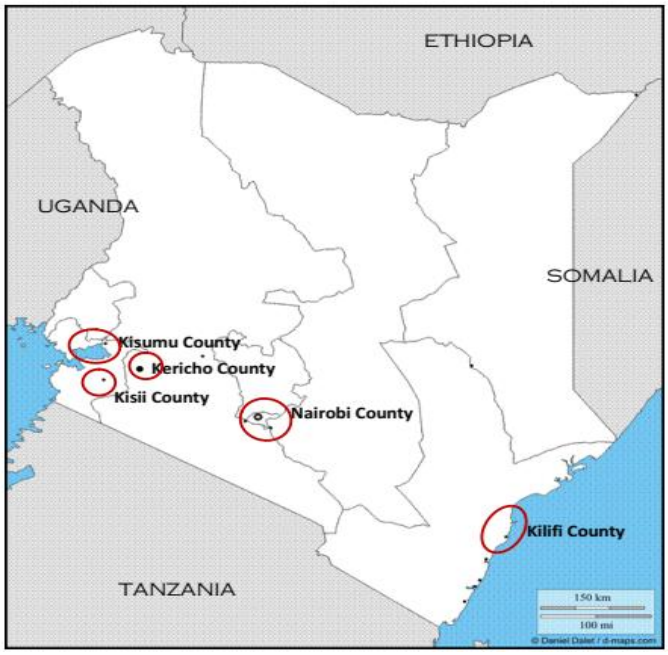

Figure 3.1: A map of Kenya showing the Counties where samples were collected

3.2 Study design

This study was a descriptive retrospective laboratory-based survey nested in a current study. Briefly, the parent protocol(KEMRI# 2767/WRAIR#2089) is a Multidrug-Resistant Organism (MDRO) surveillance study in both military and civilian hospital populations that identifies and tracks antibiotic resistance patterns in target bacteria including *E. coli* that are clinically significant and have high-levels of multidrug resistance. The study biotypes and characterizes the genetic determinants of key bacterial pathogens.

3.3 Study population

This study used archived UPEC isolates collected in a parent protocol from patients with symptoms of UTIs in hospitals from several counties, as described in section 3.1.

3.3.1 Inclusion criteria

All isolates of uropathogenic *E.coli* (UPEC) were collected between April2015 to August 2018.

3.3.2 Exclusion criteria

Other uropathogens except for*E. coli*

3.4 Sample size determination

The total number of isolates from urine samples collected between April 2015- August 2018 in the parent protocol was 527. Out of these isolates, 95 were UPEC. These were the isolates included in the study.

3.5 Sampling design

As this was a purposive descriptive survey study of UPEC isolates, all UPEC isolates obtained from the parent protocol were identified from the database at the start of the study (95 isolates) and matched to the laboratory accession numbers to aid in the retrieval of the isolates.The Antibiotic Susceptibility Testing (AST) data for each of the isolates was also obtained.

3.6 Methods of Data collection

3.6.1 Laboratory Procedure

Urine specimens had been collected from individuals older than two months presenting with symptoms of UTIs at both inpatient and outpatient departments in various health care facilities in Kenya who gave informed consent. For individuals less than 18 years old, the parent or guardian gave informed consent before participating in the parent protocol. About 9 milliliters midstream clean-catch urine samples were collected from the patients. The midstream urine samples were collected by first passing a small amount of urine into the toilet,midway through urination, a

sterile urine collection cup washalf full filled and the rest voided in the toilet.Urine collection bags were used to collect urine from infants and young childrenby attaching the sticky part of the urine collection bag on the infant's private part. After collection, the urine was transferred to a boric acid tube and kept at room temperature. Fresh urine samples from patients who had catheters in place, were collected using sterile needles and syringes from the catheter tubing and deposited into a collection cup. The urine samples were then transferred into a urine preservative tube containing boric acid and kept at room temperature. All the samples were then transported within 48 hours to the Center for Microbiology Research in Kenya Medical Research Institute (KEMRI), Nairobi, for testing.

The isolates used in this study were from the urine samples that had been cultured in CLED and MacConkey mediato detect bacterial pathogens using standard microbiology techniques and the bacteria identified on theVitek 2 (BioMerieux,France) platform. Antimicrobial susceptibility testing for gram-negative pathogens, including UPEC isolates, had been performed 2against a panel of 27 antibiotics on the automated Vitek 2 (BioMerieux, France) platform and the results interpreted as either susceptible, intermediate or resistant according to Clinical Laboratory Standard Institute guidelines(Dolinsky, 2017).The 27 antibiotics tested includes Cefoxitin, ceftazidime, cefotaxime, cefuroxime, cefixime, ceftriaxone, ciprofloxacin, clindamycin, Aztreonam, azithromycin, tetracycline, oxacillin, gentamicin, erythromycin, levofloxacin, clarithromycin, minocycline, imipenem, Amoxicillin/clavulanicacid, ampicillin/sulbactam, amikacin, trimethoprim, colistin, tigecycline, chloramphenicol, and moxifloxacin.All results were recorded in the study database. The bacterial isolates were archived in glycerol stocks at -80°C for future studies.

3.6.2 Bacterial isolate revival

The cryovial box with the identified isolates was removed from the freezer and placed on an ice pack. By use of a sterile inoculation wire loop, a scratch on the top of the frozen stock was made and inoculated on the surface of the MHA plate. The plate was incubated at 37°C for 24 hours. The cryovial was closed and returned to its slot in the freezer. This process was repeated for all the isolates in the study. The isolates were purified by bacterial culture and sub-culture to get a single pure colony.

3.7 Characterization of ESBL isolates

3.7.1 Phenotypic confirmatory test for ESBL production

The Combination Disc DiffusionTest (CDDT)was used to determine the inhibition of ESBL activity by clavulanic acid. Using the Vitek 2 (BioMerieux, France), AST results of the UPEC isolates, the isolates that showed Minimum Inhibition Concentration (MIC) ≥16µg/mL for ceftazidime as per CLSI guidelines, were subjected to ESBL confirmatory test by CDDT. The ceftazidime (a 3rd generation cephalosporin) resistance is indicative of an ESBL producing isolate.

Two discrete colonies of the suspect *E.coli* isolates from a pure culture plate were picked and emulsified in 3 ml of 0.45% sterile normal saline using a loop to create a uniform suspension. The turbidity of the suspension was adjusted to match the 0.5 McFarland standard. A sterile cotton swab was dipped into the suspension, excess liquid squeezed out by pressing the swab against the test tube, and the swab gently applied onto the surface of the MHA (Becton Dickinson,USA) on a 15x150mm culture plate. A ceftazidime disc (30µg) (Becton Dickinson,USA)and the combination ceftazidime(30µg) +clavulanic acid (30µg+10µg) (Becton Dickinson,USA) (Becton Dickinson,USA) disc wereplaced 60mm away from each other (edge to edge) and incubated at 37°C for 24 hours. A Vernier caliper was used to measure the zone size

around each disc. A difference in zone size>5mm between the ceftazidime(30µg) +clavulanic acid (30µg+10µg) (Becton Dickinson,USA to the ceftazidime(30µg) disc is confirmatory for ESBL producing strains as per CLSL guidelines because ESBL producers are inhibited by clavulanic acid.*Escherichia coli* ATCC 25922 was used as an ESBLnegative control and *Klebsiella pneumoniae* ATCC 700603 as an ESBLpositive control.

3.8 Detection of AmpC isolates

3.8.1AmpC screening using the disk diffusion method

The UPEC isolates that were found to be positive for ESBL production were also screenedfor presumptive AmpC β-lactamase production by the disk diffusion method using cefoxitin disc 30µg (Becton Dickinson, USA). AmpC producers, unlike ESBL isolates producing other enzymes, are resistant to the cephamycins, such as cefoxitin. Two discrete colonies of ESBL producing suspected AmpC producing UPEC isolates from a pure culture plate were picked and emulsified in 3 ml of 0.45% sterile normal saline by stirring with the loop to create a uniform suspension. The turbidity of the saline was adjusted to match the 0.5 McFarland standard.A sterile cotton swab was dipped into colony saline and gently applied onto the surface of the MHA on a culture plate. A sterilized forceps was used to place acefoxitin disk 30µg (Becton Dickinson, USA) on the center of the plate and pressed gently to ensure complete adhesion of the disk to agar media. The plate was incubated at 37°C for 24 hours before reading the results. A Vernier caliper was used to measure the zone size around the cefoxitin disk.Isolates with zone diameters of < 18mm indicating resistance as per CLSI guidelines, were considered positive for AmpC β-lactamases screening and were selected for confirmation of AmpC production(Madhumati *et al.,* 2015; Upadhyay *et al.,* 2015).

3.8.2 AmpC phenotypic confirmatorytest

The disk approximation test was used to detect inducible AmpC production. The principle of this test is that two antibiotics are placed next to each other, with one antibiotic acting as the inducer and the second antibiotic as a reporter substrate. A bacterium with an inducible enzyme will be resistant to a 3rd generation cephalosporin such as cefotaxime but susceptible to cefoxitin unless the AmpC is up-regulated. Exposure to the cephalosporin drug will induce the production of AmpC in bacteria around the disc, which will render those bacteria resistant to cefoxitin and create a one-sided flattening of the cefoxitin susceptibility zone. To conduct this test, two discrete colonies of suspected inducible AmpC producers of UPEC isolates, from a pure culture plate, were emulsified into 3 ml of sterile normal saline. The saline was stirred with a sterile wire loop to create a uniform suspension. The turbidity of the saline was adjusted to that of the 0.5 McFarland standard. A sterile cotton swab was dipped into colony saline and gently applied onto the surface of the MHA culture plate. Sterilized forceps were used to placea cefotaxime 30µg and a cefoxitin30µg disc (Becton Dickinson,USA) 20mm apart from the center of each disc. The plates were inverted and incubated overnight at 37°C.After overnight incubation, the plates were examined for distortion of the zone of inhibition adjacent to the cefoxitin disk, indicating the production of AmpC, which was a positive result. A negative result was indicated by the absence of distortion to cefoxitin disk (El-hady and Adel, 2015; Upadhyay *et al.,* 2015).

3.9 Genotyping of ESBL-producing isolates

3.9.1Bacterial DNA extraction

A loop full of colonies from a pure culture of each UPEC isolates that was found to be phenotypically positive for ESBL production was suspended into 500µl of DNAse/RNAse free water in a 1.5 ml Eppendorf tube (Becton Dickson,USA).A negative control containing 500µl of DNAse/RNAse free water onlywas included. The suspensions were heated at 100 $^{\circ}$C for 20

minutes to rupture the bacterial cells, cooled at room temperature and then centrifuged using a refrigerated Eppendorf centrifuge 5430R,(Germany) at 12,700 revolutions per minute (RPM). The supernatant containing chromosomal and plasmid DNA fractions plus the negative control was transferred into a sterile microcentrifuge tube (Becton Dickson, USA). Successful DNA extraction was confirmed by measuring the quantity of the DNA using a spectrophotometer NanoDrop (Thermo fisher scientific, USA) and stored at -20°C, pending the PCR reactions.

3.9.2 Detection of ESBL genes using Multiplex real-time PCR

The DNA extracts were screened for the presence of *bla*$_{TEM}$, *bla*$_{SHV,}$and*bla*$_{CTX-M,}$genes by fluorescent probe-based multiplex real-time PCR using the MIC PCR instrument (Bio Molecular systems,Australia). The sequences of the *bla*$_{TEM}$,*bla*$_{SHV}$, and *bla*$_{CTX-M}$ specific primers and probes (New England Biolabs,USA)are shown in Table 3.1. Real-time PCR amplifications were performed in reaction volumes of 20µL containing 10µL reactions Luna qPCR mix, 0.6 µL each of TEM forward and reverse primer (10 pmol), 0.8 µL each of SHV and CTX-M forward and reverse primers (10 pmol), 0.1 µL TEM TaqMan probe (5pmol), 0.2 µL of each of the SHV and CTX-M TaqMan probes (New England Biolabs, USA) (10 pmol), 3.1 µL of sterile water and 2 µL of DNA template.The real-time PCR conditions were as follows:Initial denaturation step at 95°C for 15 min; 30 cycles of denaturation at 95°C for 15 sec, annealing at 50°C for 15 sec and extension at 70°C for 20 sec.Fluorescence signals were detected in threedifferent channels: Green (465–510 nm) BHQ_1, Red (618–660 nm)/BHQ-2, and Yellow (533–580 nm)/BHQ-1Yellow. After completion of the run, a cycle threshold (Ct) was calculated by determining the signal strength at which the fluorescence exceeds a threshold limit. Samples possessing a fluorescence signal above this value were interpreted as positive. The positive controls used in this assay were

in the house, and the negative controls used were nuclease-free water and extraction negative samples.

Table 3.1: Sets of primers and probes used for multiplex PCR assay to detect the ESBL genes

Target	Primer/Probe ID	Sequences
bla_{TEM}	TEM_fwd.	5' GCATCTTACGGATGGCATGA 3'
	TEM_rev.	5' GTCCTCCGATCGTTGTCAGAA 3'
	TEM_probe	5' 6-Fam-CAGTGCTGCCATAACCATGAGTGA-BHQ-1 3'
bla_{SHV}	SHV_fwd.	5' TCCCATGATGAGCACCTTTAAA 3'
	SHV_rev.	5' TCCTGCTGGCGATAGTGGAT 3'
	SHV_probe	5' Cy5-TGCCGGTGACGAACAGCTGGAG-BBQ-650 3'
bla_{CTX-M}	CTX-M_fwd.	5' ACCGAGCCSACGCTCAA 3'
	CTX-M_rev.	5' CCGCTGCCGGTTTTATC 3'
	CTX-M-probe	5' Yakima Yellow- CCCGCGYGATACCACCACGC-BHQ1 3'

Primer sequences used in detection of ESBL genes (Table 3.1) were obtained from a study by(Roschanski *et al.*, 2014).

3.10 Detection of AmpC genes using Multiplex PCR

Multiplex PCR is considered the gold standard method for detection of AmpC β-lactamases, despite the various phenotypic test methods available (Naveen Grover 2013). Primers specific for the genes of six differentfamilies of plasmid-mediated AmpCβ-lactamases; bla_{MOX},$bla_{CIT,}$ $bla_{DHA,}$$bla_{ACC}$,$bla_{EBC,}$ and$bla_{FOX,}$ were used according to (Pérez-pérez & Hanson, 2002). The conventional PCR was performed in 25µL reaction containing 4.5µLof DNAse/RNase free water, 12.5µL reactions dream Taq master mix (New England Biolabs,USA), 0.5 µL of each of the following six forward and reverse primers (10 pmol): MOXMF,MOXMR, CITMF,CITMR,DHAMF, DHAMR, ACCMF,ACCMR, EBCMF, EBCMR and FOXMF, FORMR, specific for the genes of six different families of pAmpCβ-lactamases and 2.0 µL of DNA template. The PCR was then performed using a thermal cycler (Gene Amp PCR system 2400, Roche) with cycling condition of initial denaturation step at 94°C for 3minutes, followed by 30 cycles of DNA denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30

seconds and primer extension at 72°Cfor 1 minute. After the last cycle, a final extension step at 72°C for7 minutes was added. Ten microliter aliquots of the PCR products were separated and analyzed by gel electrophoresis with 2% agarose (Sigma Aldrich,USA). The gels were stained with SYBR Safe DNA gel and visualized under UV illumination. A 100 base pair DNA ladder (Fermentas,USA)was used as a marker. Negative controls for this assay were PCR products with water instead of template DNA.

Table 3.2: Sequences of primers used in conventional PCR to amplify six key genes that contribute to pAmpC phenotypes.

Primer	Sequence (5´ to 3´)	Target (s)	Expected amplicon size (bp)
MOXMF	GCT GCT CAA GGA GGA GCA CAG GAT	MOX-1, MOX-2, CMY-1 CMY-8 to CMY-11	520
MOXMR	CAC ATT GAC ATA GGT GTG GTG C	MOX-1, MOX-2, CMY-1 CMY-8 To CMY-11	
CITMF	TGG CCA GAA CTG ACA GGC AAA	LAT-1 to LAT-4 CMY-2 to CMY-7- BIL-1	462
CITMR	TTT CTC CTG AAC GTG GCT GGC	LAT-1 to LAT-4 CMY-2 to CMY-7- BIL-1	
DHAMF	AAC TTT CAC AGG TGT GCT GGG T	DHA-1 , DHA-2	405
DHAMR	CCG TAC GCA TAC TGG CTT TGC	DHA-1, DHA-2	
ACCMF	AAC AGC CTC AGC AGC CGG TTA	ACC	346
ACCMR	TTC GCC GCA ATC ATC CCT AGC	ACC	
EBCMF	TCG GTA AAG CCG ATG TTG CGG	MIR-1T, ACT-1	302
EBCMR	CTT CCA CTG CGG CTG CCA GTT	MIR-1T, ACT-1	
FOXMF	AAC ATG GGG TAT CAG GGA GAT G	FOX-1 to FOX-5b	190
FOXMR	CAA AGC GCG TAA CCG GAT TGG	FOX-1 to FOX-5b	

The primer sequences used in amplifications of pAmpC genes (Table 3.2) were obtained from a study by(Ghonaim & Moaety, 2018)

3.11 Phylo-grouping of uropathogenic E. coli isolates

Phylo-grouping of *E. coli* was performed by extended quadruplex PCR based phylo-type technique by (Clermont *et al*., 2013). The deoxyribonucleic genome of UPEC isolates was amplified by quadruplex PCR, using primers that target 3 markers namely: *chuA, yjaA* and TspE4.C2 and recently added *arpA*, which acts as an international control for DNA quality and distinguishes the phylo-group F formally mistaken as phylo-group D (Clermont *et al.*, 2013).

Table 3.3: Sequence of primers used for phylo-grouping of uropathogenic *E. coli.*

PCR Reaction	Primer ID	Target	Primer Sequences	PCR Product (bp)
Quadruplex	chuA.1b	chuA	5′-ATGGTACCGGACGAACCAAC-3′	288
	chuA.2		5′-TGCCGCCAGTACCAAAGACA-3′	
	yjaA.1b	yjaA	5′-CAAACGTGAAGTGTCAGGAG-3′	211
	yjaA.2b		5′-AATGCGTTCCTCAACCTGTG-3′	
	TspE4C2.1b	TspE4.C2	5′-CACTATTCGTAAGGTCATCC-3′	152
	TspE4C2.2b		5′-AGTTTATCGCTGCGGGTCGC-3′	
	AceK.f	arpA	5′-AACGCTATTCGCCAGCTTGC-3′	400
	ArpA1.r		5′-TCTCCCCATACCGTACGCTA-3′	
Group E	ArpAgpE.f	arpA	5′-GATTCCATCTTGTCAAAATATGCC-3′	301
	ArpAgpE.r		5′-GAAAAGAAAAAGAATTCCCAAGAG-3′	
Group C	trpAgpC.1	trpA	5′-AGTTTTATGCCCAGTGCGAG-3′	219
	trpAgpC.2		5′-TCTGCGCCGGTCACGCCC-3′	
Internal control	trpBA.f	trpA	5′-CGGCGATAAAGACATCTTCAC-3′	489
	trpBA.r		5′-GCAACGCGGCCTGGCGGAAG-3′	

Primer sequences used for assigning thephylo-groups of UPEC isolates (Table 3.3) were obtained from a study by(Clermont *et al.*, 2013).

A PCR reaction, as described by (Clermont *et al.*, 2013), was used to amplify the *arpA* (400bp) *ChuA* (288bp), *YjaA* (211bp),TspE4C2(152bp), and the internal control *trpA* (489bp) genes. The conventional PCR was performed in 25µL reaction containing 6.5µL of DNAse/RNase free water, 12.5µL reactions dream Taq master mix (New England Biolabs, USA), 0.5 µL of each of

the following four forward and reverse primers (10 pmol): chuA.1b, chuA.2, yjaA.1b, yjaA.2b, and TspE4C2.1b, TspE4C2.2bspecific for the genes of the six phylo-groups (A, B1, B2, A or C, D or E, and F, and 2.0 µL DNA mixture was added to the 23 µL master mixture. A negative control (reaction lacking the template DNA) was included in all performed amplifications.Using GeneAmp PCR System 9700 thermocycler (Applied Biosystem,USA),the assay was conducted under the following conditions:Initial denaturation at 94°C for 3 minutes, 30 cycles of each denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds and amplification at 72°C for 1 minute, then a final extension at 72°C for 7 minutes (Clermont *et al.*, 2013). The amplified DNA fragments were visualized after electrophoresis run in a 2% agarose gel with SYBR Safe DNA gel stain in TBE buffer for 50 minutes and visualized after electrophoresis under UV illumination, and then the gels were photographed.

The results allowed the classification of the isolates into either one of the six phylo-groups (A, B1, B2, A or C, D or E, and F,(Clermont *et al.*, 2013), All amplification procedures were repeated at least four times. Phylo-grouping was done based on the presence or absence of the DNA fragments (Table 3.4).Based on the results obtained from quadruplex genotype, an isolate was either assigned to a phylo-group or tested further using E or C specific primers E and C specific primers to classify Group D and A respectively as shown in (Table 3.4).

Table 3.4: Quadruplex PCR criteria for assigning UPEC isolates into various phylo-groups

ArpA 400bp	*chuA* 288bp	*yjaA* 211bp	TspE4. C2 152bp	Phylo-group	Next step
+	-	-	-	A	
+	-	+	-	A or C	Use C specific primers if C+ then C, else A
+	-	-	+	B1	
-	+	+	-	B2	
-	+	+	+	B2	
-	+	-	-	F	
+	+	-	-	D or E	Use E specific primers if E+ then E, else D
+	+	-	+	D or E	Use E specific primers if E+ then E, else D
+	+	+	+	Unknown	Perform MLST
-	-	-	+	Unknown	Perform MLST

UPEC isolates were assigned to different phylo-groups (Table 3.4) based on the method of(Clermont *et al.,* 2013). Phylo-grouping was based on the presence or absence of the DNA fragments. For example, *arpA*+, *chuA*-, *yjaA*– and TspE4.C2– groupA, *arpA*+, *chuA*-, *yjaA*+, and TspE4.C2–, group A or C, *arpA*+, *chuA*+, *yjaA*-, TspE4.C2+, group B1, *arpA*-, *chuA*+, *yjaA*+, TspE4.C2-, group B2, *arpA*-, *chuA*+, *yjaA*+, TspE4.C2 + group B2, *arpA*-,*chuA* +, *yjaA*-, TspE4.C2-, group F. *arpA*+*chuA*+, *yjaA*-, TspE4.C2- group D or E, *arpA*+, *chuA*+, *yjaA*-,TspE4.C2 + group D or E, *arpA*+, *chuA*+, *yjaA*+, TspE4.C2+ unknown, *arpA*-, *chuA*-, *yjaA*-,TspE4.C2 + unknown.

Table 3.5: Phylo-grouping of UPEC using E and C specific primers to classify Group D and group A, respectively

Internal control	group-specific primer	Group interpretation
trpA(489bp)	Group E primer*arpA*(301bp)	
+	-	D
trpA(489bp)	Group C specific primer *trpA*(219 bp)	
+	-	A

UPEC isolates which could not be assigned based on (Table 3.3) were further assigned (Table 3.5) according to(Clermont *et al.,* 2013).

Isolates assigned to a phylo-group or tested further using an internal control, E and C specific primers, to classify as Group D and Group A, respectively.

3.12 Statistical analysis

The data for this study were entered in Microsoft Excel and analysed using Statistical Package for Social Sciences (SPSS) version 20, where the frequencies and percentages for the occurrence of ESBL and AmpC β-lactamases plus the phylo-groups for UPEC isolates were obtained through cross-tabulation in descriptive statistics.

3.13 Ethical considerations

The primary study (KEMRI # 2767/ WRAIR #2089) protocol version 2.0 Dated 1 September 2016 was approved by the Kenya Medical Research Institute (KEMRI) /Scientific Ethics Review Unit (SERU) and the Walter Reed Army Research (WRAIR) Institutional Review Board (IRB). This study was approved by KEMRI SERU, WRAIR IRB, and Maseno University Ethical Review Committee.

3.13.1 Confidentiality

The investigator did not have access to or require identifiable subject data from the parent study.

CHAPTER FOUR

RESULTS

4.1 ESBL and AmpC resistance in the UPEC isolate

4.1.1ESBL resistance

Selection of ESBL producers among the uropathogenic *E. coli* using the ceftazidime by minimum inhibition concentration (MIC) (≥ 16µg/ml) cut-off.

Table 4. 1 : Results of UPEC isolates by ceftazidine MIC

Isolates (selected)	Ceftazidime MIC
Isolate 1	>16
Isolate 2	>16
Isolate 3	>16
Isolate 4	>16
Isolate 5	>16
Isolate 6	>16
Isolate 7	>16
Isolate 8	>16
Isolate 9	>16
Isolate 10	>16
Isolate 11	>16
Isolate 12	>16
Isolate 13	>16
Isolate 14	>16
Isolate 15	>16
Isolate 16	>16
Isolate 17	>16
Isolate 18	>16
Isolate 19	>16
Isolate 20	>16
Isolate 21	>16
Isolate 22	>16
Isolate 23	>16

Ceftazidime minimum inhibition concentration (MIC) of (> 16µg/ml) 23 of 95 UPEC isolates from the five study Counties. A MIC of >16µg/ml indicates resistance to ceftazidime typically caused by ESBL production.

4.1.1.1 Phenotypic confirmatory test of ESBL producers among the UPEC isolates

A total of 23 (24.2%) out of the 95 UPEC isolates that screened positive for ESBL production by MIC (≥ 16 ug/ml) using ceftazidime were confirmed to be ESBL producers. These isolates were from Nairobi 12/23;(52.2%), Kilifi 4/23;(17.4%), Kisumu 5/23;(21.7%), and Kericho 2/23;(8.7%). Kisii did not have any UPEC ESBL producers (Table 4.2).

Table 4.2: Distribution of the ESBL producersamong the five counties

COUNTY	Frequency per County (n)	Percent (%)
NAIROBI	12	52.2
KILIFI	4	17.4
KISUMU	5	21.7
KERICHO	2	8.7
KISII	0	0.0
Total	23	100.0

The frequency and percentage distribution of the 23 UPEC ESBL producers within the five study Counties.

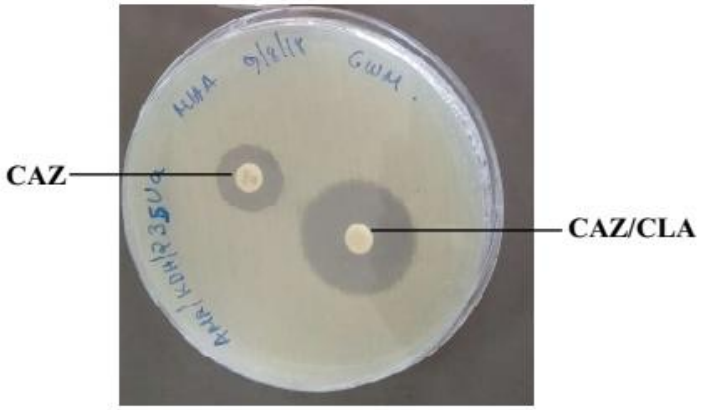

Figure 4.1: Apositive CDDT of a UPEC ESBL producer. The isolate was number 3 from Kisumu County. The figure indicates a larger zone of inhibition with versus without the inhibitor. The inhibition zone size >5mm in the disk with ceftazidime(30µg) plus clavulanic acid(10 µg) (CAZ+CLA) was compared to ceftazidime(30µg) (CAZ)disk alone.

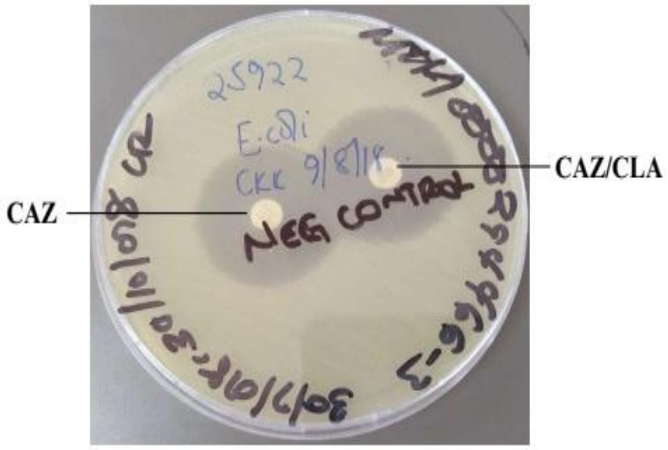

Figure 4.2: An ESBL negative control test of *Escherichia coli* ATCC 25922 using a combination disc diffusion test.The inhibition zone size of > 5mm in both the disk with ceftazidime (30 µg) plus clavulanic acid(10 µg) (CAZ/CLA) and ceftazidime (CAZ) (30µg) disk alone indicates a negative ESBL test.

Figure 4.3: An ESBL positive control test of Klebsiella pneumoniae ATCC 700603 using a combination disc diffusion test.The inhibition zone size of> 5mm in the disk with ceftazidime (30 µg) plus clavulanic acid(10µg) (CAZ/CLA) compared to ceftazidime(CAZ) (30µg) disk alone indicates a positive ESBL test.

4.1.2 AmpC resistance

4.1.2.1AmpC screening using the disk diffusion method

A total of (5/23);(21.7%) phenotypically confirmed ESBL producers were cefoxitin resistant.These five isolates were from Nairobi 2/5;(40%), Kilifi 2/5;(40%), and Kisumu 1/5;(20%) Counties. Kericho and Kisii did not have any cefoxitin resistant UPEC isolate (Table 4.3).

Table 4.3: Cefoxitin resistant UPEC isolates

COUNTY	Frequency (n)	Percent (%)
NAIROBI	2	40.0
KILIFI	2	40.0
KISUMU	1	20.0
KERICHO	0	0.0
KISII	0	0.0
Total	5	100.0

The frequency and percentage distribution of the 23 UPEC ESBL producers within the five study Counties

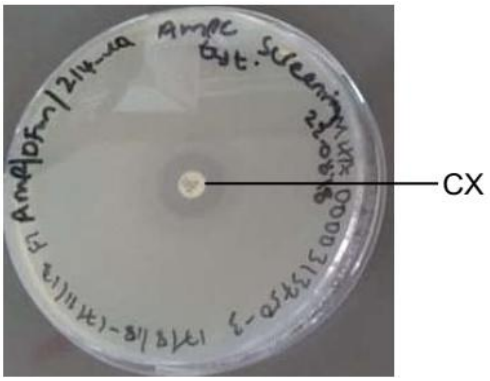

Figure 4.4:A positive AmpC screening test using the disk diffusion method. Inhibition zone size of <18mm in the disk with cefotixin (30µg) (CX)shows a positive test. The UPEC test isolate was number 2 from Nairobi County.

4.1.2.2 AmpC disk approximation test

Cefoxitin resistance is indicative of an ESBL phenotype conferred not by the typical genes but by an AmpC gene. However, none of the five isolates was an AmpC producer bythe AmpC disk approximation test (Figure 4.5B).

Figure 4.5A: Positive AmpC approximation test, **B** Negative AmpC approximation test.

Blunting of the zone of inhibition around Cefotaxime(CTX) (30µg) disc on the side adjacent to Cefoxitin (CX) (30µg) disc is a positive AmpC approximation test indicating the presence of inducible AmpC producer. (Madhumati *et al.,* 2015).**Figure 4.5B** there is a circular zone of inhibition around cefotaxime (CTX) (30µg) disc, a negative AmpC approximation test that indicates the absence of AmpC production.

4.2 Types of ESBL and AmpC resistance genes

4.2.1 ESBL resistance genes

Molecular detection of ESBL genes among the 23/95;(24.2%) phenotypically confirmed ESBL-producing UPEC isolates, showed that all of the isolates possed at least one ESBL genes. The results indicated that the bla_{TEM} and $bla_{\text{CTX-M}}$ were the predominant ESBL genes with each present in 22/23;(95.6%) isolates, followed by bla_{SHV} 5/23;(21.7%). This study found that

5/23;(21.7%) isolates had three ESBL genes (*bla*$_{TEM}$, *bla*$_{CTX-M,}$ and*bla*$_{SHV}$) each. Sixteen positive ESBL producers16/23;(69.6%) had *bla*$_{CTX-M}$ and *bla*$_{TEM}$ genes (Figure 4.6)

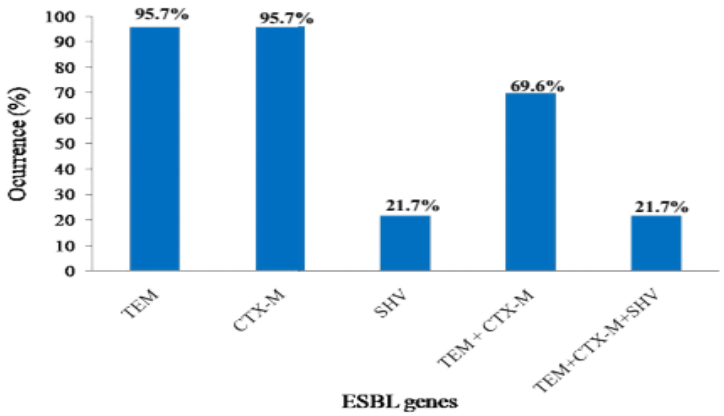

Figure 4.6: Occurrence of ESBL genes among UPEC isolates in the five study Counties**.** The occurrence of the four ESBL genes, namely: Temoneira (TEM), sulfhydryl variable (SHV), and cefotaximase-Munich (CTX-M) among the 23/95;(24.2%) UPEC isolates. The figure shows the presence of single-gene and multiple-gene combinations.

4.2.1.1 Distribution of ESBL genes among the five study counties

The *bla*$_{TEM}$and *bla*$_{CTX-M}$genes predominated among UPEC isolates in Nairobi County with 11/12;(91.7)% and 10/12;(83.3%) isolates respectively.One out of the 12;(8.3)% isolates in Nairobi Countydid not have the *bla*$_{TEM}$gene and 2/12;(16.7)% isolates lacked*bla*$_{CTX-M}$ genes. None of the 12 isolates in Nairobi County had the *bla*$_{SHV}$gene. InKisumu County,*bla*$_{TEM}$and *bla*$_{CTX-M}$ predominated each with 5/5; (21.7%) UPEC isolates studied from the County, followed by *bla*$_{SHV}$with 2/5; (40.0%)of the isolates. Three of the 5 (13.0%) isolates in Kisumu County had both *bla*$_{TEM}$and *bla*$_{CTX-M}$ genes, while 2/5;(40%) of the isolates had all the three genes (*bla*$_{TEM,}$*bla*$_{SHV,}$ and *bla*$_{CTX-M}$ genes). The 4/4;(17.3%) isolates in Kilifi county had both *bla*$_{TEM}$ and *bla*$_{CTX-M}$genes, 3/4;(75%) of the isolates had *bla*$_{TEM,}$ *bla*$_{CTX-M}$ and *bla*$_{SHV}$genes. Two isolates in Kericho county had both *bla*$_{TEM}$and *bla*$_{CTX-M}$genes, and none of the isolates in Kericho County had b*la*$_{SHV}$ genes.There were no ESBL producers in Kisii County (Table 4.4).

Table 4.4: Distribution of ESBL genes in five study Counties.

COUNTY	ESBL UPEC Isolates per county	ESBL GENES					
		bla$_{TEM}$		*bla*$_{SHV}$		*bla*$_{CTX-M}$	
		+	-	+	-	+	-
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
NAIROBI	12(52.2)	11(91.7)	1(8.3)	0(0.0)	12(100.0)	11(91.7)	2(16.7)
KISUMU	5(21.7)	5(100.0)	0(0.0)	2(40.0)	3(60.0)	5(100.0)	0(0.0)
KILIFI	4(17.4)	4(100.0)	0(0.0)	3(75.0)	1(25.0)	4(100.0)	0(0.0)
KERICHO	2(8.7)	2(100.0)	0(0.0)	0(0.0)	2(100.0)	2(100.0)	0(0.0)
KISII	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
TOTAL	23(100)	22(95.7)	1(4.3)	5(21.7)	18(78.3)	11(91.7)	2(8.7)

Nairobi County had the highest number of ESBL producing UPEC isolates (n=12),followed by Kisumu with 5/23;(52.2%) isolates, Kilifi 4/23;(17.4%),Kericho 2/23;(8.7%) zero ESBL producers in Kisii County.

4.2.2 AmpC resistance genes

4.2.2.1 Multiplex PCR for detection of pAmpC β-lactamases genes

Multiplex PCR for the detection of pAmpC genes (*bla*$_{MOX}$,*bla*$_{CIT,}$ *bla*$_{DHA,}$ *bla*$_{ACC}$, *bla*$_{EBC,}$ and *bla*$_{FOX}$), were negative for all the cefoxitin resistantUPEC isolates.The same results were obtained from the screen for chromosomal encoded AmpC. That indicates that none of the known genes conferring cefoxitin resistance in the UPECs were present either on the plasmid or chromosome.

4.3 Phylo-groups of uropathogenic*E. coli* isolates

4.3.1 Distribution of the five phylo-groups within the study Counties

Ninety-three out of the 95 UPEC isolates, were assigned to 5 of the eight phylo-groups per study County using extended quadruplex PCR method. The assigned groups were: (A, B1, B2, D, and F). Nairobi county had the highest number of the isolates 39/95;(41.1%) of which Group B2 15/39;(38.5%) predominated, followed by groups D 12/39;(30.8%), B1 5/39;(12.8%), A 4/39; (10.3%), F 1/39;(2.6%) and 2/39;(5.1%) unassigned isolates. Kisumu County had 28/95;(29.5%) isolates assigned into 4 of the five phylo-groups. Group D 12/39;(42.9%) predominated, followed by group B2 7/28; (25.0%), group A 6/28;(21.4%),and group B1 3/28;(10.7%). Finally, group F did not assign any isolate. Kericho County had 14/95;(14.7%) isolates that were assigned to four of the five phylogenetic groups. Groups A, B2, and D predominated each with 4/14; (28.6%) isolates followed by group B1 2/14;(14.3%) and no isolate assigned into group F. Kilifi County had 10/95;(10.5%) isolates assigned into five phylogenetic groups. Group B2 predominated with 4/10;(40.0%), followed by group A 3/10;(30.0%) and groups B1, D, and F each with 1/10; (10.0%). Kisii County had 4/95;(4.2%) isolates assigned into 4 of the five phylogenetic groups. Groups A, B2, D, and F each with 1/4;(25.0%) isolates. Group B1 was not assigned any isolate (Table 4.5).

Table 4.5: Distribution of the phylo-groups of UPEC isolates in five study Counties

COUNTY	Isolates per County	PHYLO-GROUP					
		A	B1	B2	D	F	Ungrouped
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
NAIROBI	39(41.1)	4(10.3)	5(12.8)	**15(38.5)**	12(30.8)	1(2.6)	**2(5.1)**
KISUMU	28(29.5)	6(21.4)	3(10.7)	7(25.0)	**12(42.9)**	0(0.0)	0(0.0)
KERICHO	14(14.7)	4(28.6)	2(14.3)	4(28.6)	4(28.6)	0(0.0)	0(0.0)
KILIFI	10(10.5)	3(30.0)	1(10.0)	4(40.0)	1(10.0)	1(10.0)	0(0.0)
KISII	4(4.2)	1(25.0)	0(0.0)	1(25.0)	1(25.0)	1(25.0)	0(0.0)
TOTAL	95(100.0)	18(18.9)	11(11.6)	31(32.6)	30(31.6)	3(3.2)	2(2.1)

4.3.2 The occurrence of ESBL producing isolates within the five phylo-groups

Phylo-group B2 predominated with 11/23;(47.8%),followed by group A 5/23;(21.7%),group D 3/23;(13.0%), group B1 2/23;(8.7%) and group F 1/23;(4.3%). One out of 23 ESBL producerscould not be grouped. (Table 4.6)

Table 4.6: Distribution of ESBL among the phylo-groups

Phylo-Groups	ESBL	
	Frequency (n)	Percent (%)
A	5	21.7
B1	2	8.7
B2	11	47.8
D	3	13.0
F	1	4.3
Ungrouped	1	4.3
Total	23	100.0

The distribution of the ESBL among the phylo-groups shows that the majority of the ESBL belonged to phylogroup B2, followed by group A, group D group B1, group F, and ungrouped isolates.

4.3.3 Determination of the phylo-groups of UPEC isolates

The phylo-groups of UPEC isolates were determined by the presence or absence of the four genes(*arp A,chu A,yja A, and* TspE4C2)(Figure 4.7)

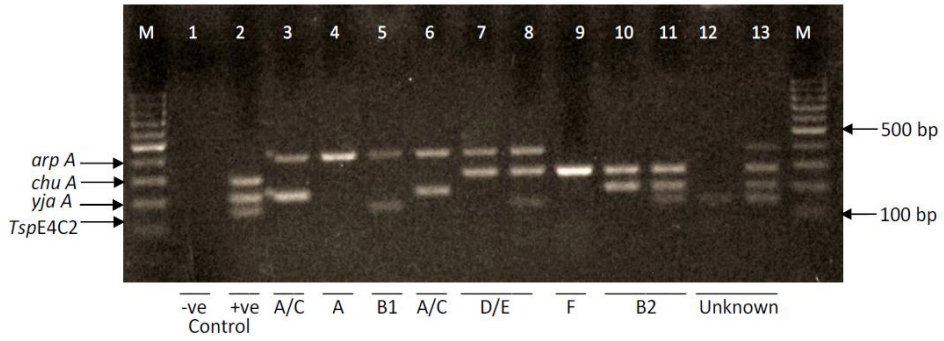

Figure 4.7: Gel electrophoresis images for the extended Quadruplex PCR profile

Extended Quadruplex PCR profiles of selected UPEC isolates representing each Clermont based phylo-group.The samples from well one to 13 were –ve,+ve controls,phylo-groups A/C,A,B1,A/C,D/E,F and B2 for wells 1,2,3, 4, 5, 6, 7, 8, 9, 10 and 11 respectively. Two isolates were ungrouped on wells 12 and 13, respectively. All the 13 isolates were from Kisumu, Kericho, Kilifi, and Kisii Counties. The isolates were assigned to a phylo-group according to the presence or absence of the following genes in the order (*arpA*, *chuA*, *yjaA*, and TspE4C2). Where, Group A is (+- - -) Groups A/C is (+ - + -), Group B1 is (+- - +) Group B2 is (- + + -) Groups D or E (+ + - -) or (+ + - +) Group F is (- + - -) and other combinations represent unknown Groups (- + ++), (- - - +) and (+ + + +).

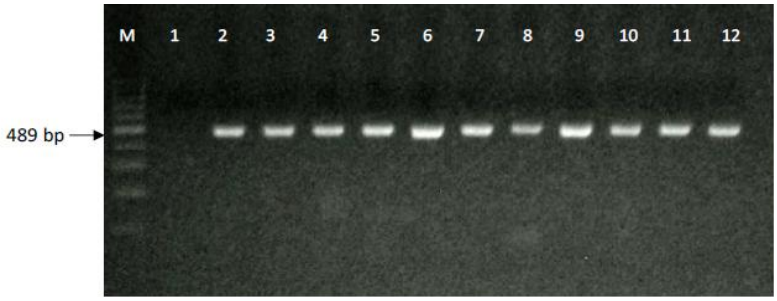

Figure 4.8: Gel electrophoresis image of the PCR amplicons used to differentiate phylo-group Group D/E. Group D or E. Lane M 100 bp molecular weight marker (England bio labs,USA); Lane 1 negative control Lane 2 to 12; only one single band for internal control was present. The presence of the internal control band only (489 bp) indicates

the Group D while amplification of the arpA (301 bp) indicates Group E. In this case, the second band for Group E specific arp A fragment (301 bp) was absent. So the isolates belong to Group D.

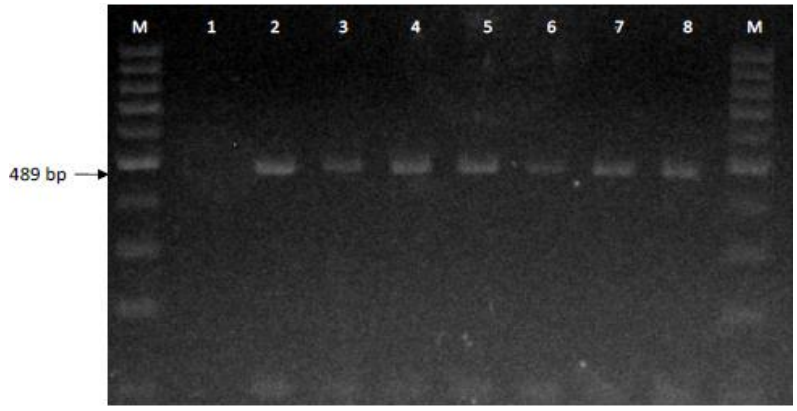

Figure 4.9: Gel electrophoresis image of the PCR amplicons used to differentiate Group A/ C. Group A or C differentiation. M 100bp molecular weight marker (England bio labs,USA); Lane 1 negative control. Lane 2 to 8, only one single band for internal control was present. The presence of the internal control band only (489bp) indicates the Group A while amplification of the trpA (219bp) indicates Group C. In this case, the second band for Group C specific trpA (219bp) was absent.

CHAPTER FIVE

DISCUSSION

5.1 Introduction

Urinary tract infections are among the most common bacterial infections affecting hospitalized and non-hospitalized patients globally (Alqasim *et al.,* 2018).The leading etiological agent responsible for causing these infections is UPEC(Al-jamei *et al.,* 2019). Urinary tract infections (UTIs) are treated with a class of antibiotics known as β-lactams. However, there is increasing resistance to antibiotics used in the treatment of UPEC infections, and this is of clinical concern(Onanuga *et al.,* 2019).The occurrence of ESBL-producing UPEC isolates varies from country to country and from region to region (Jena *et al.,* 2017).

5.2 The phenotypic occurrence of ESBL in UPEC isolates

This study showed that the general occurrence of ESBL among UPEC isolates was 23/95; (24.2%). Nairobi, Kisumu, Kilifi, Kericho, and Kisii Counties each had 12/23;(52.2%), 5/23;(21.3%), 4/23;(17.4%), 2/23;(8.7%) and zero ESBL producers respectively (Table 4.2). The high occurrence of ESBL in Nairobi County could be due to the overuse of the antibiotics both at the hospital and community levels within the County. The present study has showed a similar trend to a previous study done in Kenya (Kiiru *et al.,* 2012) on the occurrence of ESBL among UPEC isolates. That study reported the detection of 27% ESBL producers from clinical samples collected in 18 years (from 1992-2010).Kiiru *et al.,* study, aimed to determine the β-lactamases phenotypes and carriage of *bla* genes of critical importance in *E. coli* obtained from blood, stool, urine from hospitalized and non-hospitalized patients seeking treatment in Kenyan hospitals during 18 years (1992 to 2010) (Kiiru *et al.,* 2012). The similarity between Kiiru *et al.* and this study is that the existenceof the ESBL producers among the UPEC isolates is still high in Kenya. This implies, that there is ;a higher chance of developing multidrug resistance to β-lactams;

hence, increased patient treatment failure given that the presence of these genes have been associated with multidrug resistance in other studies(Lohani *et al.,* 2019; Shaikh *et al.,* 2015).However, the difference between the findings of this study and those of the Kiiru *etal*., is that the present study phylo-typed and showed the geographical distribution of the isolates per study County. Four out of the five Counties had UPEC isolates that were ESBL producers.

The findings of this study, further show a high occurrence of resistance to the 3rd generation cephalosporins, the drugs used to treat UTIs. The high occurrence of ESBL production could be attributed to the misuse of antibiotics by health care workers in hospitals in addition to the self-prescription by the community (Jaggi *et al.,* 2012). The health care workers misuse antibiotics by offering drug prescriptions without the culture and sensitivity results. Another reason for the high ESBL occurrence could be a lack of continuous antibiotic surveillance studies (Ouedraogo *et al.,* 2016). The surveillance studies would provide data that demonstrates the need for antibiotic stewardship and guide patient treatment by indicating the lower level antibiotics that are still effective for use so that the efficacy of higher level drugs efficacy can be preserved

5.3 Phenotypic occurrence of pAmpC β-lactamases

Production of plasmid-mediated AmpC β-lactamases (pAmpC) is a public health concern, since there are limited therapeutic options for infections caused by Gram-negative organisms, expressing the enzyme (Ghonaim & Moaety, 2018); The pAmpC producers are usually resistant to β-lactams except cefepime, cefepirome and carbapenems (Nasir *et al.*, 2015). According to this study, 5/23 ;(21.7%) ESBL producers were cefoxitin resistant. These isolates were from Nairobi and Kilifi Counties, each with 2/5; (40%) isolates. The remaining 1/5 ;(20%) isolate was from Kisumu County (Table 4.3). However, the phenotypic confirmatory tests performed on all the five cefoxitin resistant isolates, demonstrated that they were negative for AmpC production

(figure 4.5). These findings suggests that resistance due to pAmpC genes is still not a problem within our hospitals set up. The exhibited cefoxitin resistance despite lack of pAmpC production by the isolates could be due to the presence of other enzymes rather than pAmpC β-lactamases and are worth investigating further.

5.4 Genotypic detection of ESBL genes

The Extended-spectrum β-lactamases genes have been reported to be diverse (Jena *et al.*, 2017). In the past decade, bla_{TEM} and bla_{SHV} genes were reported to be the most common types of ESBL genes, but currently, the bla_{CTX-M} gene has been spread worldwide compared to bla_{TEM} and bla_{SHV} genotypes (Bajpai *et al.,* 2017). The TEM and SHV types of enzymes degrade penicillin but are susceptible to cephalosporins combined with β-lactam inhibitor such as clavulanic acid (Kiiru *et al.,* 2012). On the other hand, CTX-M can hydrolyzed advanced spectrum cephalosporins and monobactums (Bush, 2010). In this study, all the 23/95; (24.2%) ESBL-positive *E. coli* isolates possessed at least one of these genes. In general, the study showed that bla_{TEM} and bla_{CTX-M} genes predominated in our study population, each with 22/23 ;(95.7%) isolates followed by bla_{SHV} with (69.6%) isolates (figure 4.6). The predominance of the bla_{TEM} and bla_{CTX-M} genes indicated the possibility of rapid dissemination of these genes from both the hospital and the community.

The overall distribution of ESBL genes between Nairobi, Kisumu, Kilifi, Kericho and Kisii Counties was indicated as: 12/23;(52.2%), 5/23;(21.7)%, 4/4;(100%), 2/23;(8.7%) and zero respectively. A co-existence of bla_{TEM} and bla_{CTX-M}, genes predominated in all the five Counties as 11/12; (91.7%), 5/5; (100%), 4/4;(100%), 2/2;(100%) and 0% respectively. Two isolates from Nairobi County had each a single gene, i.e., bla_{TEM} and bla_{CTX-M}, respectively. Further, not a single isolate in this County had bla_{SHV} genes (Table 4.4). In Kisumu and Kilifi Counties 2/5;

(40%) and 3/4; (75.0%) isolates had an additional *bla*$_{SHV}$ genes respectively (Table 4.4). Lack of ESBL producer isolatesin Kisii County could be due to the few isolates studied in these study. The findings on a wide distribution of ESBL genes among the UPEC isolates in Nairobi County could implicate high chances of β-lactamase antibiotic resistance, leading to treatment failure and recurrence of infections among the UTI patients in the County. The genes detected in this study, *bla*$_{TEM}$, *bla*$_{CTX-M,}$ and *bla*$_{SHV}$ have been previously reported in Kenya (Kiiru *et al.,* 2012; Maina *et al.,*, 2017) and it's neighboring nation, Western Uganda (Nakaye *et al.,* 2014). Further, the finding of this study suggests a possible danger of UPEC resistance to the 3rd generation cephalosporins in our hospitals set up. This danger warrants a push for judicious use of the 3rd generation cephalosporin although the evidence presented is only suggestive since few isolates were tested in this study. Additional surveillance studies in larger population that includes CTX-M gene sub-typing are required to confirm these findings.

5.5 Genotypic detection of pAmpC β-lactamases

The PCR assay to detect transferable AmpC β-lactamases indicated that there were no possible pAmpC β-lactamase genes in ESBL UPEC isolates studied. These could mean that the broad-spectrum cepharosporins are still useful in Kenyan hospitals. This findings are in agreement with other previous studies that have observed that not all cefoxitin resistant isolates are AmpC β-lactamases producers (Madhumati *et al.,* 2015; Helmy and Wasfi 2014). In contrast, the findings differ with a report in Kenya that reported 10% of *E. coli* isolates were pAmpC producers (Kiiru *et al.,* 2012) and a similar study in Uganda, reporting 37% of pAmpc β-lactamases on cefoxitin resistant isolates with 30 isolates having more than one gene coding for resistance (Nakaye *et al.,* 2014). These two studies differed from this study in several important ways. The first difference was that they had a larger sample size (912 and 293 respectively), and the second was the source

of isolates, included the blood, urine, pus aspirates, and stool samples. UPEC isolates were a minority for the samples tested in these studies, so it may be that UPEC may have fewer AmpC producers than *E. coli* from other sources. Cefoxitin resistance in the absence of AmpC production can be caused by other enzymatic mechanisms such as the production of different ESBLs and non-enzymatic mechanisms such as decreased porin entry channels (Nakaye *et al.*, 2014). This warrants additional investigation of those mechanisms in the UPEC isolates.

5.6 Determination of the phylogenetic distribution of UPEC isolates

Escherichia coli strains can be classified into eight phylogenetic groups: (A, B1, B2, C, D, E, F, and *Escherichia* Clade I that differ in their geographical characteristics (Clermont *et al.*, 2013). The presence of multiple antibiotic resistance genes in different phylo-groups indicate the importance of UPEC isolates in public health (Bozcal *et al.*, 2018). In the current study,3/4; (75.0%) and2/5; (40%) UPEC isolates that had multiple ESBL genes in Kilifi and Kisumu Counties belonged to phylo-group F, A, B1, D and A respectively (Table 4.4). These findings suggests horizontal transfer of the resistant genes from the virulence groups to commensal groups that warrant close monitoring.

In this study, the overall report showed that phylo-group B2 31/95;(32.6%) predominant followed by group D 30/95;(31.6%), group A 18/95;(18.9%), group B1 11/95;(11.6%), group F 3/95;(3.2%) and an unknown group 2/95;(2.1%). The study further showed that Nairobi County had the highest number of UPEC isolates analyzed 39/95;(41.0)%. Phylo-group B2 predominated in the County with 15/39;(38.5%) followed by group D 12/39;(30.8%), group B1 5/39;(12.8%), group A 4/39;(10.3%), group F 1/39;(2.6%) and unassigned isolates 2/39;(5.1%). In Kisumu County, 28/95;(29.5)% isolates were assigned to four of the five phylo-groups. Group D predominated in the County, with 12/39;(42.9%) followed by group B2 7/28;(25.0%), group A

6/28;(21.4%), and group B1 3/39;(10.7%) and group F was not assigned any isolate. In Nairobi and Kisumu Counties, the predominant phylo-groups were different, suggesting different regional distribution (Table 4.5). Kericho County had 14/95; (14.7) % isolates that were assigned to four of the five phylo-groups. Groups A, B2, and D predominated each with 4/14; (28.6%) isolates followed by group B1 2/14; (14.3%) and no isolate assigned in group F. Kilifi County had 10/95; (10.5%) isolates assigned in five phylo-groups. Group B2 predominated with 4/10; (40.0%), followed by group A 3/10; (30.0%) and groups B1, D, and F each with 1/10; (10.0%). Kisii County had 4/95; (4.2%) isolates assigned into 4 of the five phylo-groups. Groups A, B2, D, and F each with 1/4; (25.0%) isolates. Group B1 was not assigned any isolate (Table 4.5). The difference in the distribution of isolates in various phylo-groups in this study differs significantly from County to County. This variation may be due to the health status of the host, the diet and genetic factors of the host, overuse of the antibiotic, social, environmental, and geographical climatic conditions or different areas facilities that the samples were collected from (Derakhshandeh *et al.*, 2013).

Determination of *E. coli* phylo-groups is of epidemiological importance as several reports are indicating that phylo-groups could be of potential relationship to diseases (Abd ALameer, 2015). There is a relationship between the phylogeny and the virulence and that the virulent extraintestinal strains belong to phylo-group B2 and, to a lesser extent, to group D (Iranpour *et al.*, 2015). In contrast, most commensals belong to phylo-group A and group BI.

This study has shown that the majority of the isolates that were ESBL producers belonged to phylo-group B2 11/23; (47.8%) and group A 5/23; (21.7%), followed by group D 3/23; (13%), group B1 2/23; (8.7%), group F 1/23; (4.3%) and ungrouped isolate 1/23; (4.3%) (Table 4.6).This investigation demonstrated a link between the phylo-group and

penicillin/cephalosporin resistance. Tracking of these phylo-groups will give an indication of the burden of ESBL producers among UPECs. This study indicates that the majority of the isolates in phylo-group B1 and groupA were non-ESBL producers. The presence of a few ESBL producers among the commensal groups B1, A, and F shows the potential horizontal transfer of the antimicrobial resistance genes from the phylogenetic groups associated with resistance to the typically non-ESBL producing phylo-groups.

The occurrence of phylo-group F 3/95; (3.2%) in only a few isolates in the present study was similar to that described by Iranpour *et al.*,2015, that showed the presence of only 2.9 % of group F. Isolates in groups C, E, and Clade 1 were not detected in this study. The groups C, E, and Clade 1 could be geographically restricted as have been described in previous studies of UPEC in Australia and Iran (Iranpour *et al.,* 2015).

In this study, two isolates could not be assigned to any of the eight recognized phylo-groups using the new extended quadruplex method. These may have occurred because the assay used the primers specific to currently known genes and are unable to detect novel genes. According to Clermont *et al.,* 2013), not all *E. coli* strains will be assigned to a phylo-group due to the rare occurrence of the minor or novel phylo-groups. The new strains could emerge due to large scale recombination between two or more different phylo-group, or the *E. coli* genome was affected by gain or loss of genes. These un-grouped isolates could be other species of UPEC, worth investigating in further studies.

5.7 Study limitations

This study was limited by the limited number of isolates available and the difference in the number of isolates from each County. The study was not powered for statistical analysis to analyze the distribution across Counties. This was, therefore, not an objective of the study, but

the study has provided a baseline for future studies to be conducted as the numbers of isolates increase. The study lacked all the controls to test for the presence of pAmpC genes, and the reagents for sequencing the two unassigned UPEC isolates to a phylo-group UPEC, so these provide areas of future research. The findings of this study are from only four out of the forty seven Counties in Kenya but still represents the distribution of *E. coli* isolates studied in Kenya and shows the diversity in ESBL prevalence and types of strains that can occur between Counties. This information can assist future studies should, therefore, incorporate more Counties in Kenya that would give a better national report of the occurrence of ESBL and AmpC β-lactamases, as well as phylo-groups of UPEC, isolates in Kenya

5.8 Conclusion

This study has determined that ESBL-producing UPEC isolates are present in our hospitals, with 24.2% of the isolates being resistant to the commonly prescribed cephalosporin drugs for UTIs; and that Nairobi County had the highest number of ESBL UPEC isolates producers. This indicates a strong antibiotic selection pressure in Kenyan hospitals and community settings driving resistance to these widely prescribed drugs.However, AmpC production was not detected. This is an indication of a possible other cefoxitin resistant mechanisms that warrants further investigation

The $bla_{\text{CTX-M}}$ and bla_{TEM} genes were predominant in this study with the co-existence of multiple genes in single isolates indicates increased transmission of the genetic determinants and the further increase of ESBL pathogens in Kenyan hospitals. However,the AmpC genes typically associated with cefoxitin resistance were not observed in this study. The findings suggest UPEC resistance to the 3rd generation cephalosporins in our hospitals set up and warrants call for judicious use of the 3rd generation cephalosporin.

The phylo-group B2 and D predominated in these study. Phyo-group B2 and A were identified as the high-risk clones for ESBL in UPEC. Nairobi County recorded the highest number of ESBL producers that belong to these phylo-groups likely due to the overuse of the antibiotics in the County.The typing of the UPEC phylo-groups has contributed to the understanding of the epidemiology of UPEC isolates in Kenya.

5.9 Recommendations and suggestions for further research

Continued surveillance studies to monitor the occurrence of ESBL and AmpC producers plus the genes associated with the resistance in UPEC isolates should be done in our hospitals. Such studies will provide information about the key drug-resistant isolates to guide empirical therapy with β-lactam drugs. Patients with symptoms of UTI's should take a culture and sensitivity test first, to identify if the infections are treatable to 1^{st} or 2^{nd} line cephalosporin or penicillin drugs so that a step-wise approach to treatment can be implemented and 3^{rd} and 4^{th} line drugs can be reserved for the most resistant infections. This will halt the selection of and transmission of ESBL producers in UPEC isolates.

Additional surveillance studies in larger population are required to confirm the presence of alternative cefoxitin resistant enzymes rather than the pAmpC β-lactamases.

Close monitoring of the high-risk phylo-groups B2 and A associated with ESBL producers should be continuous and where possible mapped to prevent there spread. Whole-genome sequencing of the atypical UPEC isolates should be done to expand our knowledge on novel/unassigned UPEC phylo-groups.The identification of the new uncharacterized phylo-groups emerging on the landscape opens up new avenues for the study of these important pathogens

Additional studies on UPEC isolates from other hospitals in Kenya will build on this study to provide a greater understanding of the occurrence, geographical distribution, and drug resistance profiles of UPEC phylo-group.

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APPENDICES

Appendix I: Informed consent for the parent protocol

Appendix 2a: INFORMED CONSENT FORM – Adults, Parent/Guardian (ENGLISH version)

Study Title: Antimicrobial resistance surveillance in military and civilian populations in Kenya

Study sponsor: Department of Emerging Infectious Diseases (DEID), United States Army Medical Research Directorate - Kenya (Walter Reed Project).

Principal Investigator and institution: Lillian Musila, PhD. - United States Army Medical Research Directorate – Kenya (USAMRD-K)

Other investigators and institutions:
MAJ Dr. Angela Nderi, Defense Forces Memorial Hospital (DFMH) - Kenya Defense Forces
Willie Sang, PhD. - Kenya Medical Research Institute.
Valerie Oundo - United States Army Medical Research Directorate - Kenya

Study funders: This research is paid for by the Global Emerging Infectious Disease Surveillance Systems GEIS) program of the Armed Forces Health Surveillance Branch, USA

Introduction: A study is being done by the Kenya Medical Research Institute/Walter Reed Project in partnership with the Kenya Defense Forces in Kenya looking at the types of germs that cause infections. The doctor in the hospital has found that you (your child) have/has an infection. This is why we would like to tell you more about the study, answer any of your questions and invite you (your child) to take part in the study.

Purpose of study: This study wants to find out which germs (bacteria) are causing infections in hospitals and in the community. Particular types of germs are a big problem because they cannot be killed by the drugs that are being used to treat the infections. In this study, we want to find out which drug resistant germs are causing infections at this hospital and which drugs can still kill them. We also want to find out why the germs are not being killed by the drugs, how they are spreading and what makes it easier for them to infect people. This information will help the hospital treat these infections and help us understand how to stop them from spreading in the hospitals and our communities in Kenya and the world.

Enrollment requirements: Anyone older than 2 months of age who has been identified as having an infection can join the study. Pregnant women can join the study but prisoners and mentally challenged individuals cannot take part in the study.

Study duration and enrollment: This study will be going on for 5 years and we hope to be able to test samples from up to 1000 people from this hospital every year and from other hospitals in Kenya. If you accept to be part of the study you will indicate your agreement by signing this form or providing a thumb print if you cannot write. The process of consent will be witnessed if you are unable to read this form. You (your child) will be required to participate in the study only for as long as it takes to get all the information and the sample is collected. You (your child) can participate in the study more than once during the 5 years if you/ your child come(s) to the hospital again with an infection.

Study procedure: We will get some medical information from your (your child's) doctor or medical record, take some measurements and also ask you (your child) some personal questions about yourself (your child) such as your (your child's) age, sex, where you live and if you (your child) has been sick

before. Your doctors may have taken some samples to test but we will also collect some samples from where you (your child) are infected to test for this study. We will take care to respect your (your child's) safety and privacy during sample collection.

- **Wound/throat/skin infection:** We will use either a swab or a syringe to collect some material. The swab and syringe collection will be taken quickly and you (your child) will not feel a lot of pain.
- **Blood**: If your child weighs less than 25kg we will collect a total of about half a teaspoon for every year of birth. If you (your child) weigh(s) more than 25kg we will take a total of about 2 tablespoons of blood. We will collect half this blood at one time and then after about one hour we will collect the rest. The blood collection will involve a needle prick which may cause just a little bit of pain and leave a small mark.
- **Urine:** You (your child) will pass urine into a collection cup. If you (your child) are/(is) unable to pass urine, urine will be collected via a collection bag or an existing catheter. None of these methods are painful.

Sample testing: The sample will be tested in the United States Army Medical Research Directorate/ Kenya Medical Research Institute laboratory / in Nairobi or Kericho. The germs in the sample will be grown so that they can find out which germs are causing your (your child's) infection, which drugs are able to treat the infection, why they may not be killed by some drugs and how they make you sick and spread to others.. Your (your child's) specimen will be stored until all the tests are finished for a maximum of 2 weeks. . All the germs that are grown from the samples will be stored for ever in KEMRI to allow more studies to be done, for example, to test new drugs which could kill them better. If we decide to do new studies or perform different tests we will get permission from KEMRI and WRAIR before we use the stored germs. If your (your child's) sample is found to have germs that are very difficult to treat the Multidrug-Resistant Organism Repository and Surveillance Network laboratory in Maryland, USA are able to do more tests and compare them with other similar germs from around the world. The germs from your (your child's) samples would be destroyed after testing at the MRSN and will not be sold to researchers or companies.

Participation and withdrawal from the study: You are free to choose if you (your child) will take part in this study or not. You (your child) can stop participating in the study at any time, for any reason by telling the study staff. When you (your child) are/is being asked questions you (your child) do/does not have to answer questions you (your child) are/is not comfortable answering. If we find out anything new during the study which may affect you (your child's) willingness to continue participating in the study we will let you know and give you a chance to decide if you (your child) will continue to be in the study. If you decide that you (your child) will leave the study, the normal care you (your child) are/is receiving at the hospital will continue and any information, specimens or germs collected up to the time you (your child) withdraw(s) consent/assent will not be used in the study unless you agree. If you do not agree for us to continue using the samples/germs then they will be destroyed.

Risks of the study: We will take some of your (your child's) time as we collect the sample and ask you questions. We do not expect that you will be injured by taking part in the study other than the small amount of pain you (your child) may feel when we collect a sample. In case there is an injury during the specimen collection, medical care will be provided at no cost to you at this hospital.

Benefits of the study: There will be no direct benefit to you (your child) for participating in this study because the tests can take up to 3 weeks to complete. The results of the study will not be given to you (your child) but will be provided to the hospital where you (your child are/is being treated so that they can know how to treat their patients better in future. The results of the study will help the researchers

understand how big the problem of these germs that are difficult to treat are at this time and find ways to prevent them from spreading in hospitals and the community in the future.

Compensation: Other than medical care that may be provided there is no other compensation available for your (your child's) participation in this research.

Costs of participation: There is no charge for taking part in the study

Confidentiality: Your (your child's) personal information will not be shown to anyone and only a study code will be put on all your (your child's) samples and information sheets. Only the signed agreement forms which is required to show that you agreed to participate or allowed your child to participate in the study will contain your (your child's) name. All the study information including the list that connects your (your child's) code to your (your child's) information will be kept in locked cabinets at the hospital. The consent form and questionnaires with your (your child's) information will be kept in locked cabinets and rooms and on computers with passwords in KEMRI so that only people involved in the study will be able to see the information. Other people from organizations who are supposed to check that the study is being done the right way may also see your (your child's) identifying information. These organizations are KEMRI, Walter Reed Army Institute of Research, U.S. Army Medical Research and Materiel Command/Office of research protections/Human research protection office (USAMRMC/ORP/HRPO). All the study information will be stored for up to 10 years after the study is finished. Any samples sent to other laboratories will be given a different code from the one used in the study.

Communication of results: The study results will be shared with the participating hospitals, the Kenyan Ministries of Defense and Health. The results will be written in scientific journals and discussed in scientific meetings however no information that can be used to identify you (your child) will be used.

Other studies: You may be approached to participate in another study related to this one if you meet the requirements for enrollment .

Questions: If you have any questions about the study, you (your child) being in the study or you think you (your child) have/has been hurt as a result of being in this study you may call or email the main study researcher Dr. Lillian Musila at 0728817872 or lillian.musila@usamru-k.org. If you have any questions about your (your child's) rights as a study participant you can contact: The Secretary, KEMRI Scientific and Ethics Review Unit, P. O. Box 54840-00200, Nairobi, Kenya; Telephone numbers: 0717-719477; Email address: seru@kemri.org

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Consent statement:

I have read this consent form (or it has been read to me), it has been explained to me why this study is being performed, and I feel that all my questions have been answers. The risks and benefits of being in the study have been explained to me. I have chosen freely for me (my child) to take part in this study and I can also choose to take myself (my child) out of the study at any time. It has been explained to me that my (my child's) personal information will not be shared with anyone except a few members of the study and people given the responsibility to check that the study is being done properly. It has been explained to me that when I sign this form I do not give up my (my child's) rights or release the

researchers from doing the things they should do for me (my child) as a study participant. I will be given a signed copy of this consent form for my records.

As part of this study I agree for:

a) The hospital to share my (my child's) health information with the research staff.
b) The researchers to find germs in my (my child's) specimen and find which drugs can kill it and which ones cannot and the reasons why they cannot be killed by the drugs.
c) The research staff to ask me questions about my/my child's health and other personal details.
d) Germs from my (my child's) specimen to be stored for ever at KEMRI/Walter Reed Project for future studies.
e) Results from my (my child's) specimen to be put in medical journal articles, scientific meetings, conference presentations, etc. however my (my child's) name and other information that could tell someone who you (your child) are/is will not be used.
f) Germs from my (my child's) specimen to be sent to the Multidrug-Resistant Organism Repository and Surveillance Network laboratory (MRSN) in Maryland, USA for additional testing

☐ **I agree** for the germs found in my (my child's) specimen to be shipped to and tested by the MRSN laboratory in Maryland, USA.

☐ **I do not agree** for the germs found in my (my child's) specimen to be shipped to and tested by the MRSN laboratory in Maryland, USA.

_____
Participant's name printed

_____ _____
_____
Age Date Signature/thumbprint

_____
Parent/guardian name printed

_____ _____
_____
Age Date Signature/thumbprint

Participant contact information (tel/email): _____

Appendix II: Approval letter from Maseno University

MASENO UNIVERSITY

SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref: MSC/PH/00153/014

Private Bag, MASENO, KENYA
Tel:(057)351 22/351008/351011
FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 02nd August, 2017

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR CATHERINE WAWIRA MURIUKI— MSC/PH/00153/2014

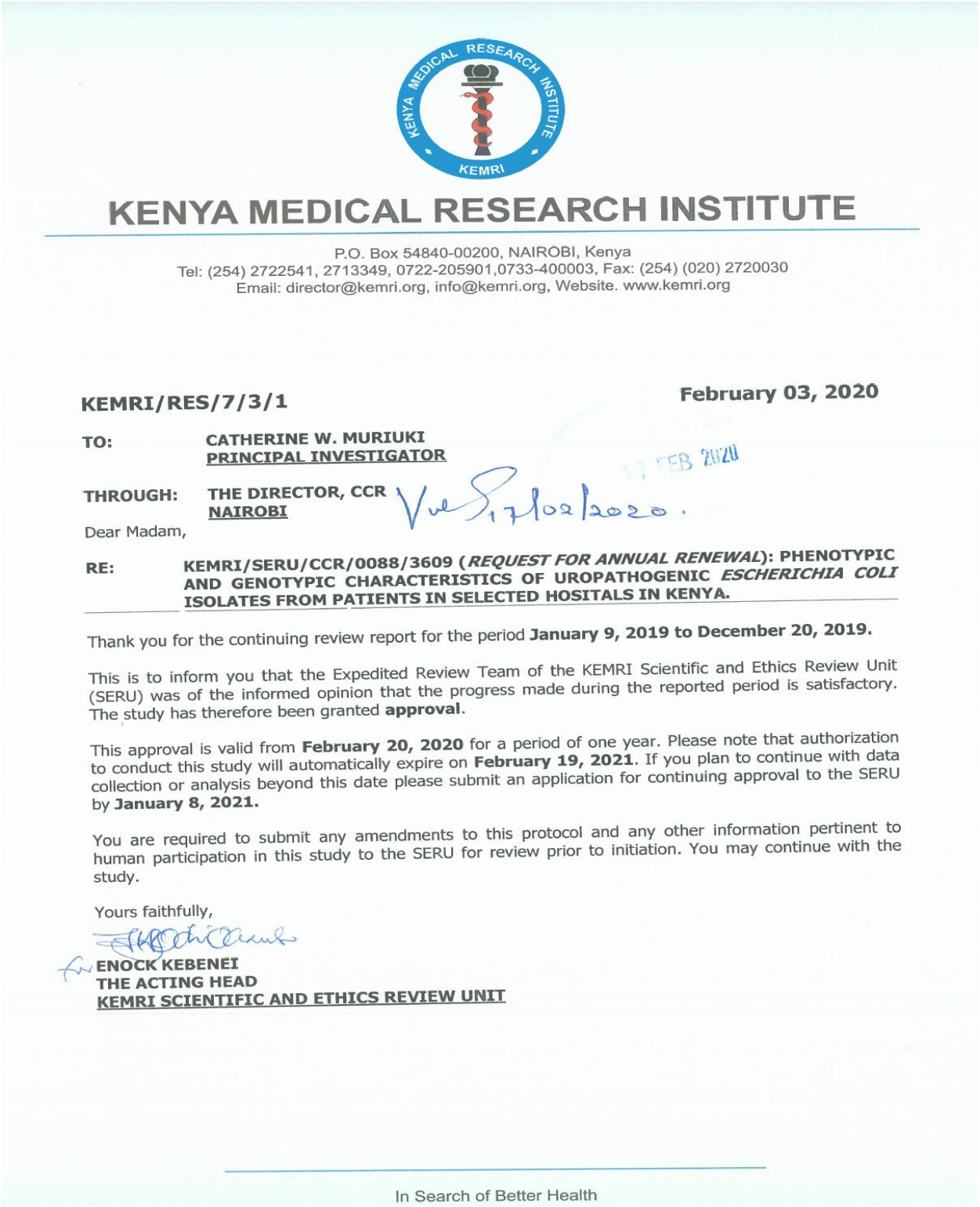
The above named is registered in the Master Science Programme of the School of Public Health and Community Development, Maseno University. This is to confirm that his research proposal titled "Phenotypic and Genotypic Characteristics of Uropathogenic *Escherichia coli* Isolates from Patients in Selected Hospitals in Kenya" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

Prof. J.O. Agure
DEAN, SCHOOL OF GRADUATE STUDIES

02 AUG 2017

Maseno University *ISO 9001:2008 Certified*

Appendix III: Approval letter from KEMRI



KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) 2722541, 2713349, 0722-205901,0733-400003, Fax: (254) (020) 2720030
Email: director@kemri.org, info@kemri.org, Website. www.kemri.org

KEMRI/RES/7/3/1 **February 03, 2020**

TO: **CATHERINE W. MURIUKI**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CCR**
NAIROBI

17 FEB 2020

17/02/2020.

Dear Madam,

RE: **KEMRI/SERU/CCR/0088/3609 (*REQUEST FOR ANNUAL RENEWAL*): PHENOTYPIC AND GENOTYPIC CHARACTERISTICS OF UROPATHOGENIC *ESCHERICHIA COLI* ISOLATES FROM PATIENTS IN SELECTED HOSITALS IN KENYA.**

Thank you for the continuing review report for the period **January 9, 2019 to December 20, 2019.**

This is to inform you that the Expedited Review Team of the KEMRI Scientific and Ethics Review Unit (SERU) was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **February 20, 2020** for a period of one year. Please note that authorization to conduct this study will automatically expire on **February 19, 2021**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the SERU by **January 8, 2021.**

You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to the SERU for review prior to initiation. You may continue with the study.

Yours faithfully,

ENOCK KEBENEI
THE ACTING HEAD
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health

Appendix IV: Parent protocol approval letter from KEMRI

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
Email: director@kemri.org, info@kemri.org, Website. www.kemri.org

KEMRI/RES/7/3/1 **October 22, 2019**

TO: **DR. LILIAN MUSILA**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CCR**
NAIROBI

Dear Madam,

RE: **SSC PROTOCOL NO. 2767 *(REQUEST FOR ANNUAL RENEWAL)*: ANTIMICROBIAL RESISTANCE SURVEILLANCE IN MILITARY AND CIVILIAN POPULATIONS IN KENYA WITH EMPHASIS ON METHICILLIN- AND VANCOMYCIN-RESISTANT *S. AUREUS***

Thank you for the continuing review report for the period **September 28, 2018** to **September 27, 2019.**

This is to inform you that the expedited review team of the KEMRI Scientific and Ethics Review Unit (SERU) conducted the annual review of the above referenced application and was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **November 15, 2019** through to **November 14, 2020**. Please note that authorization to conduct this study will automatically expire on **November 14, 2020**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the **SERU** by **October 03, 2020.**

You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to the SERU for review prior to initiation. You may continue with the study.

Yours faithfully,

ENOCK KEBENEI
ACTING HEAD
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health