ANTIMICROBIAL RESISTANT *ESCHERICHIA COLI* GENES IN CHILDREN AGED BELOW FIVE YEARS PRESENTING WITH DIARRHOEA AT THIKA LEVEL 5 HOSPITAL, KIAMBU COUNTY, KENYA

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P97/3216/09

A THESIS SUBMITTED IN FULFILMENT FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY (MEDICAL MICROBIOLOGY) IN THE SCHOOL OF MEDICINE OF KENYATTA UNIVERSITY.

OCTOBER, 2015

DECLARATION

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

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DEDICATION

I dedicate this thesis to my mother Mrs. Gladys Muthoni Kamwati, my wife and children for their love, support, and encouragement.

ACKNOWLEDGEMENTS

This thesis is a result of combined efforts of many people and Institutions who made invaluable contributions to make sure it was a success. First, I am greatly indebted to God Almighty for giving me good and health life. Thanks to my supervisors Prof. Ephantus Kabiru, Prof. Samuel Kariuki and Dr. Ng'ethe Muhoho for their commitment in guiding me through the journey of completing this thesis, motivation and dedication to details, to the late Dr. John Mbithi, who was my supervisor until his demise. The Management of Mount Kenya University (MKU), with special thanks to the Chairman Board of Trustees, Dr. Simon Gicharu and Vice-Chancellor Prof. Stanley Waudo for the commitment, moral encouragement, and material support. Many thanks to the staff members of Thika Level 5 Hospital Pediatric ward and out-patient department for support and willingness to help me in identification of study subjects during sample collection; the parent and guardians for consenting to have their children participate in the study. The Medical Superintendent and the entire hospital administration for their support and permission to have me do the study in their Institution and to Mr. Simon Kamau for assistance in sample collection from the hospital. I owe much success of this study to Dr. John Kiiru and Prof. Samuel Kariuki for providing the reagents and materials for molecular work; Dr. John Kiiru for practical guidance you made during my long hours in your laboratory, especially in updating my molecular biology knowledge and technical skills and Elijah Kiarie for all IT skills in this Thesis.

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ABBREVIATIONS AND ACROYNMS

AAF	Aggregative Adherence Fimbriae
A/E	Attaching Effacing
AMR	Antimicrobial Resistance
ANOVA	Analysis of Variance
API 20E	Analytical Profile Index
CIs	Confidence Intervals
CLSI	Clinical Laboratory Standards Institute
DEC	Diarrhoeagenic *Escherichia coli*
DNA	Deoxyribonucleic Acid
EAggEC	Enteroaggregative *E. coli*
EASTI	Enteroaggregative *Escherichia coli* Heat Stable Enterotoxin 1
E .coli	*Escherichia coli*
ECORE.	*E. coli* Reference
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic *E. coli*
ERIC	Enterobacterial Repetitive Intergenic Consensus
ESBL	Extended Spectrum Beta-Lactam
ETEC	Enterotoxigenic *E.coli*
FAO	Food Agriculture Organization
HEP-2	Human Epidermoid Cancer Cells
HIV	Human Immunodeficiency Virus

ISCR	Insertion Sequence Common Region Elements
KHDS	Kenya Health Demographic Survey
KEMRI	Kenya Medical Research Institute
LEE	Locus of Enterocyte Effacement
LIA	Lysine Iron Agar
LT	Labile Toxin
M	Molecular Weight
MDG	Millennium Development Goal
MDR	Multiple Drug Resistance
MIC	Minimum Inhibitory Concentration
NCST	National Council for Science and Technology
NACOSTI	National Commission of Science Technology and Innovation
NC	Negative Control
OR	Odds Ratio
ORT	Oral Rehydration Therapy
PAI	Pathogenic Islands
PATH	Programme for Appropriate Technology in Health
PC	Positive Control
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
pINV	Invasive Plasmid
SPSS	Statistical Package for Social Sciences

ST	Stable Toxin
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA
T3SS	Type Three Secretion System
TSI	Triple Sugar Iron
UNICEF	United Nations Children's Fund
UV	Ultra Violet
VCR	Variable Cassette Regions
WHO	World Health Organization

DEFINITION OF OPERATIONAL TERMS

Breakpoints- discriminatory antimicrobial concentration used in the interpretations of results of susceptibility testing to define isolates as susceptible, intermediate or resistant

Conjugation- is the transfer of DNA mediated by conjugal plasmids or conjugal transposons requires cell to cell contact, between distantly related bacteria or even eukaryotic cells and can transfer long fragments of DNA**.**

Crohn's Disease- a form of inflammatory bowel disease usually affecting the intestines but may occur anywhere from the mouth to the rectum.

Hemolytic Uremic Syndrome - is a disorder that usually occurs when an infection in the digestive system produces toxic substances that destroy red blood cells, causing kidney injury.

Horizontal Genetic Transfer- is the movement of genetic materials, between bacteria other than by descent in which information travels through the generation as the cell divides.

Insertion Elements- genetic are distinct elements of less than 2.5kb of DNA whose only genes are related to transposition and self-propagating.

Integrons- are mobile genetic elements with the ability to capture genes notably, those encoding antibiotic resistance**.**

Irritable Bowel Syndrome- a disorder that leads to abdominal pain and cramping, changes in bowel movements, and other symptoms**.**

Mobile Genetic Elements- Are segments of DNA that encode enzymes and proteins that mediate the movement of DNA within genes or between bacterial cells that is intracellular and intercellular mobility respectively.

Pathogenicity Islands- are distinct class of genomic islands acquired by microorganisms through horizontal gene transfer.

Plasmids- circular pieces of DNA found in bacteria separate from genome that is capable of replicating itself independently.

Thrombocytopenia- any disorder in which there is an abnormally low amount of platelets. Thrombocytopenic purpura- a blood disorder that causes blood clots to form in small blood vessels around the body leading to low platelet counts**.**

Transduction – is the transfer of DNA by phage and requires that the donor and recipient share cell surface receptor for the phage binding and is thus usually limited to closely related bacteria.

Transformation- is the uptake of naked DNA, typically only in short DNA fragments are exchanged.

Transposons- A short mobile genetic DNA sequence that can replicate and of which copies can be inserted at random sites within chromosomes.

Ulcerative Colitis- is a type of inflammatory bowel disease that causes long lasting inflammation and ulcers in the digestive tract.

Virulence- the degree of pathogenicity of a microorganism as indicated by severity of the disease produced and the ability to invade the tissue if the host**.**

ABSTRACT

Diarrhoea is one of the leading causes of illness and death among children in developing countries, where an estimated 1.3 billion episodes and 4 to 10 million deaths occur each year in children less than 5 years of age. The study envisioned to determine the genetic basis of extended spectrum β-lactam (ESBL) resistance in *E. coli* isolated from children aged below 5 years attending Thika Level 5 Hospital Kiambu County in Kenya. Faecal samples were obtained from 384 children presenting with diarrhoea. A questionnaire was used to collect data concerning the age of the child, gender and additional information recorded included on history of hospitalization and antibiotic use. Samples were sub-cultured on MacConkey agar and suspect *E. coli* isolates identified. Antimicrobial susceptibility profiles were determined for the most common antimicrobials against Gram-negative bacteria. The types and prevalence of Extended-Spectrum βeta-Lactamases were also determined. Polymerase Chain Reaction methods were used for detection of the Extended-Spectrum βeta-Lactamases genes and carriage of integrons. Conjugation experiments were performed to determine the potential of horizontal transfer of resistance genes. The plasmid content for defining diversity of plasmid replicon types was also determined among donors and transconjugants. Approximately 25% of all isolates were resistant to ampicillin, tetracycline, chloramphenicol, streptomycin, amikacin sulphamethoxazole-trimethoprim and amoxicillin-clavulanic acid (p=0.0051; OR=2.27; 95% CI: 2.01-2.33). Isolates (50%) had a Minimum Inhibitory Concentration $mode_{50}$ value within the resistance range except for ceftazidime, Gentamicin, chloramphenicol, Nalidixic acid, Ciproflocin and Kanamycin. Factors identified to predispose children to carry highly resistant isolates included recent history of hospitalization and antimicrobial use and having acute diarrhoea (p=0.0026; OR=4.21; 95% CI: 3.76-4.98) There was no significant difference in antimicrobial resistance patterns for isolates from rural and urban children (p=0.049). Carriage of a combination of $bla_{\text{CTX-M}}$+ bla_{TEM} or a combination of $bla_{\text{CTX-M}}$+bla_{OXA} genes was highly associated with resistance to multiple β-lactams (p=0.00015; OR=2.31; 95% CI: 1.99-2.65). Isolates with multiple β-lactamases were also likely to carry integrons encoding resistance to other antimicrobials. Plasmids that mediated resistance to most antimicrobials belonged to incL/M, HI2 and F-type. In conclusion, most of isolates recovered from these children were resistant to first line treatment for diarrhoea and therefore a need to revise the existing empiric treatment of *E. coli* infections. Resistance was transferrable via conjugation and integrons were significantly implicated in resistance dispersion hence increase in the prevalence of multidrug resistant isolates (p=0.0017; OR: 4.03; 95% CI: 3.81- 4.04). There is a need to encourage parents and doctors to follow the treatment guidelines for diarrhoea in which case, the oral re-hydration therapy zinc and vitamin C rather than antibiotics should be administered in cases of watery bacillary diarrhoea, implement proper antimicrobial use polices and launch national surveillance programs to monitor emergence and spread of MDR isolates.

CHAPTER ONE: INTRODUCTION

1.1 Background to the Study

Diarrhoeal diseases continue to be an important cause of morbidity and mortality worldwide especially in children under five years of age. It is estimated that children under 5 years' experience between two and three episodes of diarrhoea per year globally, (Kosek, *et al.,* 2003). Diarrhoea is the second leading cause of death in children under five years, and is responsible for 1.5 million deaths in children every year especially in developing countries (*Bla*ck *et al.,* 2010:UNICEF/WHO, 2009). It accounts for approximately 1.6 to 2.5 million deaths in children annually and a leading cause of malnutrition in children fewer than five years of age (UNICEF/WHO, 2009)

In Kenya, diarrhoea is among the five main causes of mortality in children younger than five years. Bacterial diarrhoea contributes to 30% of all cases of infantile diarrhoea and is the most common cause of travelers' diarrhoea (Oundo, *et al.,* 2008). In light of this, the fourth Millennium Development Goal (MDG) aims at reducing by two thirds the mortality rate associated with diarrhoea among children under five by the year 2015 (UNICEF/WHO, 2009). This target may not be achieved due to the observed persistence of high diarrhoeal disease rates and also increase in resistance to drugs that are usually used in the management of diarrhoea in children under five years (Okeke *et al.,* 2007).

Diarrhoeal diseases have been associated with diverse bacterial, viral and protozoan pathogens which are transmitted mainly through the faecal-oral route

and malnutrition. These micro-organisms include *Salmonella spp*., *Shigella spp*., *Campylobacter jejuni*, *Vibrio cholerae*, rotavirus, norovirus, Giardia lamblia, *Cryptosporidium sp*., and *Entamoeba histolytica* with the most common pathogen being *Escherichia coli* (Merson *et al.,* 2005). *Escherichia coli* were first identified in faeces obtained from a child by Theodor Escherich in 1885 (Whitfield and Valvano, 1993). *Escherichia coli* belong to Enterobacteriaceae that are anaerobic, facultative and gram negative rods known to inhabit the gastrointestinal tract of humans and animals. *Escherichia coli* strains are of biological significance to humans. On the basis of genetic and clinical criteria *E. coli* can be broadly classified into three major groups; commensal E coli, intestinal pathogenic (diarrhoeagenic) *E.coli*, and extra intestinal *E. coli* (ExPEC) (Nunes *et al.,* 2011; Kaper *et al.,* 2004).

Diarrhoeagenic *E. coli* is rarely present in the faecal flora of healthy hosts. These are obligate pathogens and responsible for gastroenteritis or colitis, when ingested in sufficient quantities by naïve host. They usually cause secretory diarrhoea which can result to dehydration and death. Diarrhoeagenic *E. coli* isolates characteristically cause diarrhoea through either the action of toxins or expression of intestinal adhesions. Both of these virulence traits are typically plasmid encoded (Friederike-Feldmann, 2008).Young children in areas of the world with limited resources and travelers to these regions are the most affected human populations (Farthing, 2012; Salyers and Whitt, 2012). In the very young, disease can lead to

death if patients are not properly treated while in older children and adults it generally results in temporary incapacitation (Cravioto, 2013).

Pathogenic types of *E. coli* have evolved over time leading to virulent isolates; these virulent isolates can be distinguished from each other based on their distribution in different regions, clinical symptoms they manifest in their host, microscopic features, biotypes and the unique gene markers they express. Although evolution of *E. coli* is unclear, studies have linked it to the concomitant evolution of diverse mammalian hosts (Tourneur and Chassin, 2013; Chow, *et al.*, 2010; Ochman and Selander, 1984).

The advancement and rapid dissemination of different pathogenic *E. coli* is a major concern in developing countries where factors such as harsh climatic conditions, poor sanitation, malnutrition and immunosuppression related to Human Immune Virus (HIV) and Acquired Immune Deficiency Syndrome (AIDS) have contributed to its enhancement (Sinha *et al.,* 2011). According to a study carried out by (Paton and Paton, 2002), diarrhoeagenic *E. coli* (DEC) has been demonstrated to be a potential public health risk, with EHEC O157: H7 causing life-threatening diseases mainly; hemolytic uremic-syndrome and thrombocytopenic purpura that result in kidney failure, hemolytic anemia, and thrombocytopenia (Wei *et al.,* 2011; WHO 2011).

Diversity in virulence amongst DEC pathotypes might be attributed to the presence of specific pathogenicity islands (PAIs) (Schmidt and Hensel, 2004) which enhance gene mobility within various mobile elements such as plasmids, and from

chromosomal location to mobile elements, which has implications for the spread of virulence factors from DEC to commensal *E. coli* ; thus the PAIs play a significant role in pathogenesis of DEC and increased virulence in disease presentation (Taha *et al.,* 2014,2004; WHO 2011) The pathotypes can easily be cultured in the laboratory; however identification of genotypes entails molecular methods which are expensive and not available in most clinical laboratories especially in developing countries (Vernet *et al.,* 2014). Faecal contamination by *E. coli* can result in the pathogen being deposited in soil and water, this can occur in cases where there is lack of proper human-waste disposal or contamination of clean water by waste-water. *Escherichia coli* have been used as an indicator organism in the bacteriological analysis of water (Onyango and Gerba, 2011).

The control of infectious diseases which includes diarrhoea is badly endangered by the rise in the number of microorganisms that are resistant to antimicrobial agents. This is because infections caused by resistant microorganisms often fail to respond to conventional treatment resulting in prolonged illness and greater risk of death. (Laxminarayan, 2003). The greater the duration of exposure of antibiotic, the greater is the risk of the development of resistance, irrespective of the severity of the need for the antibiotic. People infected with antimicrobial-resistant organisms are more likely to have longer, more expensive hospital stays, and may be likely to die as a result of the infection (Gaude and Hattiholi, 2013). The primary cause of antibiotic resistance is genetic mutation in bacteria, The indiscriminate use antibiotics which promotes antibiotic resistance, results from patients'

incompliance use of recommended treatment and demand, prescribers', irrational use of antibiotics in human, drug advertisement, dispensing doctor's and antibiotic use in agriculture, poor quality antibiotics, inadequate surveillance and susceptibility testing, antibiotic resistance therefore pose a significant problem (Landecker, 2015)

While antibiotics are not recommended for treatment of watery diarrhoea especially that caused by *E. coli,* diarrhoeagenic isolates may contain antimicrobial resistance genes that may be from other isolates that are resistant within the same genus or other genera through horizontal transfer mechanisms (Baidouri *et al.*, 2014). In paediatric settings, ESBLs are the most often encountered in the hospital setting. The ESBL enzymes are plasmid mediated enzymes capable of hydrolyzing and inactivating a wide variety of beta lactams, including, third generation cephalosporins, penicillins, and aztreonam. These enzymes are result of mutations of TEM-1 and TEM-2 (Temoniera enzymes) and SHV-1 (Sulfhhdryl variable enzyme) (Odonkor and Addo, 2011). All these beta-lactamases enzymes are commonly found in the Enterobacteriaceae family. Widespread use of cephalosporins and aztreonam is believed to be a major cause of mutation in these enzymes that has led to the emergence of ESBLs (Langerndorf *et al.*, 2015). Children, especially those below the age of 5 years are significantly exposed to diarrhoea than adults (Singh, 2014; Mamo and Hailu, 2014) and there is a need to investigate prevalence of antimicrobial resistance among isolates obtained from children of different clinical backgrounds and responsible beta-lactam enzymes.

This study investigated carriage of MDR *E. coli* isolates from children under the age of five presenting with diarrhoea at Thika Level 5 Hospital.

1.2 Statement of the Problem

Notable drug-resistant Enteropathogenic *E. coli* outbreaks and sporadic cases have been reported in Kenya (Benet-Martínez and Haritatos, 2005; Bii *et al.,* 2005: Sang *et al.,* 1997; Senerwa, *et al.*,1991). In Thika Level 5 Hospital, treatment of diarrhoea is largely empirical as aetiological agents' isolation and antimicrobial susceptibility testing from children below five years are rarely performed. Such practices pose a problem presenting a scenario with a great potential to compromise treatment options especially if the aetiologic agent is resistant to the antimicrobials used, thus leading to build up of resistant populations of enteric bacteria.

1.3 Justification

Children under the age of five are most prone to diarrhoeal disease worldwide. Diarrhoeagenic *Escherichia coli* (DEC) pathotypes are leading cause of diarrhoea in children in developing countries (Singh 2014; Mamo and Hailu, 2014), Kenya included (KDHS, 2008-09). There is paucity of data on the genetic basis for antimicrobial resistant in *E. coli* from children aged below 5 years presenting with diarrhoea as the capacity of clinical laboratory to perform molecular studies on diarrhoeagenic *E. coli* remains wanting in Thika Level 5 Hospital. Currently routine detection of DEC in Thika Level 5 hospital is based on a combination of biochemical tests and serotyping and there is need for molecular characterization

to detect virulence genes. This study investigated the diversity of *E. coli* strains recoverable from stool samples of children presenting with diarrhoea in Thika Level 5 Hospital. This study provided baseline information on resistance phenotypes and conjugal transfer of resistance plasmids among *E. coli* isolates from children with diarrhoea. This study also provides baseline data on resistance mechanisms to various antimicrobials as mediated by β-lactamases and integrons. The data therefore provide important information that could guide therapy, development of antibiotic-use policies and may also provide vital information regarding the epidemiology of MDR isolates in rural and urban settings. In Thika Level 5 Hospital stool culturing is not a routine practice and data is unavailable on antimicrobial resistance genes in the pediatric population within the catchment area of the hospital and this study is the first to provide this crucial data. The data from this study could find important applications such as providing evidence of important *E. coli* resistance profiles that may in turn be useful in formulation and revision of antimicrobial use policies availed to doctors for management of diarrhoea cases in Thika Level 5 Hospital.

1.4 Research Questions

i. What is the prevalence of antimicrobial resistance in diarrhoeagenic *E. coli* from rural and urban population of children under the age of 5 years presenting with diarrhoea at Thika Level 5 hospital.

ii. What are the circulating resistant genes in *E. coli* isolates obtained from children under the age of five years presenting with diarrhoea at Thika Level 5 Hospital?

iii. Is there any diversity in extended beta-lactamases from *E. coli* isolates from rural versus urban children under the age of five years presenting with diarrhoea at Thika Level 5 hospital?

1.5 Objectives

1.5.1 General Objective

To establish antimicrobial resistance and responsible genes in *Escherichia coli* faecal isolates from children aged below 5 years presenting with diarrhoea at Thika Level 5 Hospital, Kiambu County, Kenya.

1.5.2 Specific Objectives

i. To establish the antimicrobial resistance in *Escherichia coli* isolates in rural and urban population of children under the age of 5 years presenting with diarrhoea at Thika Level 5 Hospital.

ii. To establish the antimicrobial resistance in *Escherichia coli* isolates in inpatient and outpatient children under five years

iii. To establish resistance genes in the *E. coli* isolates obtained from children under the age of five years presenting with diarrhoea at Thika Level 5 hospital.

iv. To establish relationship diversity between extended beta-lactamase spectrums *E. coli* isolates using ERIC-PCR.

CHAPTER TWO: LITERATURE REVIEW

2.1 Diarrhoea

Diarrhoea is an enteric infection especially in developing world leading to the passing of watery stool more than three times a day and is often a symptom of an infection or long term condition, (Dutta *et al.,* 2014). In order to guide optimal case management, and for the purposes of epidemiological tracking, a diarrhoeal episode is often diagnosed according to symptoms into one of the following three categories: acute watery diarrhea, dysentery or bloody diarrhoea, or persistent diarrhea. Each of these categories has unique considerations that direct the clinician to provide appropriate care (Farthing, 2012; WHO, 2005)

2.2 Types and causes of Diarrhoea

2.2.1 Acute Watery Diarrhoea

Majority of episodes would be classified as acute diarrhea. Secretory organisms lead to diarrhoea symptoms described as acute watery diarrhoea. The agents causing this non-inflammatory type of enteric illness often produce enterotoxins as in the case of *Vibrio cholerae* or ETEC Alternatively, they may disrupt the normal absorptive or secretory processes of the enterocyte as in the case of viruses or Giardia, without destroying the mucosa or causing inflammation. Though typically mild, this type of illness can rapidly lead to dehydration. In such cases, the infection may be due to rotavirus, ETEC or *V. cholerae* (Keusch, *et al.,* 2006). Cases of watery diarrhoea lasting seven days or longer can lead to malnutrition (Moore *et al.,* 2010).

2.2.2 Acute Bloody Diarrhoea or Dysentery

Clinically, bloody diarrhoea is the term used to describe stools that may either visibly contain blood or that contain blood detectable by microscopy (Oundo, *et al.,* 2008). Dysentery describes characteristically small-volume bloody or mucous stools accompanied by abdominal cramping and tenesmus and is the result of inflammatory or invasive organisms, faecal leukocytes or lactoferrin are usually present (Pawlowski, *et al.*, 2009). Pathogens causing dysentery or bloody diarrhoea may destroy cells in the gut or invade the mucosa and result in inflammation or ulceration. Inflammatory organisms include cytotoxin-producing noninvasive bacteria such as EAEC, and invasive pathogens like Salmonella, Shigella and Campylobacter and amoeba. Fever can be the result of an enterotoxin, or indication of colonic tissue damage by invasive organisms (Ahs, *et al.*, 2010).

2.2.3 Persistent Diarrhoea

The distinguishing characteristic of persistent diarrhoea is solely the duration of illness; persistent diarrhoea lasts longer than fourteen (14) days (Guandalini *et al.*, 1982). The causes of persistent diarrhoea include multiple successive gastrointestinal infections or an infection that has not resolved (Didierlaurent, 2014). Though persistent diarrhoea can be the result of any of the many different enteric pathogens the most important are Giardia, Cryptosporidium, EAEC and EPEC (Mbae *et al.,* 2013: Pawlowski *et al.,* 2009: Kang'ethe, 2008). When persistent diarrhoea is the result of bloody diarrhoea, as is frequently the case, the associated risk of death is ten times greater than in cases of bloody diarrhoea that

are less than two weeks in duration (Keusch *et al.*, 2006). In the case of a child, the risk of death from persistent diarrhoea can increase if he or she was malnourished prior to contracting the illness or suffered other systemic infections (Ochoa *et al.*, 2004; Tarbert *et al.*, 2013)

2.2.4 Malnutrition In Diarrhoea

A patient with diarrhoea who is suffering from severe malnutrition (marasmus or kwashiorkor) is at risk of not only dehydration, but also severe systemic infection, heart failure and vitamin and mineral deficiency (WHO, 2005). The rehydration of a severely malnourished patient with diarrhoea should take place immediately and in a hospital. In a case of severe malnutrition, it may be difficult to differentiate the signs of minor dehydration from those indicating severe dehydration, and the signs of severe dehydration could be those of septic shock. A malnourished patient should be rehydrated orally at a slow rate, with hypo-osmolar ORS (Tarbert *et al.*, 2012).

2.3 Management and Control of Diarrhoea

During the diagnosis of diarrhoea in children, clinical features should be documented **t**o exclude the presence of blood or mucus in the stools, if these are present, the antibiotic-therapy adapted to the causative pathogen may be required (Faure, 2013). However, in the case of acute infectious diarrhoea, systematic use of antimicrobial therapy is not recommended because the etiology may not be bacterial, and the disease is generally self-limiting. (WHO 2014; Literak *et al.*, 2011). In such cases, no laboratory tests are necessary to identify the pathogenic

agent and a symptomatic treatment can be prescribed straight away. Only if diarrhoea persists despite appropriate symptomatic treatment should the patient be evaluated further (Farthing, 2012; World Gastroenterology Organization, 2008). The WHO has set the following therapeutic goals for the treatment of acute diarrhoea (WHO/UNICEF 2009; WHO 2008; Thapar and Sanderson, 2004) to prevent dehydration, treat dehydration and reduce the duration and severity of diarrhoea and occurrence of future episodes.

2.3.1 Chemotherapy

Parasitic diarrhoea (such as that caused by amoeba and helminthes) are treated with antimicrobials. Antimotility agents such as Loperamide can reduce diarrhoea in patients with Shigella, but not in patients with traveler's diarrhoea due to enterotoxigenic *E. coli* or children less than 3 years old (Marsano, 2001). Antisecretory agents such as racecadotril, an enkephalinase inhibitor, may be used to reduce the volume of watery diarrhoea. Antibiotics may also be used to reduce the duration of acute diarrhoea due to bacterial pathogens , even though there are concerns that this causes the enteropathogens to develop resistance to drugs (Chart and Cheasty, 2008; Salyers and Whitt, 2012).

The widespread use of antibiotic prophylaxis has been criticized both on the grounds of toxicity and because of the possibility that they present a selection pressure that favour emergence and spread of drug resistant strains (Chart and Cheasty, 2008). It is also, has been suggested that the administration of certain antibiotics such as Fluoroquinolones and Trimethoprim may be counterproductive

(Salyers and Whitt, 2012). The emergence of multi-drug resistant diarrhoeagenic *E. coli* strains is now a major public health concern and presents a major challenge when dealing with infections in children (Okeke, *et al.,* 2007). Chemotherapeutic interventions in diarrhoea management depend on the cause of the diarrhoea and severity (Stein *et al.,* 2010).

2.3.2 Oral Rehydration Therapy

The mainstay of symptomatic treatment of acute infectious diarrhoea, particularly in young children at risk for dehydration, is to provide rehydration and to continue feeding (WHO/UNICEF, 2009) The WHO guidelines for treatment of acute diarrhoea in children recommend the immediate rehydration comprising of corrective electrolyte therapy. Rehydration is achieved by oral rehydration solutions (ORS), a mixture of glucose and electrolytes dissolved in water. The composition of ORS has been fixed by WHO and UNICEF (2009).

The treatment of diarrhoea requires a clear understanding of the underlying causative agent. Although many patients with mild diarrhoea can prevent dehydration by ingesting extra fluids (such as juices or soups), more severe diarrhoea and reduced urination signify the need for more rehydration fluids (Ghassemi *et al.,* 1995). The most critical treatment must include rehydration, which can be accomplished with an oral glucose or starch-containing electrolyte solution. Oral Rehydration Therapy (ORT) solutions contain specific

concentrations of sodium, glucose, potassium, chloride, and alkali bicarbonate or citrate in water (Thapar and Sanderson, 2004; Guerrant *et al.*, 2001).

The rationale for this treatment stems from the observation that in most causes of acute infectious diarrhoea , including cholera, the coupled transport of sodium to glucose or other solutes is largely unaffected (Lamberti *et al.*, 2014) It is now widely accepted that water supplies and sanitation, though necessary for the prevention of diarrhoea in young children, are not sufficient unless they are accompanied by changes in domestic hygiene behavior (Merten *et al.*, 2004). It is likely that improving hygiene practices is potentially one of the most effective means of reducing the global burden of diarrhoeal diseases in children. (Karambu *et al.*, 2013; Williams *et al.*, 2008)

2.3.3 Breastfeeding

For infants, exclusive breastfeeding during the first six- months of life can reduce the possibility for the ingestion of enteric bacteria from contaminated water, food, formula, and bottles. (Shah *et al.*, 2012) Breastfeeding has the potential of preventing 13% of under-five deaths in developing countries (Jones *et al.*, 2003). The immunological properties of breast milk protect the infant from infection, especially diarrhoea The anti-infective components of breast milk include secretory IgA , lactoferrin, and immune cells that produce lysozyme, which destroy bacteria, and cells that produce interferon, which have antiviral properties (Orlando, 1995). Studies from all settings have consistently documented the protective effect of exclusive breastfeeding on diarrhoea morbidity incidence and

prevalence (Shah *et al.*, 2012). The evidence in favour of breastfeeding is of intermediate quality as randomized controlled trials directly evaluating breastfeeding versus no breastfeeding are not available due to ethical reasons (WHO, 2009). However, observational studies and breastfeeding promotion trials conclusively support the protective role of exclusive breastfeeding in prevention of diarrhoeal morbidity and mortality (WHO, 2009).

2.3.4 Zinc Supplementation

Zinc supplementation as part of the treatment; have the potential to further reduce diarrhoeal morbidity and mortality by reducing the duration and severity of diarrhoeal episodes and lower their incidence (Shah *et al.*, 2012). Zinc has been shown to reduce the severity and diarrhoea episode period. Zinc reduces the incidence of diarrhoea in the following 2-3 months (WHO, 2005; Bhutta *et al.*, 1999). It is therefore recommended that children below the age of 5 years be supplemented with zinc immediately they experience diarrhoea (Fontaine, 2001). In India, the Ministry of Health recommends the use of, 20 mg of elemental zinc to all children older than 6 months with diarrhoea, and should be started as soon as diarrhoea starts and continued for a total period of 14 days. The WHO and UNICEF recommend that all children under five years with diarrhoea receive 10-20 mg of zinc each day for 10-14 days (WHO/UNCICEF, 2004; Bhatnagar *et al.*, 2007).

2.4. Control of Diarrhoea

2.4.1 Water, sanitation and hygiene

Increased water availability and quantity, associated with improved hygiene, may reduce faecal contamination of the hands, regardless of water quality wide spread unhygienic water handling and limited access to sanitation facilities perpetuates transmission of diarrhoea (Onyango and Angienda, 2010). Proper cleaning of utensils, food, and home environments is also likely to reduce transmission of faecal matter. The transmission of all the main diarrhoea-causing agents is probably influenced to some degree by increased water availability and quantity, but it is Shigella transmission that has been particularly associated with poor personal and domestic hygiene (Wittenberg, 2012). This may be because of the low infectious dose of Shigella relative to other bacterial enteric pathogens, or it may be only because Shigella has been most studied.

The relationship between personal hygiene and the newly-recognized diarrhoea agents (especially Campylobacter jejuni, enterotoxigenic *E. coli* , and rotavirus) should be studied. All the major infectious agents of diarrhoea are shed by infected persons via the faeces, and therefore hygienic disposal of human excreta plays a role in controlling them in yards, gardens and the neighborhood to avoid infection and spread (Clasen, 2007). In addition, proper treatment and disposal of human excreta would prevent faecal contamination of fields, crops, and receiving water-bodies, which would in turn further reduce the transmission of faecal pathogens.

The use of toilets by all members of the community should reduce faecal contamination of houses, The hygienic disposal of the faeces of children too young to use the toilet is of the utmost importance, because such children constitute an important reservoir of several agents of diarrhoea; for instance, rotavirus and enterotoxigenic *E. coli* (Clasen, 2010).

2.4.2 Vaccines strategies and opportunities

There are two main approaches to primary prevention of enteric infections, (a) improved water and sanitation and (b) vaccination. Because most acute diarrhoea is associated with faecal-oral transmission, improved sanitation and water quality are crucial to decrease the transmission of enteric pathogens. In a broad sense, better sanitation is meant to include improved personal hygiene practices as well as community sanitation (Ahmed and Pulendran, 2011). Vaccines would take care of causes that are microbial in nature. Despite the many causes of diarrhoea few vaccines have been developed or are in developmental stages. Vaccines such as rotavirus, Salmonella and ETEC and Shigella have been formulated (Ahmed and Pulendran, 2011). Rotavirus vaccines were first introduced in the developed countries in 2006 and in developing countries in 2012 (Tate *et al.*, 2011, 2008). This vaccine has been approved by most countries and implemented as a control measure. The Global Alliance Vaccine Initiative works closely with national governments to subsidize the cost of rotavirus vaccines support their introduction and expand routine immunization of young children against diarrhoeal diseases. Vaccination underpins two of the United Nations' Millennium Development Goals

(MDGs) namely reduction of child mortality (Goal 4) and improvement of maternal health (Goal 5) (Delany *et al.*, 2013; UNDP, 2009). This public-private partnership has a mission to save children's life and improve global health by increasing access in vaccines in low income countries (Dutta *et al.*, 2014). It is believed that for enteric vaccines to be effective mucosal immunity requires boosting up. Ideally, mucosal protection achieved by a vaccine through the mucosa and oral route has been found to be the best choice. (MacLennan, 2013) The long-term goal is to see the rotavirus vaccine introduced in at least 50 lower- and middle-income countries. Along with improved sanitation and the discovery and use of antibiotics, vaccination is the intervention that has had the greatest impact on human health and the standard of living in recent history (Rappuoli *et al.*, 2011).

Typhoid vaccine is needed to control typhoid and paratyphoid, which have been reported to kill up to 250,000 people a year, mostly children. Typhoid vaccine is an effective and affordable vaccine that provides short-term solution to control enteric fever in countries with poor access to clean water, sanitation, and hygiene as well as high rates of antibiotic resistance (WHO 2007; Deroeck, 2005). The International Vaccine Institute works together with Shantha Biotechnics, the Sabin Vaccine Institute, and others to develop a conjugate vaccine that should have a longer duration than the current vaccine and can be used in children under age 5 (UNICEF, 2012). Enterotoxigenic *Escherichia coli* and Shigella vaccines target

these ubiquitous bacterial pathogens; ETEC and Shigella in most regions of the world, particularly in developing countries, where they are a constant risk to both children and adults. ETEC and Shigella kill an estimated 200,000 children under age 5 every year. Even a single episode of Shigella will cause severe damage to the gastrointestinal system (WHO, 2013).

2.5 Global Burden of Diarrhoea Caused by Bacteria

Infectious diarrhoea is one of the leading causes of morbidity and mortality worldwide, affecting mainly infants in developing countries. It constitutes a major disease burden in low and middle-income countries (Parashar *et al.,* 2003). The average number of episodes per child in 1993 was reported to be 3.5 per child per year (Sousa, 2006). Globally diarrhoea is a still a cause of significant mortality in children according to Lancet Review of 2003 (*Bla*ck *et al.,* 2003). Approximately 22% of 10.8 million deaths in children under 5 years old are due to diarrhoea (*Bla*ck *et al.,* 2003). Diarrhoea accounts to 12% of deaths due to infectious diseases in the world. Of the 12% diarrhoeal cases 90% are from children below 5 years of age (Kallander, 2007). For a long period of time, diarrhoea has been the leading cause of death for the world's children, accounting for 4.6 million deaths annually from around 1 billion episodes of diarrhoea every year in children less than 5 years of age (Snyder and Merson, 1982) and the estimate in 1999 for children and adults together was 2.2 million deaths per year (Davidson *et al.,* 2002).

Most recent estimates suggest the number of death is closer to 2.5 million per year and majority of these cases are reported in Africa (Lawn *et al* 2006). The annual incidence of diarrhoea is 3.5 to 4.6 episodes per child. Diarrhoeal diseases among children under five (an age-group that comprise 20 per cent of the Kenyan population) account for over 4.7 percent of all outpatient cases in Kenya (Rukunga, 2008). Diarrhoeal diseases have significant negative impacts on economies and households. Therefore, diarrhoea is also considered a poverty-related disease (Creel, 2002).

2.6 Burden of Diarrhoea in sub-Saharan Africa

Diarrhoeal diseases account for the high childhood mortality and morbidity reported in sub-Saharan Africa and is responsible for 1.5 million child deaths every year in sub-Saharan Africa (Sambe-Ba *et al.,* 2013). In spite of the continuous efforts put in place in the understanding of the pathogenesis and management of these diseases (Schiller, 2007) reported that the risk of contracting diarrhoeal diseases is currently five-fold higher in sub-Saharan Africa than in other developed countries of the world. Though most studies on diarrhoeal diseases have shown that adults are less likely to contract diarrhoea, it is unlikely to be life threatening. However, adults contribute to the transmission of enteric pathogens to susceptible patients, and in particular children below the age of five years (Sambe-Ba *et al.,* 2013).

Diarrhoea is characterized by four clinical features namely; acute watery diarrhoea, acute bloody diarrhoea, persistent diarrhoea and diarrhoea with severe malnutrition of which 50% of worldwide cases of the condition present with watery diarrhoea. Approximately 35% are persistent diarrhoea and 15% dysentery-diarrhoea with blood stains (UNICEF/WHO, 2009: WHO, 2002). Diarrhoea is a major cause of death and disease, especially among young children in low-income countries. Dehydration is the major threat, though diarrhoea also reduces the absorption of nutrients, causing poor growth in children, reduced resistance to infection, and potentially long-term gut disorders (UNICEF/WHO, 2009: WHO, 2002). Diarrhoea can cause abdominal cramping, fever, nausea (with or without vomiting), chills, appetite loss, headache, muscle aches, and bloating can also occur, but are less common. Illness develops 1 – 3 days after exposure to the disease agent and usually lasts 3 – 4 days. Some infections may take a week or longer to resolve. Symptoms rarely last more than 3 weeks. Most patients recover with supportive measures alone and do not require hospitalization or antibiotics (Faure, 2013; Qadri *et al.,* 2005).

2.7 Prevalence and Etiologies of Diarrhoea in Children in Kenya

Although there are other causes of diarrhoea including dietary factors, infectious pathogens are the major cause of diarrhoea with bacteria causing up to 80% of traveler's diarrhoea. Bacteria such as *E. coli* , Vibrio cholerae, Salmonella and Shigella, viruses such as rotavirus and protozoa such as Cryptosporidium and Giardia have been shown to cause diarrhoea especially in developing countries

(Keusch *et al.,* 2006; Crum *et al.,* 2005). Among these pathogens, the most common causes are rotavirus, *E. coli* and V. cholerae. Diarrhoea due to viral agents is normally not treated with antibiotics as they are unaffected by such antibiotic therapy and the illness is usually self-limiting. For bacteria Enterotoxigenic *E. coli* (ETEC) and Enteroaggregative *E. coli* (EAEC) is the most common causative agent isolated in most countries. Other bacterial causes are Shigella, Salmonella, Campylobacter, Yersinia, Aeromonas, and Plesiomonas, are less frequently associated with diarrhoea (Karambu *et al.,* 2013; Ansari *et al.,* 2012). While some bacteria cause diarrhoea by directly attaching the lining of the gut, others use toxins as effectors. The mechanisms may include alterations of in ion transport and tight junctions as well as virulence factors (Boyd *et al.,* 2014; Hodges and Gill, 2010).

Diarrhoea has negative effects on the growth and development of children, this happens when the balance in electrolytes and water transport is upset in favour of net secretion because of decreased absorption from the intestinal lumen or increased secretion or water loss into the lumen (Thapar and Sanderson, 2004).These are induced by different action mechanisms of enteric pathogens. In many cases, the patients have accompanying symptoms such as fever, vomiting, and abdominal pain. Dehydration is the consequence of massive water/fluid loss due to diarrhoea and vomiting and is life-threatening for children (UNICEF/WHO, 2009).

Insufficient nutrition, poor hygiene, contaminated water and inadequate sanitation contribute to the rising cases of diarrhoea in children under the age of five years (Shaheen *et al.,* 2015). Meat and meat products have been implicated in disease outbreaks caused by various diarrhoeagenic *E. coli* worldwide (Karmali *et al.,* 2010; Rhoades *et al.,* 2009). Raw meat sold at open markets in developing countries; contain diarrhoeagenic *E. coli* pathogroups due to contamination during the meat processing at slaughterhouse or to the retailers' poor handling of meat (Adeyanju and Shola, 2014). While antibiotics are not recommended for treatment of watery diarrhoea especially that caused by *E. coli* diarrhoeagenic isolates may contain antimicrobial resistance genes that may be from other isolates that are resistant within the same genus or other genera through horizontal transfer mechanisms (Baidouri *et al.,* 2014).

2.8 Diarrhoeagenic *Escherichia coli*

The bacteria is part of the Enterobacteriaceae family of gamma-proteobacteria (Todar, 2007) *E. coli* is a Gram-negative bacillus measuring 0.5 μm in diameter and 1.0-3.0 μm in length possessing an extracytoplasmic outer membrane that consists of a lipid bilayer, lipoproteins, and a capsule of lipopolysaccharide (LPS). The outer membrane interfaces with the bacterial and host environment. A variety of components of the outer membrane are critical determinants in antimicrobial resistance and pathogenesis (Chagnot *et al.,* 2013): *Escherichia coli* are motile in liquid by means of peritrichous flagella. Isolates vary with many combinations of somatic (O and K) and flagella (H) antigens. Pathogenic *E. coli* isolates have few

patterns of these antigens and phylogenetic groupings. For instance there are over 150 antigenically unique O-antigens (Pereira and Giuglianano, 2013; Whitfield and Valvano, 1993), two or four forms of K capsular material depending on the physical, biochemical and genetic characteristics (Jann and Jann, 1990) and 53 H-antigens (Wang *et al.,* 2003).

Genetic analysis of the 5S and 16S ribosomal RNA sequences indicate that the genera Escherichia together with Salmonella originated from a common ancestor between 120 and 160 million years ago (Sousa, 2006; Ochman and Wilson, 1987). Escherichia and Shigella fall within the same family Enterobacteriaceae but they both belong to different genera although, within the DNA sequence analysis of their genomes there is a high degree of sequence similarity (Wei *et al.,* 2003; Jin *et al.,* 2002; Pupo *et al.,* 2000). Despite the fact that this study mainly focuses on *E.coli,* other species exist such as*; E. adecarboxylata, E. blattae, E. fergusonii, E. hermanii and E. vulneris* but these rarely cause disease in humans. *Escherichia coli* strains belong to a number of different phylogenetic groups, which vary in their lifestyles characteristics, ecological niches and propensity to cause disease (Gao *et al.,* 2014).

Phylogenetic analyses on the nucleotide sequence of *E. coli* have led to the identification of clonal phylogenetic groupings. Within each pathotypes phylogenetic diversity exists (Tenaillon *et al.,* 2008; Ochman and Wilson, 1987). Biotype clustering such as; raffinose fermentation and sorbose fermentation are

universal to B2 isolates; though there is no link between hosts isolates source and clonal designation (Pupo *et al.,* 2008). *Escherichia coli* are not always confined to the intestine, and their ability to survive for brief periods outside the body makes them an ideal indicator organism to test environmental samples for faecal contamination. Most *E. coli* strains are non-pathogenic, but some, such as serotype O157:H7 can cause serious food poisoning in humans (Sanjar, 2014).

Diarrhoeagenic *E. coli* strains are classified on the basis of serological characteristics and virulence properties (Alikhani *et al*., 2012). Currently at least six virotypes have been adequately studied (Todar, 2007): Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAggEC), Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* EHEC (Goldwater and Bettelheim, 2012). Enterotoxigenic *Escherichia coli* are responsible for community-acquired diarrhoeal disease in areas of poor sanitation and are the commonest cause of travelers' diarrhoea (Sanjar, 2014), EAggEC causes chronic diarrhoeal disease in developing countries while EPEC causes infantile enteritis especially in tropical countries, and EHEC strains cause a disease that is similar to dysentery (bloody diarrhoea) (Sanjar, 2014). These pathotypes are responsible for diarrhoeal diseases which can be fatal particularly in children in developing countries (Lanata *et al.,* 2002). The *E. coli* has several pathotypes which are involved in causing diseases. These diseases manifest different symptoms in the gastrointestinal tract and extra intestinal sites. Approximately six intestinal and two extra intestinal pathotypes exist (Kaper *et al.,* 2004; Aslani *et al.,* 2012).

2.8.1 Enterotoxigenic *Escherichia coli* (ETEC)

Enterotoxigenic *E. coli* is known to cause diarrhoea in both humans and animals **(reference)**. It is estimated that this pathotype causes 600 million cases of human diarrhoea and 800,000 deaths worldwide particularly in children below 5 years (WHO, 1999). Enterotoxigenic *Escherichia coli* causes watery diarrhoea and cholera-like illness which have the potential of causing severe illness and is the most common cause of travelers' diarrhoea in children under the age of 5 years in endemic areas. Enterotoxigenic *E. coli* comprise of virulence factors heat-labile enterotoxin (LT), which is similar to the *Vibrio cholerae* cholera toxin in structure and function (Gonzales *et al.,* 2013; Fleckenstein *et al.,* 2000). LT consists of an AB toxin subunit holotoxin structure the A subunit enzymatically ADP-ribosylates the α-subunit of stimulatory G protein whereas the B subunits bind to host cell surface GM1 and GD1b gangliosides (Harris *et al.,* 2011)

The G protein regulates host cell adenylate cyclase and LT-mediated modification leads to its permanent activation and an increase in intracellular cAMP levels. This eventually leads to activation of the chloride ion channel of the intoxicated cells, increased chloride ion secretion into the intestinal lumen, and decreased sodium and chloride absorption (William *et al.,* 2008; Kaper *et al.,* 2004; Nataro *et al.,* 1988). The overall result is to reverse the normal intestinal osmotic gradient and cause a net water loss into the gut lumen. Enterotoxigenic *Escherichia coli* strains have heat-stable enterotoxins (STs) that are associated with watery diarrhoea. Stable toxins belong to two distinct groups namely; STa and STb, STa binds to the

extracellular domain of plasma membrane-embedded guanylate cyclase (Nagy and Fekete, 2005).

2.8.2 Enteropathogenic *Escherichia coli* (EPEC)

Enteropathogenic *E. coli* (EPEC) is the major cause of infant diarrhoea in developing nations, EPEC are classified according to their serotypes such as O55:H6 and O127:H6. They are diarrhoeagenic *E. coli* strains that causes a histopathological lesion known as attaching and effacing (A/E) lesions (Bueris *et al.,* 2015) on intestinal epithelium but are devoid of Shiga toxins (Vero toxins). They cause watery diarrhoea with mucus and symptoms such as; vomiting, fever, malaise and dehydration which may last up to several days, although instances of long, chronic disease have been reported to occur. The pathogenesis of EPEC involves an A/E lesion which is a result of a complex system of EPEC proteins that are injected into the host intestinal epithelial cell (Aslani *et al.,* 2008). The A/E lesion represents a rearrangement of the epithelial cytoskeleton where there is an accumulation of actin below the attached EPEC cell. EPEC has a specific pathogenicity island, termed the "locus of enterocyte effacement" (LEE) that encodes the genes responsible for the A/E lesion (McDaniel *et al.,* 1998). The LEE encodes a type III secretion system which provides intimate adhesin, its receptor and the injected proteins responsible for changes in host cell signaling mechanisms including actin pedestal formation; (Nougayrède *et al.,* 2003). EPEC have EAF (EPEC adherence factor) plasmids which encode an adherence factor, the bundle-forming pilus (bfp) (Masi *et al.,* 2012; Donnenberg and Kaper, 1992).

2.8.3 Enterohemorrhagic *Escherichia coli* (EHEC)

Enterohemorrhagic *E. coli* cause watery diarrhoea which progresses to bloody stools with ulcerations of the bowel. This can cause severe life threatening illness called hemolytic-uremic syndrome (HUS) which manifests with the following symptoms; hemolytic anemia, thrombocytopenia and renal failure (Loirat, *et al.*, 2011; Nataro and Kaper, 1988). Enterohemorrhagic *Escherichia coli* can be transmitted through ingestion of contaminated beef or foods contaminated with cattle faeces with a low infection dose of 10-100 organisms and has special acid-tolerance ability compared to other enteric bacterial pathogens. Children under the age of five are the major victims of EHEC disease, although the elderly may also exhibit bloody diarrhoea and HUS. Epidemiologically serotype O157:H7 is the most common in developed countries whereas serotypes like O26 and O111 (Aslani *et al.*, 2008) are found in developing countries though they cause a similar disease. This pathotype which primarily refers to *E. coli* O157;H7 is not only dependent on the virulence factors but partially also on the pathogen's ability to survive the environmental stress conditions such as resistance to low pH levels found in the gastrointestinal tract contributing to its very low infectious dose of 50-100 bacteria or lower. (Viazis and Diez-Gonzalez, 2011).

2.8.4 Enteroaggregative *Escherichia coli* (EAEC)

Enteroaggregative *E. coli* are pathotypes that lack the LT enterotoxin or Shiga toxins but adhere to cultured HEp-2 cells in self-aggregates that are classically referred to as "stacked bricks" (Loirat *et al.*, 2011; Nataro *et al.*, 1987), causes

watery diarrhoea that occurs in some cases with abdominal cramps, with no fever (Nataro *et al.,* 1995), does not invade the bloodstream and is found in both developed and developing countries. This pathotype is the second most common cause of traveler's diarrhoea after ETEC in the developed and developing countries and commonly being recognized as a cause of endemic and epidemic worldwide diarrhoea and recently has been shown to cause acute diarrhoeal illness in newborns and children in industrialized world. The pathotype has also been associated with persistent diarrhoea (Croxen and Finlay, 2010; Okeke, 2009; Weintraub, 2007).

Pathogenesis is not clearly understood due to lack of animal models and the heterogeneity of the virulence factors causing the paucity of details regarding the EAEC transmission, pathogenicity and epidemiology (Croxen and Finlay, 2010), Colonization of the intestinal mucosa by the EAEC occurs via aggregative adherence fimbriae (AAF) encoded by a 55-65 MDa plasmid named pAA (Czeczullin, *et al.,* 1997; Scaletsy *et al.,* 1984). Several potential virulence factors are common to EAEC isolates and express fimbrial adhesin called "aggregative adherence fimbriae" ("AAF). Enteroaggregative *Escherichia coli* isolates often produce a mucinase called "Pic" whose gene has the ability to express from its nonencoding DNA strand a smaller gene that encodes an enterotoxin Shigella enterotoxin [ShET1]) first described in Shigella strains, often produce a heat stable enterotoxin EAST1 that is homologous to the ST1 of ETEC (Boisen *et al.,* 2009; Huang *et al.,* 2006; Sheikh *et al.,* 2001).

2.8.5 Diffusely Adherent *Escherichia coli* (DAEC)

This a heterogeneous group of that generates a diffuse pattern of Hela and Hep-2 cells and is associated with watery diarrhoea and can become persistent in young children in developed and developing countries respectively. (Croxen and Finlay, 2010) The relative risks associated with DAEC increases with age with children from 18 months to 5 years, carriage of these strains have been reported to be widespread in older children and adults. These persistence consequences have a potential role in the development of chronic inflammatory intestinal disease (Servin, 2005).Two types of adhesin have so far been described dividing the DAEC strains into AIDA-1-dependent group and those that their adhesin is encoded by a family of related operons which include both fimbrial and fimbrial adhesin. These groups of proteins are collectively designated alfa-Dr adhesin (Croxen and Finlay, 2010; Nowicki *et al.,* 1990). The pathogenesis of DAEC is predominantly mediated through Afa/Dr adhesin interactions with host cells. In addition a secreted auto transporter toxin (Sat) has also been implicated in pathogenesis, but nevertheless, the implication of Afa/Dr DAEC strains remain controversial. (Servin, 2005)

2.8.6 Enteroinvasive *Escherichia coli* (EIEC)

The Enteroinvasive *E. coli* (EIEC) is related to Shigella species and share principal virulence genes (Van de Beid, 2012). It has been shown that EIEC strains and Shigella spp biochemically and pathogenetically very closely related so much so that that it has been proposed that they should be classified as one species in genus

Escherichia coli (Lan *et al.,* 2004; Benner *et al.,* 1972). The acquisition of the invasive plasmid (pINV) encoding the ability to invade host tissues (Parsot, 2005; Silva *et al.,* 1980) is the most important event that could have given rise to the evolution of Shigella and EIEC respectively from non-pathogenic *E.coli*. The pathotype causes invasive inflammatory colitis and dysentery with a clinical presentation when it invades the intestinal epithelium, principally in the large intestine where it lyses the phagocytic vesicle and replicates freely in the host cell cytoplasm. There is spreading to neighboring host cells by a motility process whereby actin is nucleated on one pole of the bacillus and subsequent actin polymerization propels the bacterial cell (Goldberg and Theriot, 1995).

Many components of type three secretion system (T3SS) important for delivery of modifiers of host cell signaling and membrane lysis are found on plasmids. In addition, the plasmid encodes an outer membrane protein (IcsA) that is localized on one pole of the bacterium and directs the actin microfilament polymerization necessary for spread of bacteria to other host cells (Sansonetti, 1992). They rarely invade the bloodstream, but they do invade the lamina propria immediately under the intestinal epithelium, where interaction with macrophages causes the release of pro-inflammatory mediators and even induction of apoptosis. Interestingly, the inability to decarboxylate lysine, a trait shared by EIEC and Shigella, is the result of mutations and gene rearrangements at the cadC gene (Maurelli *et al.,* 1998). The decarboxylation of lysine results in cadverine, which acts as an inhibitor of inflammation and migration of neutrophils into the lamina propria. The lack of this

function is hypothesized to be a pathoadaptive trait that enables EIEC/Shigella to cause disease (Casalino *et al.*, 2003; Fernandez *et al.*, 2001)

2.9 Phylogenetic Diversity and Epidemiology of Diarrhoeagenic *Escherichia coli*

Phylogenetic analysis of *E. coli* type is important since it helps in the determination of the relatedness between the genetic background and the type of the extended-spectrum ß-lactamase Branger *et al*., (2005) and Pupo *et al.*, (1997) found that intestinal pathogenic strains of *E. coli* are derived from phylogenetic groups namely A, B1 or D or from ungrouped lineages. These groups relate to each other on the basis of phylogeny and virulence. The virulent extra intestinal strains belong mainly to B2 and D group, whereas most commensal strains belong to groups A and B1 (Desjardins *et al.*, 1995). Evidence indicates a possibility of bla_{SHV} and bla_{TEM}-type being found in B2 phylogenetic strains, while bla_{CTX}-M-type in D phylogenetic group strains (Branger *et al.*, 2005). The multilocus enzyme electrophoresis and ribotyping are known to be the main reference techniques for phylogenetic grouping although these methods have been shown to be complex and time-consuming (Desjardins *et al.*, 1995; Bingen *et al.*, 1994; Selander *et al.*, 1986). To overcome these drawbacks, (Clermont *et al.*, 2000) described a simple and rapid technique for determination of the phylogenetic group of *E. coli* strains based on PCR detection of three specific phylogenetic group markers namely *chuA*, *yjaA*, and TSPE4C2, chuA is a gene required for heme transport, *yjaA* is a gene has been identified after complete genome

sequencing of *E. coli* K-12 and its function is still unknown. TSPE4C2 is an anonymous DNA fragment (Clermont *et al.,* 2000).

2.10 Antibiotic Resistance

Emergence of antibiotic resistance against commonly used antibiotic has become a serious global concern. Antimicrobial resistance is a significant public health concern and particularly so in developing countries (Zhang *et al.,* 2006; Kaper *et al.,* 2004), this consequently leads to challenges in the treatment of infectious diseases among the diarrhoea (Usha *et al.,* .2010).This is as a result of overuse and misuse (Jafari *et al.,* 2009; Nguyen *et al.,* 2006). Multi drug resistant clinical isolates of *E. coli* pathogenic strains are becoming common in clinics representing a major healthcare problem with increased morbidity and mortality worldwide (Mohamadi *et al.,* 2013; Lockhart *et al.,* 2007) This is made more complicated as a result of emergence of multidrug resistant strains to three (3) or even more classes of antibacterial agents belonging to different chemical classes by using various mechanisms (Falags *et al.,* 2010)

Antibiotics resistance can be intrinsic or acquired. Intrinsic resistance refers to bacteria that are resistant to an antibiotic in their natural state without acquiring resistance factors, for example, reduced sensitivity of gram –negative bacteria to penicillin. It is an inherent structural or functional characteristic which allow tolerance of particular or antimicrobial agent. This type of resistance may lead treatment with certain antibiotics becoming unsuccessful (Vernet *et al.,* 2014). It occurs due to the lack of the target for the action of drug and the inability of drug

to penetrate inside bacterial cell (Greenwood *et al.,* 2006; Normark and Normark, 2002). Acquired resistance to antibiotics involves different mechanisms of drug resistance. Acquired resistance involves two types that are; mutational resistance which occurs by point mutation, deletion, inversion or insertion in the bacterial genome and transferable resistance in which a resistance gene is transferred from a resistant to susceptible bacterial cell (Greenwood *et al.,* 2006: Normark and Normark, 2002).

2.11 The mechanisms of antibiotic resistance

A variety of mechanisms are responsible for acquired bacterial resistance to various antibiotics (Fluit *et al.,* 2001). Bacteria utilize these resistance mechanisms for protection against antibiotics; inactivation or destruction of the antimicrobial agent; alteration or protection of the target site; blocking the active transport mechanism, decreasing the cell surface permeability or (efflux) removal from the cell and the creation of alternative metabolic pathway instead of that was inhibited by antimicrobial agent (McManus, 1997). Beta symbol-lactam agents such, as penicillins, cephalosporin's and carbapenems are molecules of choice to treat a variety of infections. Their introductions into therapy were rapidly followed with development of resistances being increasingly being reported from different geographical regions (WHO, 2013).

The acquisition and accumulation of resistant determinants has given rise to multidrug ESBL producers, further limiting therapeutic options since the

identification of ESBL microorganisms in the early 1980s and shortly after the introduction of oxyimino-beta-lactam (Crémet *et al.,* 2010; Bonnet, 2004). In many European countries a rapid dissemination of *E. coli* and others entrobacteria producing ESBLs have been reported (Usein *et al.,* 2009; Woodford and Ellington, 2007; Coque *et al.,* 2008). In Latin America significant studies on resistance in *E. coli* isolates have been reported showing an increase in trends among the children (Ochoa *et al.,* 2004; Vilchez *et al.,* 2014; Estrada-Garcia *et al.,* 2005).In Africa the *E. coli* isolates, the resistance is seen in both pathogenic and nonpathogenic isolates, in Morocco (Bourjilat *et al.,* 2013), Madagascar (Rakotonirina *et al*.,2013) Egypt (Marwa *et al* 2012) Central Africa (Vlieghe *et al*.,2009) Nigeria (Okeke *et al*.,2000) in Gabon.

In Kenya several studies have shown similar trends being observed as in other regions pointed out in the above studies of an increase in *E. coli* strains showing resistance,(Sang *et al.,* 2011, 2012; Kiiru, 2011; Kariuki *et al.,* 2006; Bii *et al.,* 2005). Recognizing the potential for emergence of resistance and an upward trend of ESBLs should garner newfound respect for the discovery of new agents so urgently needed to cure infectious diseases which currently on the decline before the world get to the prebiotic era (Logan, 2014).

2.11.1 Genetic Basis of Resistance

Genetic basis of microbial resistance is vital since it provides information on the evolution and identity of different DNA elements that play a major role in resistance (Tourneur *et al.,* 2013). These elements include; Transposons, integrons,

and plasmids among others (Chen, 2009; Greenwood *et al.*, 2006; Heinemann, 2001; Carattoli, 2001).Although gene transfer among organisms within the same genus is common, the process has also been observed between very different genera, including transfer between very transfer between such evolutionary distant organisms as gram-positive and gram-negative bacteria (Tenover , 2006)

2.11.2 Transposons

These are mobile genetic elements that can exist on plasmids or integrate into the other transposons or the host's chromosomes. In general, these are pieces of DNA which contain terminal regions that participate in recombination and specify a protein(s) (for example transposase or recombinase) that facilitates incorporation into and from specific genomic regions. Conjugative trasnspons are unique in having qualities of plasmids of endogenous plasmids from one organism to another (Shapiro, 2010). They possess two main characteristics that differentiate them from other genetic elements, as they are mobile they can change their genetic environment and so doing they alter the genetic environment of the locus into which they insert. They have the intrinsic ability and multiply during the transposition process, they are automatically amplified, with a large copy number, restricted only by the carrying capacity of their environment for example the genome (Wicker *et al.*, 2007). Two classes based on their mechanism of transposition are; Class 1 (Retro-Transposons) which function by copying themselves from DNA to RNA by transcription and vice versa by reverses transcription. Class 11 (DNA Transposons), an RNA intermediate is not needed

for the cut and paste of transposable elements which are catalysed by various transposase enzymes (Wicker *et al.*, 2007).

2.11.3 Integrons

Integrons are bacterial genetic elements able to promote acquisition and expression of genes embedded within gene cassettes and characteristically constitutes a small genetic system possessing the ability to capture and co-express a set of resistance determinants with different functions (Ravi *et al.*, 2014; Stokes and Hall, 1989). They are generally non-mobile and are often located on mobile genetic elements like transposons and plasmids that could serve as vehicles for inter-and intra-species transmission of genes (Szmolka and Nagy, 2013).Integrons contain collections of genes (gene cassettes) that are generally classified according to the sequence of the protein (intergrase) that imparts the recombination function (Mazel, 2006).

The gene cassettes in the integrons are mainly antibiotic resistant genes which are expressed by a common promoter that ensures the correct expression of these cassettes (Szmolka and Nagy, 2013). They have the ability to integrate stably into regions of other DNAs where they deliver, in single exchange, multiple new genes, knowledge of the integrons residing in the microbiota can potentially aid in controlling the spread of antibiotic resistance genes to pathogens (Ravi *et al.*, 2014) and are classification is based on sequence and similarity. There are five classes of integrons with class1integrons being the most studied and characterised, they are reported in many gram-negative genera associated with gut microbiota

like Acinetobacter, *Escherichia coli* Salmonella (Mahmoudi-Aznaveh *et al.,* 2013; Meervenne *et al.,* 2013; Kiiru, 2011) among many more.

The class 11 integrons are associated often associated with Tn7 family of transposons and also found in Salmonella and Shigella (Kiiru, 2011). Class 111 integrons are very similar to other classes but are related to Tn402 transposons (Mazel, 2006). The class 1V and V studies have been identified in association with the development of trimethoprim resistance (Li *et al.,* 2013; Chen *et al.,* 2013). Integrons are composed two conserved regions flanking a variable region containing one or more of resistance genes. The essential components of any integron include the intergrase gene *(intl*), the attachment site (*attl*) and the promoter (Carattoli, 2001)

2.11.4 Plasmids

DNA transfer among bacteria is critical to the dissemination of resistance. Plasmids coding for antibiotic resistance were first recognized in Japan in 1959 following an outbreak of dysentery caused by multiple resistant multiple drug resistant (MDR) strains of Shigella (Datta, 1977). Transfer of DNA is most often via plasmids. A plasmid is a circular body of double stranded DNA which is separate from the chromosome and carries genes that encode various traits such as virulence and antimicrobial resistance (Kaye *et al.,* 2000). Plasmids have proved to be the ideal vehicles for recruitment and dissemination of resistance genes. There are two types of plasmids; conjugative and non-conjugative.

Conjugative plasmids transfer resistance via the sex pili whereas the non-conjugative plasmids must have direct contact for the transfer to occur (Svara and Rankin, 2011). In non-conjugative transfer, both the donor and the recipient bacteria have a copy of the transferred plasmid. Conjugative transfer is an important mechanism in antimicrobial resistant because transfer can occur in a wide range of bacterial species and can spread to unrelated organisms. A single plasmid can contain genes conferring resistance to multiple classes of antimicrobials (Szmolka and Nagy, 2013). Plasmids are extraordinarily versatile in that they are readily mobilazible by transformation, a factor that may be important in soil-dwelling organisms (Sikorski *et al.,* 2002) and represents an obvious mechanism for the spread of non-conjugative plasmids.

2.11.5 Insertion Sequence Common Region Elements (ISCR)

These are gene capture systems in organisms (Chen, 2009). They are closely related to usual family of insertion sequences called IS91 family. ISCR elements, as novel gene-capturing systems, are capable of mobilizing any piece of adjacent DNA sequences (Toleman *et al.,* 2006). This powerful gene mobilization mechanism serves as a highly mobile vector or milieu in transferring antibiotic resistance genes between different species of bacteria (Ooka *et al.,* 2009). Studies have highlighted the environmental resistance as a source of resistance genes of clinical interest (Marti *et al.,* 2014; Aminov and Mackie, 2007). Insertion Sequence Common Region (ISCR) elements are presently recognized as powerful antibiotic resistance gene capture and movements systems that also possess the

ability to construct extended clusters of antibiotic resistance genes on plasmids as well as on chromosomes (Toleman and Walsh, 2010).

2.12 Study Gaps in study area from literature review

Escherichia coli and the other entrobacteria are important reservoirs of transferrable antibiotic resistance (Salyers, *et al.,* 2004). Resistance to Beta-lactam antibiotics has become a particular problem in recent decades, as strains of bacteria that produce extended-spectrum Beta-lactamases have become more common. (Paterson and Bono, 2005). The Beta-lactamase enzymes make many, if not all, of the penicillins and cephalosporins ineffective in therapy. Extended-spectrum Beta-lactamase–producing *E. coli* is highly resistant to an array of antibiotics, and infections by these strains are difficult to treat. In many instances, only two oral antibiotics and a very limited group of intravenous antibiotics remain effective. Susceptibility testing should guide treatment in all infections in which the organism can be isolated for culture. The aetiology of diarrhoea includes both emerging and re-emerging pathogens. It is important to bear in mind that the principal purpose of monitoring antibiotic resistance trends among enteric pathogens is to provide clinicians with data that can be used to select appropriate treatment regimens. Molecular studies have to be considered as a part of diagnosis for prompt and accurate identification along with epidemiological monitoring.

Further investigations are therefore required to further explore acquiring resistance, particularly among animals and human to isolates to establish whether similarities exist. *Escherichia coli* isolates between animal and human groups can

possess relatively distinct profiles. This suggests that the number and diversity of genes driving phenotypic resistance are dynamic and have evolved through selection by antimicrobial use. There is urgent need to monitor antimicrobial resistance trends in the study site hospital.

Recent investigations suggest that integrons are common among MDR isolates and they can be used as a marker for the identification of MDR isolates. This could lead to a serious threat of an outbreak of antimicrobial resistance development, which complicates the treatment of infections and in the future, therefore precautionary measurements must be adopted to prevent spread of integrons. Eventually, considering the number of isolates bearing class 1 and class 2 integrons lacking any gene cassette, it is suggested that integrons structures should be further investigated at preserved regions in future researches.

The screening of ESBL production is not routinely done in many clinical laboratories in this country and the impact of treatment failure associated with these infections when treated with cephalosporins is not well documented. Failure or inability to screen for ESBL put into question the reliability and usefulness of susceptibility testing results generated in health facilities.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Location of Study Site

Thika Sub-county of Kiambu County covers an area of 1960.2 square kilometers. It bonders Ruiru Sub-County to the South, Gatundu Sub-County to the West, Murang'a County to the North and Machakos County to the East. It lies between latitudes 3^0 53' and 1^0 45' South of Equator and longitudes 36^0 35' and 37^0 25' East (Appendix VII). This study was conducted at Thika Level 5 Hospital from September 2010 to December 2012. This hospital serves the wider Thika District Community with an estimated population of 674,868 people by year 2010 at the start of the study (Thika District Strategic Plan 2005-2010). Thika Level 5 Hospital has a capacity of bed of 245 in the general wards and 51 beds in the pediatric ward but patients may share beds. It is estimated that 700 new outpatients visit the hospital each day. From hospital pediatric data between ten to twenty children per day visit the hospital due to diarrhoeal related illnesses. The outpatient department treats an average of 4,200 children per month.

3.2 Research Design

This was a cross-sectional laboratory based analytical study that sought to investigate antimicrobial resistance genes of faecal bacterial isolates from September 2010 to December 2012. The laboratory determination of antimicrobial resistance carriage of resistant genes patterns of *E. coli* isolates was carried out in this study.

3.3 Study Population

The target populations of this study were children under five years seeking treatment for diarrhoea treatment at the Thika Level 5 Hospital. The study population was comprised of both hospitalised children and those presenting to the outpatient department. A biographical data about each child was obtained and registered in the questionnaire (Appendix VII). Data included age, gender, outpatient or inpatient, disease history and taking antibiotic at the time of sampling and two (2) weeks of sampling. In 2010 during the study period, Thika District Hospital treated over 64, 004 children aged below 5 years (Thika District Strategic Plan, 2005-2010). The under-five years of age mortality rate was estimated at 36.7 per 1000 children.

3.3.1 Inclusion criteria

i. Children under five years of age presenting with diarrhoea at Thika Level 5 Hospital.
ii. Children with diarrhoea who have not taken antibiotics at least 2 weeks prior to sampling at Thika Level 5 Hospital.
iii. Children with diarrhoea whose parents/guardians give consent for their participation at Thika Level 5 Hospital

3.3.2 Exclusion criteria

i. Children above 5 years of age with or without diarrhea at Thika Level 5 Hospital

ii. Children with diarrhoea who had taken antibiotics within last 2 weeks to sampling at Thika Level 5 Hospital

iii. Children with/without diarrhoea whose parents/guardians did not give consent for their participation at Thika Level 5 Hospital

3.4 Sampling Technique and Sample Collection

Consecutive children under five years of age with laboratory requests for stool culture and sensitivity, visiting Thika Level 5 Hospital for diarrhoeal treatment during the study period and meeting the inclusion criteria were recruited into the study until the minimum sample size was attained. Stool samples were collected from recruited children in sterile plastic containers from those attending outpatients' clinic and inpatients respectively. For children who could not readily produce stool specimen, faecal material was collected from the anal opening using sterile moist cotton swabs. All samples collected were transported to the laboratory immediately for processing.

3.5 Sample Size Determination

The minimum sample size for this study was calculated following Lwanga and Lemeshow formulae (1992) as follows:

$n = pq\ (z^2)/\ d^2 = (.5)\ (.5)\ (1.96)^2\ /\ (0.05)^2$

Where n is the desired sample size, p is the proportion of patients with resistant diarrhoea. Since this proportion is not known, an estimate of 0.5 was made.

q = 1– p, z^2 is the standard normal deviate and d is the error of margin allowed (0.05 in this study), = 384 patients.

3.6 Proportional probability

The proportions of the 384 sampled children were 106 (27.6%) outpatients and inpatient 278 (72.4%) distributed resident wise, those who were outpatients: urban 43 (40.5%), Semi urban 30 (28.3%), rural 33 (31.13%) and from inpatient: Out patients: urban 95 (34.17%), semi urban 72 (25.9%) and rural 111 (39.93%)

3.7 Patient information and categorization diarrhoea data collection

Information on age, sex, residence (urban or rural), onset of diarrhoea, clinical symptoms and history of treatment and hospitalization was collected through interviews to mothers or guardians and the information recorded and filled in the questionnaire (Appendix IX).

Residence clustering was based on sub locations of former Thika municipality and divisions of Thika district, municipality boundaries being categorized as urban and surrounding areas as rural respectively.

For consistency, diarrhoea was defined as defecation of liquid or semi-liquid stool three or more times a day. Acute diarrhoea was defined as an abnormally frequent discharge of semisolid or fluid faecal matter from the bowel, lasting less than 14 days. Chronic diarrhoea was defined as diarrhoea lasting for more than four weeks. Specimens were only collected from children whose parents or guardians gave a written consent. In addition, specimens were only obtained from patients who had

not taken antibiotics at least two weeks before the onset and during the period of diarrhoea.

3.7.1 Culture and identification of *E. coli*

All collected stool samples were macroscopically observed and recorded on colour, mucoid, bloody or blood stained, soft or liquid, and were processed immediately on arrival at the Mount Kenya University Microbiology laboratory. The specimen were plated on MacConkey agar within one to three hours of collection and incubated at 37°C for up to 24 h. A maximum of three (3) colonies that morphologically resembled lactose fermenting *E. coli* were selected and sub-cultured on MacConkey agar again to obtain pure *E. coli* isolates. Pure growth of all sub cultured *E. coli* isolates were confirmed by the biochemical standard tests as previously described (Behiry *et al.,* 2011) These tests included oxidase test, citrate and urease utilization, and growth characteristic on lysine iron agar (LIA), triple sugar iron agar (TSI), and motility test on indole ornithine media (Oxoid LTD, Basingstoke, Hampshire, England). Colonies of pure strains were transferred into vials containing Tripicase Soy Broth supplemented with 15% glycerol and stored at -80^{o}C for future use.

3.7.2 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was done on Mueller-Hinton agar using the disc diffusion method according to the recommendation of Clinical and Laboratory Standards Institute (CLSI, 2012). Each of the three isolates obtained from each patient were subjected to antimicrobial susceptibility testing. The results were

interpreted according to guidelines of Clinical and Laboratory Standards Institute (CLSI, 2012). The choice of antibiotics was based on routine antimicrobials used for gram negatives and beta lactamases detection antibiotics. The following antibiotics classes: Beta-lactams, Quinolones, Aminoglycosides, Tetracycline, Sulfonamide+timethoprim (Marwa *et al.*, 2012) were tested at the concentrations indicated: Ampicillin (10 µg), Ceftazidime (CAZ, 30 µg), and Cefepime (FEP, 30 µg) Sulphamethoxazole-Trimethoprim 30:5.2 µg, Amoxicillin-Clavulanic acid, (AMC, in the ratios of 20:10 µg) respectively, Ceftriaxone (CRO, 30 µg), Tetracycline (TET, 30µg) Chloramphenicol (C, 30 µg), Nalidixic Acid (NA, 30 µg), Ciprofloxacin (CIP, 5 µg), Kanamycin (K, 30µg) Streptomycin (S, 30µg), Amikacin (AK, 30µg) and Gentamicin (CN, 10 µg). All the antibiotic discs were obtained from Oxoid (Basingstoke, Hampshire, England). In order to identify potential β-lactamase-producers, one plate was inoculated with only β-lactam antibiotics while the second plate contained all other antimicrobials.

The β-lactam antibiotics were placed adjacent to the Amoxicillin-Clavulanic (AMC) disc at inter-disc distances (centre to centre) of 20 mm. A clear extension of the edge of a cephalosporin disc zone towards the AMC (also described as an appearance of a ghost zone) was interpreted as positive for Extended Spectrum β-lactamase (ESBL) production. After the susceptibility to different antimicrobials was determined, the profiles of the three sets of isolates per patient were

compared. Any two isolates with an identical profile were regarded as duplicates in which case, only one of these isolates was selected for further analysis.

3.7.3 Minimum Inhibitory Concentrations (MIC) tests

MICs were determined for the following β-lactam antibiotics: - Amoxicillin-Clavulanic (AMC), Ceftazidime (CAZ), Sulphamethoxazole-Trimethoprim (SUL, TRIM) - Ampicillin (AMP), Ceftriaxone (CRO), Gentamicin (CN), Chloramphenicol (C), Nalidixic Acid (NA), Ciprofloxacin (CIP), Kanamycin (K), Streptomycin (S). MICs were tested using E-test susceptibility strips following manufacturer's recommendations (AB bioMérieux, Solna-Sweden) on Mueller Hinton Agar plates (Oxoid, Basingstoke, UK). *E. coli* ATCC 25922 was used as a control. The MIC breakpoints used were those recommended by the Clinical and Laboratory Standards Institute guidelines (CLSI, 2012).

3.7.4 Polymerase Chain Reaction for detection of antibiotic resistance genes

The method used in this study was that by Unno *et al.* (2010). Deoxybolic Nucleic Acid used as template in PCR reactions was obtained through boiling at 95^{o}C for 5 minutes. Briefly the bacterial cultures were grown overnight in LB broth at 37^{o}C with continuous shaking and 150rpm. The cells are collected by centrifugation at 14000rpm for 5 minutes before the supernatant was discarded. The pellet was re-suspended in 2 mL of sterile distilled water and transferred to 2 mL Eppendorf tubes. The tubes were placed in a heating block and the suspension allowed boiling at 95^{o}C for minimum 5 min. The mixture was allowed to cool down before centrifugation at 14000 rpm for 2 min. The supernatant was transferred to a new

tube and stored at -20^{o}C until further use. PCR amplifications were carried out in a final volume of 25 µL with 5 µL of template DNA and 1 µL of 10 mM concentration of both forward and reverse primers, see primers used in Table 3.1. Appropriate positive control strains were used for each reaction. PCR products were analyzed by electrophoresis in 1.5 % agarose gels, stained with ethidium bromide, visualized under UV light and the image recorded with the aid of a digital camera.

3.7.5 *In-vitro* Conjugation tests

Mating experiments were done as previously described (Jeong *et al.,* 2005). Selected MDR strains were used as donors in conjugation experiments. Selection of the strains was based on their antimicrobial resistance profiles. The azide resistant strain of *E. coli* J53 was used as a recipient. Transconjugants were recovered on Mueller-Hinton agar containing Sodium azide (200 µg/ml) and appropriate antimicrobials such as ampicillin (32 µg/ml) depending with the resistance profile of the donor. The susceptibility profiles of putative Transconjugants were done using the same panel of antimicrobials used for the donor strains.

3.7.6 Plasmid replicon typing by Polymerase Chain Reaction

For the determination of the role of plasmids in horizontal transfer of antimicrobial resistance, characterization of the diversity of plasmids among donors and Transconjugants was done. Plasmid DNA for replicon typing was obtained from cultures grown in brain heart infusion broth for 6-8 h at 37^{o}C with shaking. A

commercial plasmid extraction kit was used for purification of plasmid DNA following manufacturer's instruction (Qiagen Ltd., West Sussex, U.K). The protocol of (Carattoli *et al.,* 2005) was used for detection of plasmid replicon types. The lists of primers used for replicon typing are presented in Table 3.1.

Table 3.1: List of primers used for plasmid-replicon typing

Plasmid Replicon	Primer Name	5'-3' Sequence	Annealing Temperature	Expected product size (bp)	Gene (Accession Number)	
Multiplex I	HI1	H1-F	GGAGCGATGGATTACTTCAGTAC	60	471	parA-parB (AF250878)
		H1-R	TGCCGTTTCACCTCGTGAGTA-			
	HI2	H2-F	TTTCTCCTGAGTCACCTGTTAACAC	60	644	iterons (BX664015)
		H2-R	GGCTCACTACCGTTGTCATCCT			
	I1-Iγ	I1-F	CGAAAGCCGGACGGCAGAA	60	139	RNAI (M20413)
		I1-R	TCGTCGTTCCGCCAAGTTCGT			
Multiplex 2	X	X-F	AACCTTAGAGGCTATTTAAGTTGCTGAT	60	376	ori g (Y00768)
		X-R	TGAGAGTCAATTTTTATCTCATGTTTTAGC			
	L/M	L/M-F	GGATGAAAACTATCAGCATCTGAAG	60	785	repA,B,C (U27345)
		L/M-R	CTGCAGGGGCGATTCTTTAGG			
	N	N-F	GTCTAACGAGCTTACCGAAG	60	559	repA (NC_003292)
		N-R	GTTTCAACTCTGCCAAGTTC			

Plasmid Replicon	Primer Name	5'-3' Sequence	Annealing Temperature	Expected product size (bp)	Gene (Accession Number)	
Multiplex 3	FIA	FIA-F	CCATGCTGGTTCTAGAGAAGGTG	60	462	iterons (J01724)
		FIA-R	GTATATCCTTACTGGCTTCCGCAG			
	FIB	FIB-F	GGAGTTCTGACACACGATTTTCTG	60	702	repA (M26308)
		FIB-R	CTCCCGTCGCTTCAGGGCATT			
	W	W-F	CCTAAGAACAACAAAGCCCCCG	60	242	repA (U12441)
		W-R	GGTGCGCGGCATAGAACCGT			
Multiplex 4	Y	Y-F	AATTCAAACAACACTGTGCAGCCTG	60	765	repA (K02380)
		Y-R	GCGAGAATGGACGATTACAAAACTTT			
	P	P-F	CTATGGCCCTGCAAACGCGCCAGAAA	60	534	iterons (M20134)
		P-R	TCACGCGCCAGGGCGCAGCC			
	FIC	FIC-F	GTGAACTGGCAGATGAGGAAGG	60	262	repA2 (AH003523)
		FIC-R	TTCTCCTCGTCGCCAAACTAGAT			
	A/C	A/C-F	GAGAACCAAAGACAAAGACCTGGA	60	465	repA (X73674)

Plasmid Replicon	**Primer Name**	**5'-3' Sequence**	**Annealing Temperature**	**Expected product size (bp)**	**Gene (Accession Number)**	
Multiplex 5		A/C-R	ACGACAAACCTGAA TTGCCTCCTT			
	T	T-F	TTGGCCTGTTTGTGC CTAAACCAT	60	750	repA (K00053)
		T-R	CGTTGATTACACTTA GCTTTGGAC			
	FII	FII-F	CTGTCGTAAGCTGAT GGC	60	270	repA (AE006471)
		FII-R	CTCTGCCACAAACTT CAGC			
Simplex PCR**s**	FIB	FIB-F	TGATCGTTTAAGGAA TTTTG	52	270	RNAI/repA (AY234375)
		FIB-R	GAAGATCAGTCACA CCATCC			
	K	K/B-F	GCGGTCCGGAAAGC CAGAAAAC			
		K –rev	TCTTTCACGAGCCCG CCAAA	60	160	RNAI (M93063)
	B/O	B/O-rev	TCTGCGTTCCGCCAA GTTCGA	60	159	RNAI (M28718)

3.7.7 Determination of Plasmid Profiles

The plasmid profiles of the donors and Transconjugants were determined using the method of Kado and Liu (1981). Pure isolates were inoculated in 10 mL of LB broth at 37°C overnight with shaking. The cells were then harvested by centrifugation at 14000 rpm for 5 min. The supernatant was decanted and the pellet resuspended in 300 µL suspension buffers consisting of 50 mM glucose, 50 mM Tris-HCl, and pH 8.0, 10 mM ethylene diamine tetra acetic acid (EDTA). The cells were then lysed by adding 300 µL of lysis buffer consisting of 200 mM NaOH, and 1% SDS. The suspension was mixed by inverting the tube several times. The lysis was allowed to happen for another 2-3 minutes before adding 350 µL of neutralizing solution containing 3M potassium (pH 8.0). The suspension was centrifuged at 14000 rpm for 2 min before the supernatant was transferred to a clean Eppendorf tube. To these tubes, 250 µL of isopropanol was added before precipitating the DNA by centrifugation at 14000rpm for 2 min. The supernatant was decanted and the pellet washed by adding 750 µL of ice-cold 70% ethanol. The suspension was vortexed briefly, and centrifuged at high speed for 30 s. The supernatant was decanted and the excess ethanol left to air-dry for 5 min. To the DNA pellet, 50 µL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) was added and the pellet resuspended. Plasmid DNA was separated by electrophoresis on 0.8% agarose gel for a minimum of 2 hrs.' at 4 Volt/cm. Plasmids of known sizes isolated from *E. coli* V517 and 39R861 were used as size markers.

3.7.8 Detection of Integrons

Screening of Integrons was done for isolates from children with different clinical backgrounds. Detection of class 1 integron, the 3'-CS and amplification of the integron was done using the procedure described by Dillon *et al.,* (2005). The primers that were used for this test are listed in Table 3.2.

Detection of class 3 was done as previously described by Rayes *et al.* (2010) and Senda *et al*. (2006) and Falbo *et al*. (1999). Amplification of integrons class 1 VCRs was done using a forward primer (IN-F) that target the attI sequence on the 5'-CS and a reverse primer (IN-B) that target the 3'-CS as previously described by (Dalsgaard *et al*., 2000).

Table 3.2: Primers utilized for amplification and characterization of integron class 1, 2 and 3

Target Gene	Primer Name	5'-3' Sequence	Annealing Temperature	Expected Product Size (bp)	Gene Accession Number
intI1	int-1U	GTTCGGTCAAGGTTCTG	50	923	U12338
	int-1D	GCCAACTTTCAGCACATG			
intI2	int-2U	ATGTCTAACAGTCCATTTT	50	450	AJ001816.1
	int-2D	AAATCTTTAACCCGCAAAC			
intI3	IntI3-F	GCAGGGTGTGGACGAATACG	57	760	AY219651

	IntI3-R	ACAGACCGAGAAGGCTTATG			
integron class 1 3'-CS	qacED1	ATCGCAATAGTTGGCGAAGT	56	800	X15370
	sul1-B	GCAAGGCGGAAACCCGCGCC			X12869
Integron class 1 VCR	in-F	GGCATACAAGCAGCAAGC	52	Variable	U12338
	in-B	AAGCAGACTTGACCTGAT			
integron class 2 VCR	hep74	CGGGATCCCGGACGGCATGCACGATTTGTA	52-60	Variable	EU780012
	hep51	GATGCCATCGCAAGTACGAG			AJ002782

3.7.9 Testing for selected Extended Spectrum β-Lactamases (ESBL) genes

Isolates identified to be ESBL-producers were selected and screened for selected β-lactamase genes as described by Lartigue *et al*. (2004). The genes selected are the ones previously been reported to be harbored on conjugative plasmids and such genes are therefore of a greater clinical and epidemiologic significance. A total of 10 isolates susceptible to all β-lactams were also use as negative controls. Polymerase Chain Reactions were done using consensus sequence primers previously published for bla_{SHV} and bla_{TEM} (Arlet *et al.,* 1997), bla_{CTX-M}, (Lartigue *et al.,* 2004) and bla_{oxa} (Winokur *et al.,* 2000). These genes are common among members of family Enterobacteriaceae (Knox, 1995). The list of primers used for screening and sequencing various β-lactamase genes is presented in Table 3.3.

Table 3.3 List of primers used for screening for various β-lactamase genes

Target Gene	Primer Name	5'-3' Sequence	Annealing Temperature	Expected Product Size (bp)	Gene Accession Number
*bla*TEM	TEM-F	GCGGAACCCCTATTTG	50	964	EF125012-related
	TEM-R	TCTAAAGTATATATGAGTAAACTTGGTCTGAC			
*bla*SHV	SHV-F	TTCGCCTGTGTATTATCTCCCTG	50	854	AF148850-related
	SHV-R	TTAGCGTTGCCAGTGYTCG			
*bla*CTX-M (consensus)	CTX-F	ATGTGCAGYACCAGTAARGTKATGGC	60	593	Y10278-related
	CTX-R	TGGGTRAARTARGTSACCAGAAYCAGCGG			
	CTXM-IVR	GTA AGC TGA CGC AAC GTC TG			
*bla*OXA-1	OXA-1F	ATGAAAAACACAATACATATCAACTTCGC	62	820	JO2967-related
	OXA-1R	GTGTGTTTAGAATGGTGATCGCATT			

3.8 Genetic relationship of extended spectrum beta-lactamase *E. coli* isolates

Genetic relationship of ESBLs isolates was investigated using the Enterobacterial Repetitive Intergenic Consensus Sequence PCR (ERIC-PCR) (Ranjbar *et al.*,

2013). Amplification reactions were be carried out in 50-µl volumes using the Takara Ex Taq DNA polymerase and buffer system (Takara Mirus Bio Corporation, Madison, WI). The final PCR mixture comprised of 1× Ex Taq buffer (with 2 mM $MgCl_2$), a 200 µM concentration of each deoxynucleoside triphosphate, an 800 nM concentration of each ERIC primer, 1.25 units of Ex Taq DNA polymerase, and 150 ng of template DNA. Primer sequences used were the universal ERIC primers of Versalovic *et al.,* (1999), as follows: ERIC-1, 5′-ATG TAA GCT CCT GGG GAT TCA C-3′; and ERIC-2, 5′-AAG TAA GTG ACT GGG GTG AGC G-3′. Polyacrylamide gel electrophoresis-purified primers (Sigma-Genosys, Woodlands, TX) were be used to ensure reproducibility and to avoid variability among batches of primers.

The PCR cycle program was carried out using a Peltier PTC-200 thermal cycler (MJ Research Inc., Watertown, MA) and employing the calculated temperature control option, with initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 1 min, and extension at 65°C for 10 min and a final extension step at 65°C for 20 min.

3.8.1 Analysis of Enterobacterial Repetitive Intergenic Consensus Sequence Polymerase Chain Reaction (ERIC-PCR) patterns

After the PCR was completed, each 50-µl reaction mixture was be mixed with 10 µl of 6× loading buffer (15% Ficoll 400 in water, 0.01% bromophenol blue), a modification from the work of Sambrook *et al.* (2001) to avoid interference in band imaging by dye in the gel, and 5 µl of the mixture was used for gel

electrophoresis. Three microliters of molecular weight marker (HyperLadderI; Bioline USA Inc., Canton, MA) was loaded in the first, middle, and last lanes of the gel for gel image normalization. One percent (wt/vol) agarose was selected as the appropriate concentration for electrophoresis separation of the long-range ERIC-PCR products. Electrophoresis was performed at 120 V for 2.25 h, using a Bio-Rad GT Subcell electrophoresis system (electrode distance, 30 cm) with a 15-cm by 20-cm tray. Tris-borate with EDTA (TBE; 89 mM Tris-borate, 1 mM EDTA), or Tris-acetate with EDTA (TAE; 40 mM Tris-acetate, 1 mM EDTA) was used. After electrophoresis, the gels were stained in ethidium bromide solution (5 μg ml^{-1}) for 3 to 5 min and destained in tap water for 20 min, with shaking. The fingerprint banding patterns was recorded using ditigal image capture (Molecular Dynamics Inc., Sunnyvale, CA).

3.8.2 Analysis of Cefotaxime-Munich (CTX-M) genes

To determine the restriction patterns of CTX-M genes, the PCR amplicons were subjected to digestion polymorphism using HindII restriction enzyme (New England Biolabs Ltd, Hertfordshire, UK) as described in a previous study (Yu *et al.,* 2004). Strains shown to carry various CTX-M genes in previous studies using sequencing techniques were also digested alongside the test amplicons and used as standards. The restriction patterns were run on a 0.8% agarose gel for 2 hours at 80 volt. CTX-Ms with identical restriction fragment patterns were assigned to the same restriction patterns.

3.9 Ethical Considerations

Permission to conduct the study was obtained the hospital authorities of Thika Level 5 Hospital (Appendix V), authority from Mount Kenya University Ethical Review Committee and Board of Postgraduate Studies (Appendix VI), Kenyatta University proposal approval and research authorization respectively, (Appendix I and II), then National Council of Science and Technology (NSCT) (Appendix III and IV) and present National Commission of Science Technology and Innovation (NACOSTI). The identity of patients enrolled in the study and the data collected were treated in confidence following informed consent from the parents/guardian of the study child (Appendix VIII). All identifiers were removed prior to analysis. The susceptibility results were conveyed to the hospital to support diagnosis and treatment. Patient data was treated with utmost confidence.

3.10 Data Analysis

Key variables, as captured in the questionnaire, (Appendix IX) were summarized, cross-tabulated and analyzed for selected proportions which were reported together with their associated 95% Confidence Intervals (CIs). Both intermediate and resistant results for antibiotic susceptibility testing were grouped together as either “resistant” or “sensitive”. Inferential analysis of the dissimilarities in proportions of isolates bearing different elements were analyzed using the Chi (χ^2) tests while Fisher’s exact test was used for smaller sample sizes. The Odds Ratios (OR) and 95% Confidence Intervals (CIs) accompanying the χ^2 tests were determined using the approximation of Woolf. Associated P-values were also computed and the null

hypothesis was rejected (implying significant result) for values of $p<0.05$. Statistical analysis was performed using Stat graphics plus Version 5 (Stat Point Technologies, INC, Warrenton, VA, USA).

CHAPTER FOUR: RESULTS

4.1 Respondents' profile

A total of 384 patients both inpatient and outpatient were recruited in the study, represented as percentage frequency Figure 4.1.

4.1.1 Type of patient (Out-patient/In-patient) (n = 384)

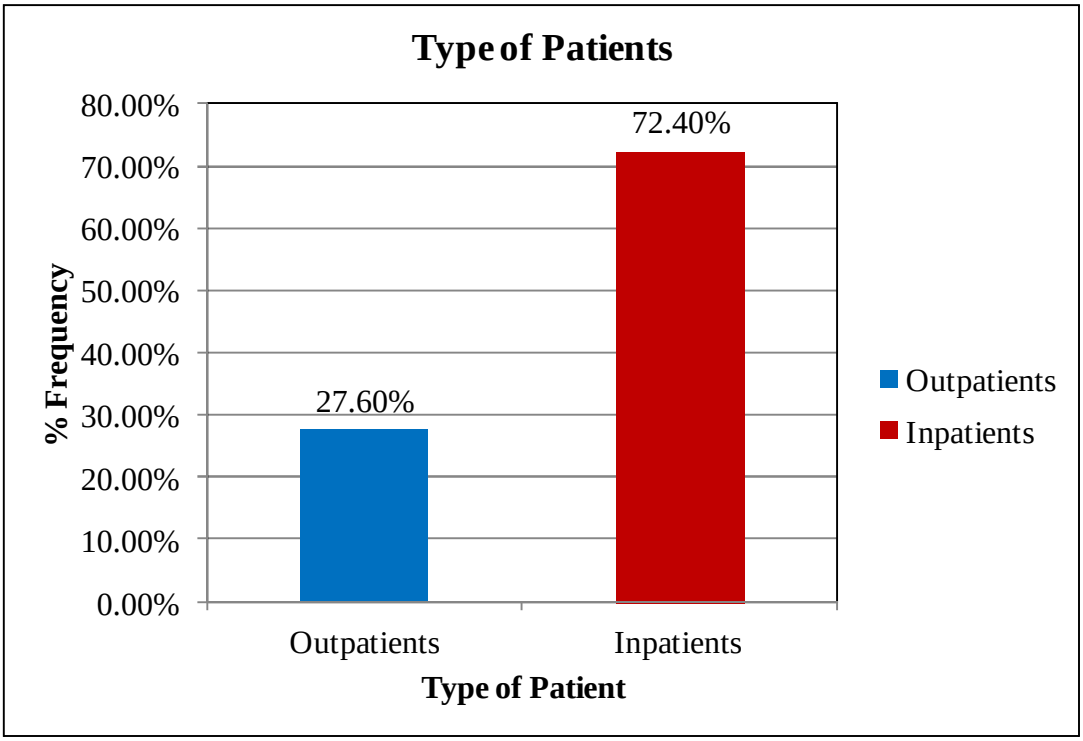

Figure 4. 1: Type of patient (Out-patient/In-patient) Overall proportion

Outpatients: 27.60 % (95% CI: 23.13-32.07%); In-patients: 72.40 % (95% CI: 67.93-76.87%).

The ages of study patients ranged from <1 month as the highest and between 36 and 60 months as the lowest as shown in Figure 4.2, with confidence intervals (CI) distributed in the following order: **0 - <1 m:** 35.94% (CI 95%: 31.14% - 40.74%); **1 m - <12 m:** 30.47% (CI 95%: 25.87% - 35.07%); **12 m- < 36 m:** 23.70% (CI 95%: 19.45% - 27.95%) and **36 m - <60 m:** 9.90% (CI 95%: 6.91% - 12.89%).

4.1.2 Age distribution (n = 384)

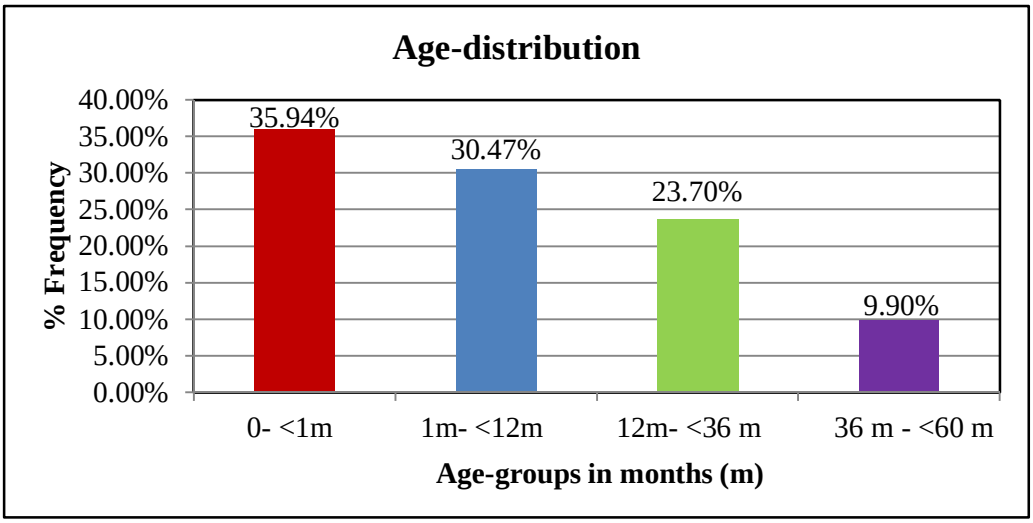

Figure 4. 2 Age distribution

A higher percentage of female than male children were examined in this study Figure 4.3

4.1.3 Sex distribution (n = 384)

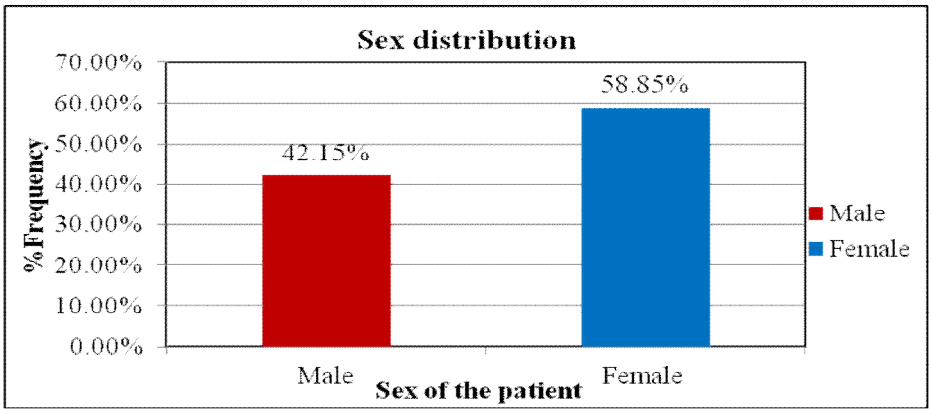

Figure 4. 3: Sex distribution

The study recruited more urban children than rural by close to a half of the studied patients as indicated in Figure 4.4

4.1.4 Type of residence (n = 384)

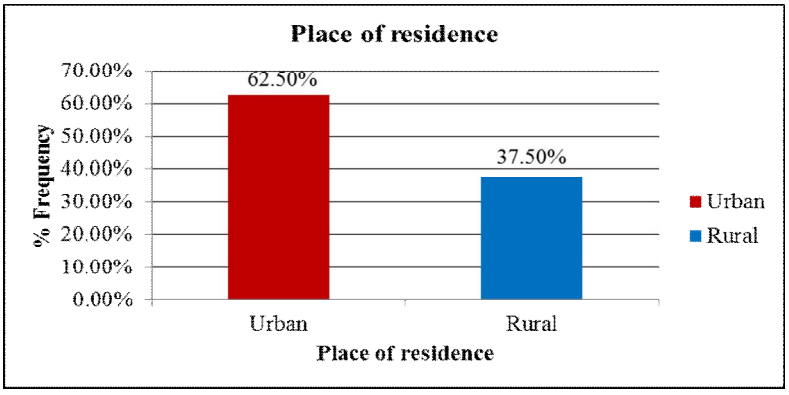

Figure 4. 4 Place of residence Urban.

4.1.5 Clinical Backgrounds

4.1.5.1 Diarrhoeal episodes (n=384)

Inpatients had significantly higher number of diarrhoeal episodes p=0.00013 and as per the non-overlapping 95% confidence intervals of CI: 3.51-3.92 for inpatients and CI: 1.97-2.41 for out patients with mean number of diarrhoeal episodes of 3.6 for inpatients and 2.3 for outpatients

4.1.5.2 Type of Diarrhoea, Vomiting and Abdominal Swelling (n=384)

All the variables in Table 4.1 below are categorical and therefore Chi-square test of independence was used to investigate possible associations. There was statistically significant association between type of patient (Outpatient/Inpatient) and various clinical states of the patients.

Table 4.1

Clinical Background			**Type of Patient**		**Total**	**χ^2 and p-values**
			Outpatient	**Inpatient**		
1	**Diarrhoea**	Acute	74	227	301	3.0172; < 0.05
		Chronic	32	51	83	
		Total	106	278	384	
2	**Vomiting**	Present	83	181	264	6.0190; < 0.05
		Absent	23	97	120	
		Total	106	278	384	
3	**Abdominal Swelling**	Present	27	60	87	1.9150; < 0.05
		Absent	79	218	297	
		Total	106	278	384	

4.1.5.3 History of Antibiotic use (n=384)

There are more outpatient antibiotic users than the inpatients (Average percentage difference: 18.83%; $\chi^2 = 2.7116$; p=0.00021) as shown in Figure 4.5

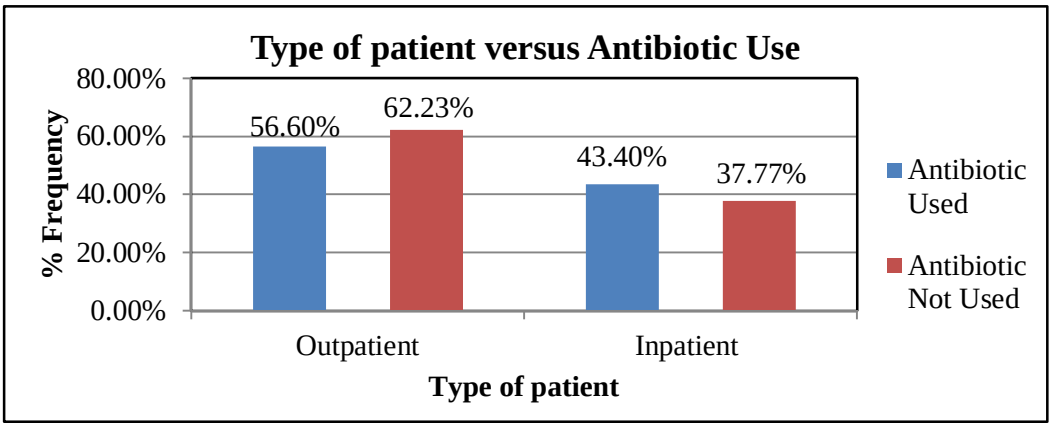

Figure 4. 5 History of Antibiotic use (Last 3 months)

4.2 Results of microbiological investigations

The isolates analyzed in this study are summarized in Table 4.3 and only one strain per sample per participant was analyzed. Isolates were grouped according to their resistance combinations to major classes of antimicrobials. The percentages were calculated by dividing the number of isolates with a given phenotype by the total number of isolates in that column. The symbol (*****) indicates χ^2 values for dichotomous variables (such as chronic versus acute Diarrhoea) that were statistically significant (p=0.05).

Table 4.2: Multidrug Resistance phenotypes combinations of *E. coli* isolates from different clinical backgrounds

Resistance Dimension	**Episodes of Diarrhoea**		**History of hospitalization**		**Antibiotic use**	
	Acute Diarrhoea	Chronic Diarrhoea	Visited hospital or hospitalised in last 6 months	Not hospitalised for last 3 months	Antibiotic used in the last 3 months	No antibiotic used for the last 3 months
Number of isolates tested	338	46	158	226	174	210
Resistance to 4th generation β-lactams (FEB)	17 (5)	0	**14 (9)***	3 (1)	12 (7)	5 (2)
Resistance to NA or CIP	14 (4)	32 (7)	**31 (20)***	15 (7)	**32 (18)***	14 (7)
Resistance to at least one Aminoglycosides	**223 (66)***	13 (29)	**137 (87)***	99 (44)	**131 (75)***	105 (50)
Resistance to SXT/FEP / NA or CIP/ one or more Aminoglycosides	**3 (1)***	3 (7)	5 (3)	1 (<1)	5 (3)	1 (<1)
ESBL phenotype	**47 (14)***	0	**28 (18)***	19 (8)	**36 (21)***	11 (5)

4.2.1 Multidrug resistance among *E. coli* isolates

Multidrug resistance (resistance to at least three classes of antimicrobials) was observed in 36% (CI: 28.01-39.35) of all isolates tested in this study. A total of 32% of *E. coli* isolates were resistant to Amoxicillin-Clavulanic acid combinations (AMC) while 63% were resistant to ampicillin (p=0.00072). Resistance to other β-lactam antibiotics was much lower and only 47 (12%) of the 384 isolates exhibited the ESBL phenotype. The study showed that 5% of isolates were resistant to cefepime (FEP). Isolates that were resistant to β-lactams were also resistant to Sulphamethoxazole and Trimethoprim (SXT) (cross-resistance) and to at least one more class of antimicrobial especially Tetracycline (TET) and Chloramphenicol (C). Streptomycin (S) was the least effective aminoglycosides while Gentamicin (CN) and amikacin (AK) were effective against at least 70% of all isolates. In addition, all isolates resistant to either Nalidixic Acid (NA) or Ciprofloxacin (CIP) were also resistant to at least a 3rd-generation cephalosporin. Overall, there was a statistically significant multi-drug resistance among *E. coli* isolates from the U5s studied (χ^2 = 11.717; p=0.00604). Such multi-drug resistant isolates were from both children who had or had not taken antibiotics, and resident in both urban and rural settings.

4.2.2 Resistance to tested antimicrobials

Resistance to Ampicillin, Sulfonamides, Tetracycline, Streptomycin and Amoxicillin-Clavulanic acid was noted in over 30% of all isolates (χ^2 = 6.0018; p= 0.00017); Figure 4.6 shows that at least 52% (χ^2 = 3.4918; p=0.00651) of the

isolates were resistant to more than three different classes of antimicrobials tested and were thus classified as multi-drug resistant (MDR). Resistance was lowest for Ciprofloxacin (4%), Nalidixic Acid (5%), Cefepime (5%), Ceftriaxone (7%) and Ceftazidime (7%).

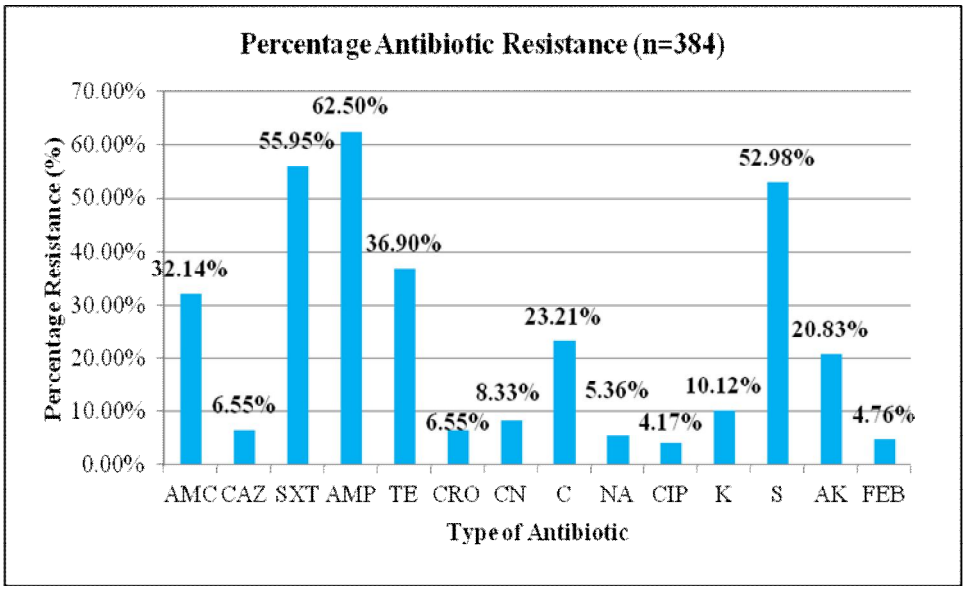

Figure 4.6: Percentage Antibiotic Resistance among the 384 *E. coli* strains

Amoxicillin-Clavulanic acid (AMC), Ceftazidime (CAZ) Sulphamethoxazole-Trimethoprim (SXT), Ampicillin (AMP), Tetracycline (TE), Ceftriaxone (CRO), Gentamicin (CN), Chloramphenicol (C), Nalidixic Acid (NA), Ciprofloxacin (CIP), Kanamycin (K), Streptomycin (S), Amikacin (AK), and Cefepime (FEP).

4.2.3 Resistance among isolates from inpatient and outpatients

Resistance to most antimicrobials tested was recorded among isolates from in patients (hospitalized) and out patients (non-hospitalised) Figure 4.7. Resistance prevalence was higher for isolates from hospitalised patients than those from

outpatients. However, a prevalence of 37% resistance to Tetracycline was recorded for isolates from outpatients compared to a prevalence of 25% among *E. coli* from inpatients, no resistances were recorded among isolates from inpatients against Cephalosporin such as CAZ, and FEP and against CN, and CIP but resistance to these antimicrobials was between 4% and 9% among strains from outpatient.

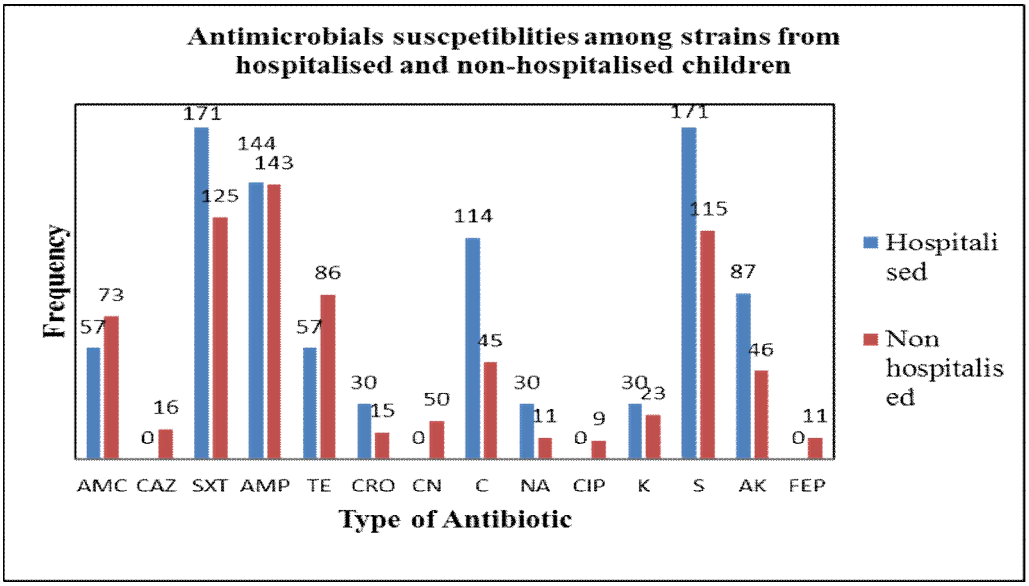

Figure 4.7: Resistance among isolates from inpatient and outpatient children

Amoxicillin-Clavulanic acid (AMC), Ceftazidime (CAZ) Sulphamethoxazole-Trimethoprim (SXT), Ampicillin (AMP), Tetracycline (TE), Ceftriaxone (CRO), Gentamicin (CN), Chloramphenicol (C), Nalidixic Acid (NA), Ciprofloxacin (CIP), Kanamycin (K), Streptomycin (S), Amikacin (AK), and Cefepime (FEP).

4.2.4 Antimicrobial susceptibilities among strains from children with different number of diarrhoeal episodes per day

Isolates from children who had more than three episodes of diarrhoea had a higher proportion of isolates showing resistance to cephalosporin (CAZ, CRO, and FEP) and to Quinolones such as NA, and CIP, than those from children with fewer than three episodes of diarrhea (Figure 4.8). However, resistance to SXT, TET, AMC, S, C, and AMP was relatively high (above 30%) in both populations. A higher proportion of isolates from children with acute diarrhoea were resistant to AMC, SXT, TET, C, S and AK, compared to those from children with chronic diarrhoea (p=0.0026). Figure 4.10 none of the isolates with chronic diarrhoea were resistant to CAZ, CRO, K, and FEP.

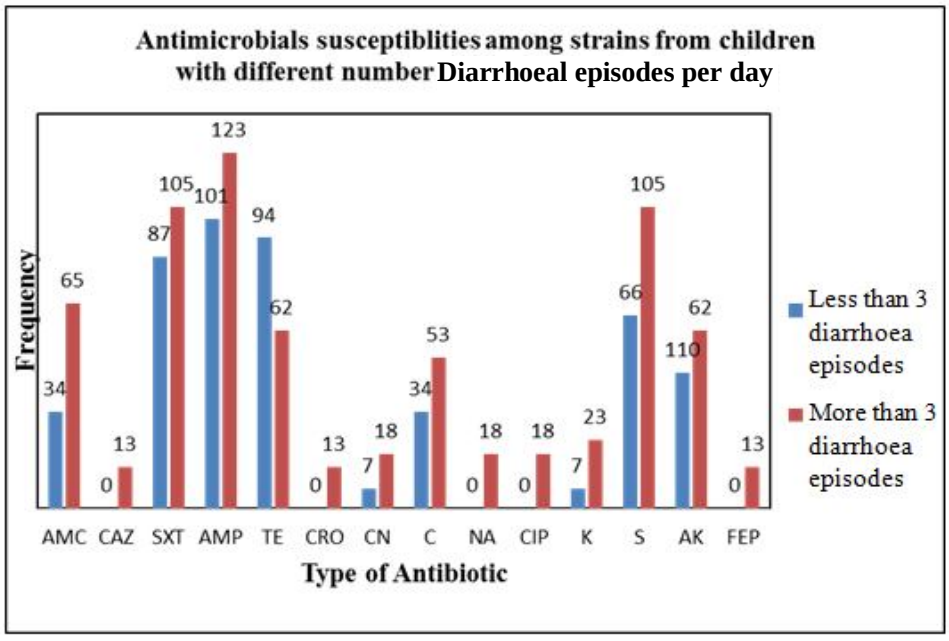

Figure 4.8: Resistance among isolates from children who had experienced different number of diarrhoea episodes

4.2.5 Resistance among isolates from rural and urban children

In general, differences in prevalence of resistance to antimicrobials among strains from urban and those from rural settings were not statistically different (OR=2.680; CI=1.41-5.113; p=0.55). However, over 30% of strains from children living in rural and urban settings were resistant to AMC, SXT, TET, and S, - a statistically significant finding (χ^2 = 3.713; p= 0.0026), Figure 4.9. The difference between the prevalence resistance of isolates from urban settings and those from rural settings to SXT, CRO, C, NA, and S was at least 5% with those from peri-urban settings recording higher values. Isolates from urban settings registered higher resistance prevalence to all antimicrobials compared to those from rural settings except for AMC in which case a prevalence of 32% was recorded for isolates from both settings. On the other hand, strains from rural settings had a resistance prevalence of 24% to K compared to a prevalence of 19% among strains from peri-urban settings, this was statistically significant (χ^2 = 5.517; p=0.000315).

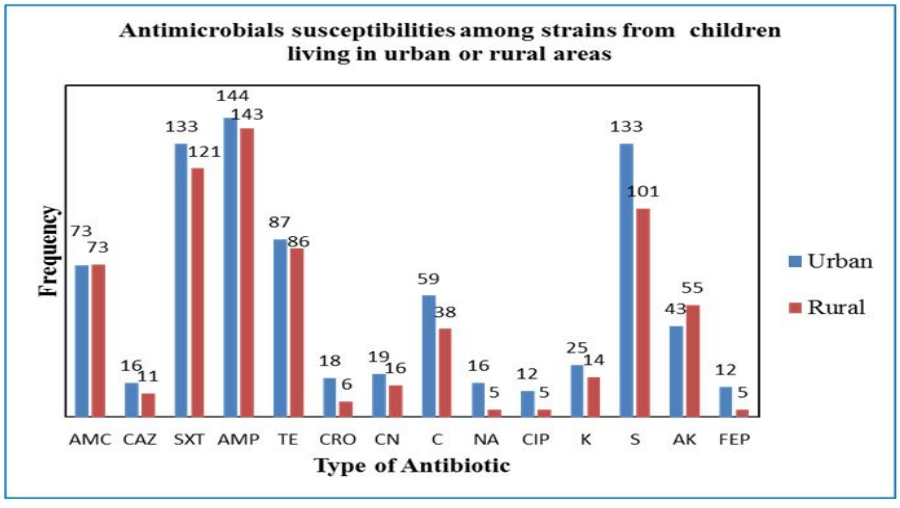

Figure 4. 9 Resistance among isolates from rural and urban settings

4.2.6 Occurrence of Multi-drug Resistant *E. coli* among isolates from children with acute or chronic diarrhoea

The isolates from acute diarrhoea stools were significantly resistant to a broader spectrum of antimicrobials than those from children with chronic diarrhea (p=0.003; OR: 2.68l; 95% CI:1.41-5.113), Figure 4.10. Resistance to fourth generation cephalosporin like cefepime or to Nalidixic acid and ciprofloxacin among isolates from these two categories of patients was not significantly different. Isolates obtained from children with acute diarrhoea were more likely to exhibit resistance to at least one or more amino glycosides than those from children with chronic diarrhoea (p=0.014; OR: 3.24; 95% CI: 1.63-6.12). All the 47 ESBL isolates identified in this study were from stool specimen obtained from children with acute diarrhoea. Isolates from acute diarrhoea were also more likely to exhibit combined resistance to SXT/FEP/NA or CIP/ and to at least one amino glycoside than those from chronic diarrhoea (p=0.024, CI=0.025-0.66, OR=0.13).

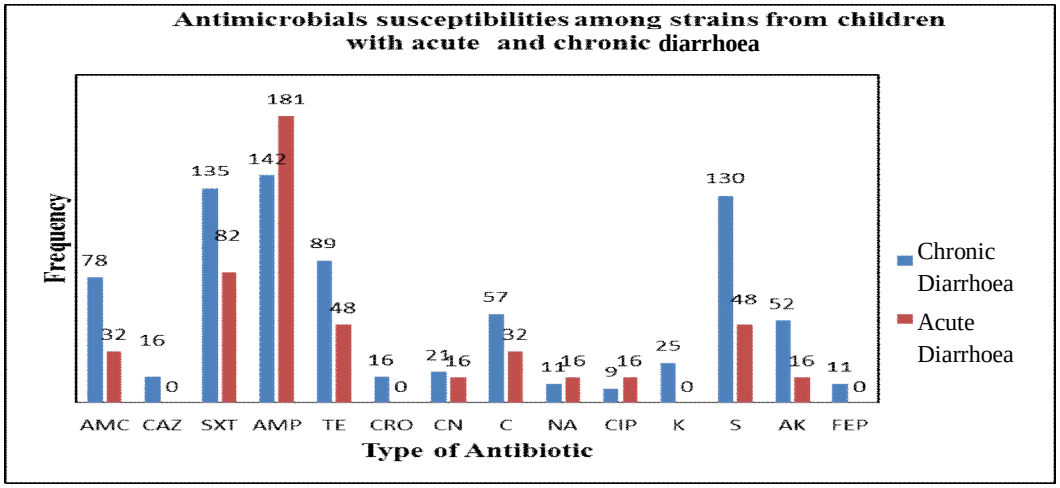

Figure 4. 10: Resistance among isolates from children with acute or chronic diarrhoea

4.2.7 Occurrence of Multi-drug Resistant strains among isolates from inpatient and outpatient children

Isolates from children who had been hospitalised in the last 6 months were likely to be more resistant than those from children not previously hospitalised, isolates from children who had used antibiotics in the last 3 months before sampling were more likely to be resistant to multiple antimicrobials compared to those from children who had not (p=0.01; OR: 0.9; 95% CI=0.016-1.22). In addition majority of the children presenting with acute diarrhoea received β-lactam antibiotics including AMC while others received Co-Trimoxazole syrup. There was no case where Ciprofloxacin, Nalidixic Acid, Tetracycline, Chloramphenicol or amino glycosides were prescribed for diarrhoea. Close to 95% of patients with a prescription had not been requested to submit faecal samples for culture and susceptibility testing indicating that these prescriptions were issued without establishing the causative agent for diarrhoea or the susceptibility profile of the isolate.

4.2.8 Minimum Inhibitory Concentration values of selected isolates

Isolates 3-6 representing all the major phenotypes were selected as shown in Table 4.3. At least 50% of the isolates had an MIC mode$_{50}$ value that was within resistance range except for CAZ, CN, C, NA, CIP and K. Isolates were resistant to trimethoprim and ampicillin that had a mode MIC of 128 and 32 respectively.

Table 4.3 shows the summary for MIC values for 45 isolates from different clinical backgrounds.

Table 4.3 MIC values for *E. coli* investigated

CLSI breakpoints (R, I, S)	**AMC**	**CAZ**	**SUL**	**TRIM**	**AMP**	**CRO**	**CN**	**C**	**NA**	**CIP**	**K**	**S**
	(8, 16, 32)	**(8, 16-32, 64)**	**(ND)**	**(8, 16)**	**(8, 16, 32)**	**(8, 16-32, 64)**	**(4, 8, 16)**	**(8, 16, 32)**	**(16, 32)**	**(1, 2, 4)**	**(16, 32, 64)**	**(ND)**
MIC Ranges	2 to 128	4 to 128	4 to 256	8 to 256	2 to >256	0.002 to 128	0.02 to 64	0.02 to 64	0.02 to 32	0.01 to 8	2 to 128	8 to >1024
Mode 50	8	6	128	64	32	16	2	6	1	0.02	6	16
Mode 90	32	64	256	256	> 256	128	16	32	24	4	64	64
Median	8	6	128	64	32	16	2	6	1	0.02	6	16

4.3 Carriage of beta lactamase (*bla*) genes

Of the 10 isolates that were susceptible to all β-lactams, one tested positive for bla_{TEM} but the rest contained no *bla* gene. All isolates resistant to Penicillins but not to Cephalosporin did not test positive for $bla_{CTX\text{-}Ms}$ but carried bla_{TEM}, bla_{SHV}, bla_{OXA} in different combinations of these genes. Strains in this study exhibited resistance to Cephalosporin (66%) but not FEP carried combinations of $bla_{CTX\text{-}M}$+bla_{TEM} while 75% of those resistant to Cephalosporin including FEP carried $bla_{CTX\text{-}M}$+$bla_{OXA.}$ Table 4.5 shows the combination of *bla* genes detected in strains exhibiting resistance to different antimicrobials.

Table 4.4: Carriage of *bla* genes (number, %) among strains with different susceptibilities to β-lactams

Resistance Dimension		**Type of Antibiotic or Antibiotic combinations**						
		CTX-M Only	**OXA Only**	**SHV Only**	**TEM Only**	**SHV + TEM**	**CTX-M + OXA**	**CTX-M + TEM**
Resistance to β-lactam	**β-lactam Susceptible (n=10)**	0	0	0	1 (10)	0	0	0
	Penicillins Only (n=42)	0	3 (8)	9 (21)	28 (66)	14 (34)	0	0

The gel electrophoresis picture for tested *bla*$_{CTXMs}$ is shown in Figures 4.11

Isolate number 1-5 were resistant to β-lactams Ampicillin (AMP), Cefotaxime (CTX) and Ceftriaxone (CRO), but susceptible to Fluoroquinolones. Isolate number 6 was only susceptible to penicillins; isolates 9-12 were resistant to β-lactams AMP, CTX, and CRO Fluoroquinolones while isolates 13-15 were resistant to β-lactams AMP, CTX, CRO and Cefepime (FEP).

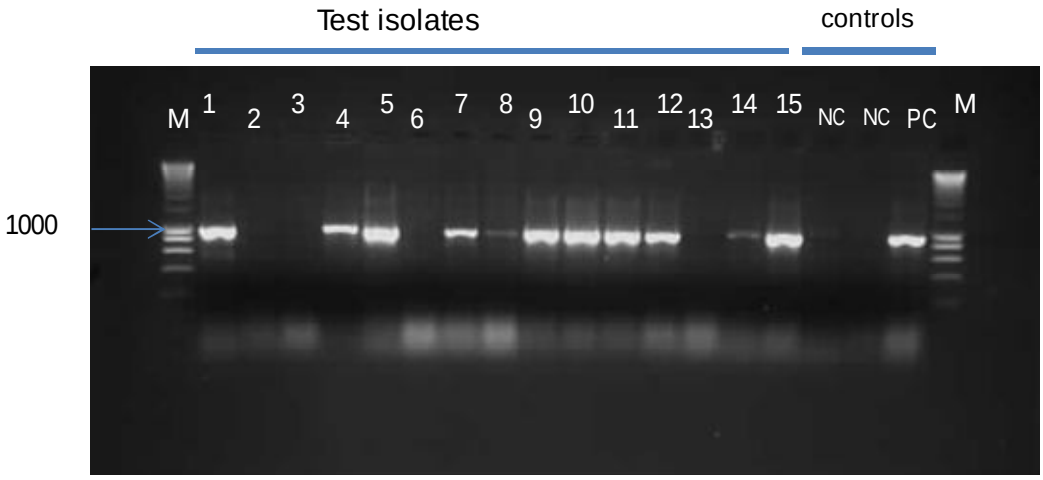

Figure 4. 11: Gel electrophoresis showing PCR **results for selected isolates tested for *bla*$_{CTXMs}$.**

M= molecular weight markers, NC: negative controls, PC: positive control for *bla*$_{CTX-}$

Gel electrophoresis showing PCR results for selected strains tested for *bla*$_{TEM}$ genes. All isolates were resistant to one class of β-lactams. Isolates numbers 1-9 were obtained from hospitalised patients while the rest were from outpatient departments.

Detection of *bla*$_{OXA,}$ and *bla*$_{SHV}$ genes, among strains exhibiting resistance to different combinations of β-lactams A: isolates 2 was resistant only to Penicillins while the isolates 1-8 were resistant to Penicillins and to at least 1 Cephalosporin. Isolates 7 and 8 were resistant to Cefepime (FEP). B: isolate `1-12 were resistant to at least to penicillins. Isolates 1-3 were not resistant to any cephalosporin, isolates 4-6 were resistant to different cephalosporin but not to FEP. Isolate 7-12 were resistant to different cephalosporin including FEP and Amoxicillin-Clavulanic acid (AMC). Isolates 13-16 were only resistant more than two classes of Cephalosporin.

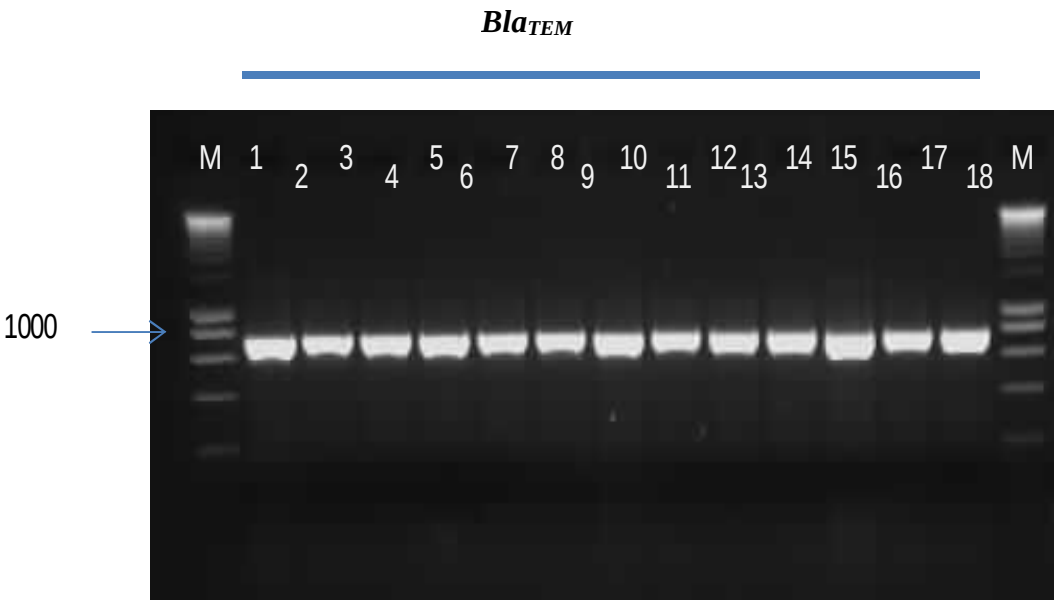

Figure 4.12

M= molecular weight markers.

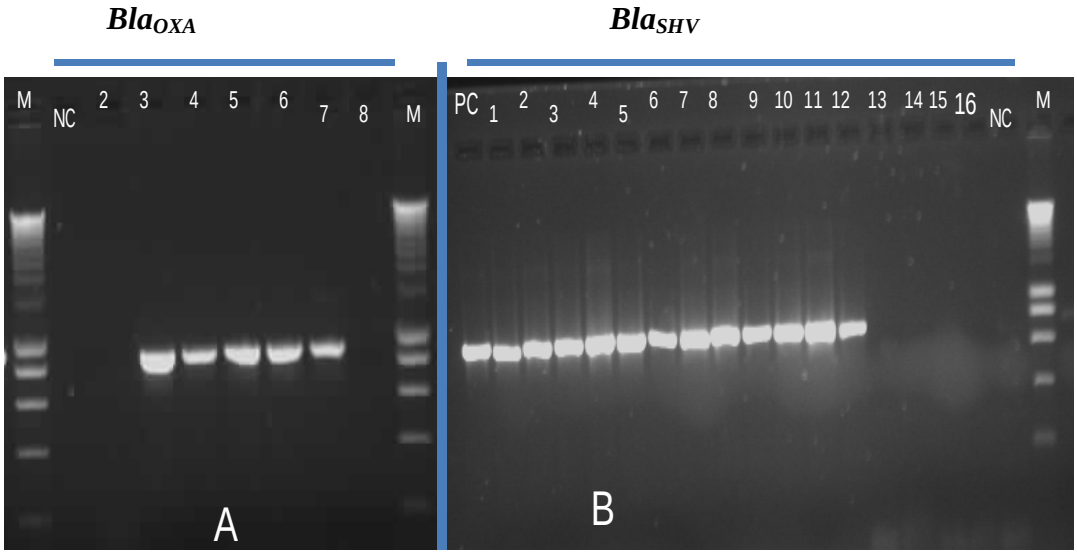

Figure 4. 13: PCR **detection of *bla*$_{OXA,}$ (A) and *bla*$_{SHV}$ genes (B), among strains exhibiting resistance to different combinations of β-lactams**

NC: negative controls, PC: positive controls, M=molecular weight markers.

4.3.2 Occurrence of integrons class 1 among isolates of different backgrounds

Polymerase Chain Reaction was used for determination of carriage of integrons among isolates selected based on their clinical background, the socio-demographic background of the source children and on their antimicrobials susceptibility profiles. Table 4.6 shows the carriage of these elements among isolates of different backgrounds. None of the isolates tested positive for class 2 or three integrons. Statistical significance was detected in isolates with MDR phenotypes (OR: 0.0194; 95% CI: 0.006-0.065; p-value: 0.0001). The rest of the variables were not statistically significant. Isolates with the ESBL phenotype were also more likely to harbor integrons compared to non-ESBL producers (p=0.0001, CI: 3.61 to 18.37, OR: 8.14). At least 50% of all the integrons detected in this study had Variable

Cassette Region (VCR) of more than 1kb except for 3 isolates that were not MDR. In these 3 isolates, the VCRs measured below 1 kb. Table 4.6 shows examples of VCR sizes of integrons detected in this study.

Table 4.5 Occurrence of integron class 1 among isolates from different backgrounds.

Category of isolates	**Number and % Frequency**		**VCR sizes (number, %) of the positive strains)**	
	Number of isolates tested	**Number (%) positive for intI1**	**≤ 1kb**	**>1kb**
1.(a) From Hospitalised children vs.	80	28 (35)	8 (29)	20 (71)
1.(b) From non-hospitalised children	68	15 (22)	6 (40)	9 (60)
2. (a) From chronic diarrhoea vs.	46	14 (30)	6 (43)	8 (57)
2. (b) From acute diarrhoea	110	36 (33)	12 (33)	24 (67)
3.(a) From rural children vs.	110	23 (21	8 (35)	15 (65)
3.(b)From urban children	110	31 (28)	12 (39)	19 (61)
4.(a)From those who Used antibiotics vs.	90	25 (28)	8 (32)	17 (68)
4.(b)From those who did not use antibiotics	86	28 (33)	13 (46)	15 (54)
5.(a)Among non MDR strains vs.	110	3 (3)	3 (100)	0
5.(b)Among MDR strains	110*	65 (59)	26 (40)	39 (60)
6.(a)Among ESBL-producers vs.	62	50 (81)	10 (20)	40 (80)
6.(b)Among non-ESBL-producers	65*	22 (34)	4 (18)	18 (82)

Distributions of class 1 integrons between two binary variables (“a” versus “b”) Categories that were statistically significant are indicated using * symbol.

Detection of class 1 integron (a): these isolates were resistant to ≤ 3 combinations of antimicrobials including sulphamethoxazole-trimethoprim (SXT), Tetracycline (TE) and chloramphenicol (C) but not to β-lactams. (b): isolates were resistant to at > 3 antimicrobials including sulphamethoxazole (SXT), tetracylines (TE), Chloramphenicol (C) and β-lactams. Isolate 4, 9 and 11 were also resistant to Fluoroquinolones. Isolates labeled C were resistant to TE and ampicillin (AMP) but were susceptible to SXT, Fluoroquinolones and cephalosporins.

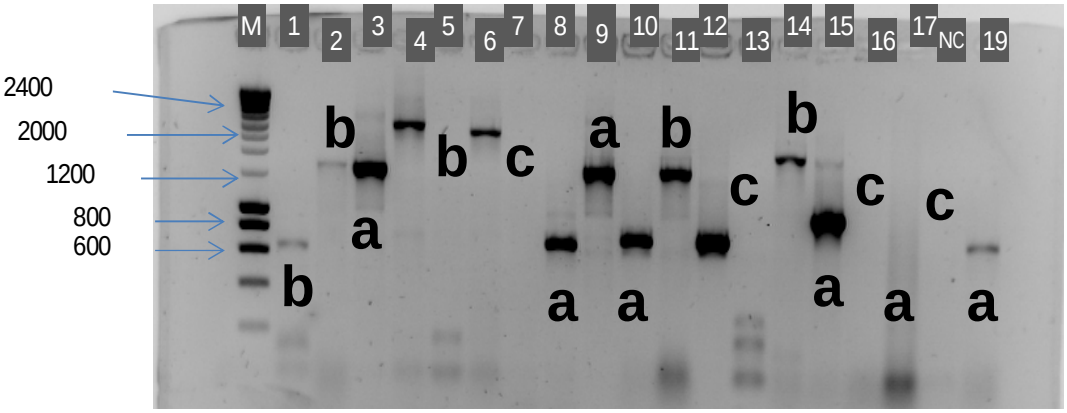

Figure 4.14: PCR **detection of class 1 integron VCR region**

4.3.3 Conjugative transfer of resistance phenotypes

Resistance to AMP and S were the most tranferrable phenotypes while determinants encoding resistance to C, CN,and CIP were the least transferrable (Table 4.7). Of the 120 isolates exhibting resistance to AMP and S, resistance determinants to these antimicrobials were transferred in 86% of tests while transfer of C, C, CN and CIP occured in 24% of 40 isolates exhibiting different combinations to these antimicrobials. All transconjugants resistant to CIP were

also resistant to NA. Resistance to AMC was always transferred together with resistance to other cephalosporins. Most transconjugants resistant to CRO were also resistant to CAZ. Among the 40 isolates with combined resistance to AMP and other cephaloporins, the AMP resistance gene was conjugatively transferred independent of the cephalosporin in 29 isolates. Plasmids incL/M, HI2 and F-type plasmids were the most commonly implicated in transferrable resistance for ESBLs. In 89% of 118 strains resistant to both SUL and TRIM, resistance to these antimicrobials were co-transferred but in some isolates carrying FIA, FIB and L/M plasmids transferred resistance to SUL occurred independently but not those conferring resistance to TRIM alone. One isolate containing L/M transferred resistance to at least 10 antimicrobials en bloc (Table 4.7).

Table 4.6: Conjugative transfer of resistance determinants from donors to susceptible recipients

S/n	**Hospitalisation**	**Diarrhoea**	**Use of antibiotics**	**Home**	**Susceptibility of donor and transconjugants (in bold)**	**Plasmid replicon types**
1	Inpatient	Chronic	Yes	Rural	AMC, CAZ, NA, SUL, TRIM, **AMP**, CRO, CN, **K**, CIP, **S**, C	A/C
2	Outpatient	Acute	No	Urban	AMC, SUL, **TRIM**, AMP, CRO, **K**, **S**, **C**	L/M

3	Inpatient	Acute	Yes	Rural	**AMC**, **CAZ**, **SUL**, TRIM, **AMP**, **CRO**, CN, K, S, C	L/M
4	Outpatient	Chronic	No	Urban	AMC, CAZ, NA, SUL, **TRIM**, AMP, CRO, CN, K, CIP, **S**, **C**	A/C
5	Inpatient	Acute	Yes	Urban	**CAZ**, **NA**, SUL, **TRIM**, **AMP**, **CRO**, CN, **K**, **S**, **C**	HI2
6	Outpatient	Acute	No	Rural	SUL, **TRIM**, **AMP**, **S**, C	HI2
7	Inpatient	Chronic	Yes	Urban	AMC , SUL, **TRIM**, **AMP**, **CRO**, K, **S**, C	FIB
8	Outpatient	Acute	No	Rural	**AMC**, **CAZ**, NA, SUL, TRIM, **AMP**, **CRO**, S, C	FIA
9	Inpatient	Acute	No	Urban	AMC, NA, **TRIM**, AMP, K, **S**,	FIA
10	Outpatient	Chronic	Yes	Rural	AMC, NA, SUL, **TRIM**, **AMP**, CN, **K**, **S**	A/C
11	Inpatient	Acute	No	Urban	**AMC**, **NA,** SUL, **TRIM**, **AMP**, **CRO**, **K**, **CIP**, **S**, C	HI2
12	Outpatient	Acute	Yes	Rural	NA, SUL, **TRIM**, **AMP**, CRO, **K**, S, C	P

13	Inpatient	Chronic	No	Urban	**AMC**, NA, SUL, **TRIM**, **AMP**, CN, K, S,	X
14	Outpatient	Acute	Yes	Rural	**TRIM**, AMP, CRO, K, **S**, C	HI2
15	Inpatient	Acute	No	Urban	AMC, CAZ, NA, SUL, TRIM, **AMP**, **CRO**, S,	FIB
16	Outpatient	Chronic	Yes	Rural	AMC , **TRIM**, AMP, **CN**, **K**, **S**, C	A/C
17	Inpatient	Acute	No	Urban	NA, SUL, **TRIM**, AMP, **CRO**, CN, K, CIP, **S**, C	FIB
18	Outpatient	Acute	Yes	Urban	**CAZ, NA,** SUL**, TRIM, AMP, CRO, CN, K, CIP, S, C**	L/M

Donor strains were selected from patients with different clinical and socio-demographic characteristics. Resistance phenotypes transferred are indicated in bold**.** The plasmid replicon types are also shown.

4.3.4 Detection of Plasmid types

Donor isolates in experiments 1, 4 and 5 were from rural patients while the rest were from urban patients. Isolates 1, 2, 5, and 8 were from inpatients while the others were from outpatients. Isolates 1 and 6 were from patients who had not

taken antimicrobials while the rest were from patents who had received antimicrobials. Weak bands are shown using arrows.

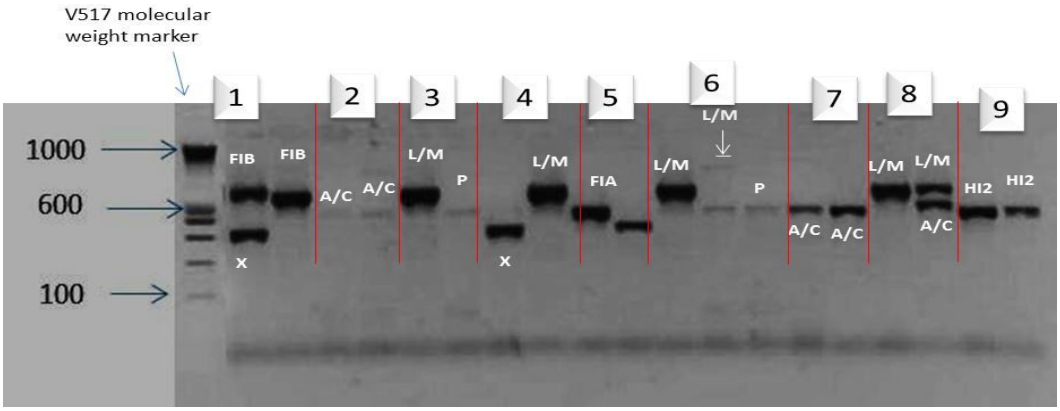

Figure 4.15: Shows Plasmid types detected in the donor (first lane after each vertical line) and transconjugants.

1. In experiment set 1 the donor contained plasmid type X and FIB but only FIB plasmid was transferred.
2. In experiment set 2 the donor contained plasmid type A/C and this plasmid was transferred to the recipient
3. In experiment set 3, the donor tested positive for plasmid type L/M while the transconjugants carried P-type plasmid.
4. In experiment set 4, the donor tested positive for plasmid type X while the transconjugants carried L/M-type plasmid.
5. In experiment set 5, the donor tested positive for plasmid type FIA while the plasmid type in the transconjugants could not be determined
6. In experiment set 6, the donor tested positive for plasmid type L/M while one transconjugants carried the L/M and the other P type of plasmid

7. In experiment set 7, the donor and the transconjugants carried A/C type plasmids
8. In experiment set 8, the transconjugants (left) tested positive for L/M plasmid while the donor carried the L/M and A/C type plasmid
9. In experiment set 9, the donor and the transconjugants carried HI2 type plasmids

4.3.5 Plasmid Gel electrophoresis of strains with different plasmid replicon types

Twelve isolates tested positive for plasmids using replicon typing detection although no plasmids were detected in gel electrophoresis. Eight isolates with a single plasmid band tested for more than one replicon types while some isolates with multiple bands tested positive for more than one plasmid group. Isolates contained plasmids of more than 54kb (Figure 4.16). These relatively large plasmids were conjugatively transferred while smaller plasmids of less than 7 Kb were not detected. There were no major differences in plasmid content and replicon types among the 49 of 61 isolated from hospitalised patients compared to the 51 of 72 isolates from non-hospitalised patients. Similarly, no differences in plasmid content were detected between 37 of the 41 isolates from patients who had taken antibiotics and 28 of 42 isolates from those who had not taken antibiotics. The study did not find any unique or unusual plasmids in 12 of the 42 MDR isolates in comparison to 22 of 53 the non-MDR strains tested.

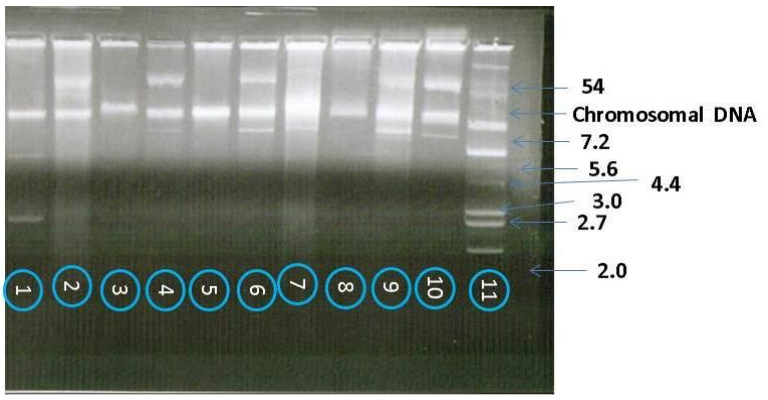

Figure: 4. 16: Plasmid profiles of donors and transconjugants.

The clinical details of the isolates and the replicon types detected using PCR **are shown**

1. Urban, hospitalised, used antibiotics (types plasmids:-FIB, X)
2. Rural, inpatient used antibiotics (types plasmids:-L/M, A/C)
3. Negative control without a plasmid
4. Transconjugants using donor from Urban, hospitalised, used antibiotics (types plasmids:-FIB)
5. Transconjugants/derived from rural/hospitalised/used antibiotics (types plasmids:-FIA)
6. Donor/rural/hospitalised/used antibiotics (types plasmids:-HI2, P)
7. Transconjugants/donor from rural/not hospitalised/not used antibiotics (types plasmids:-HI2)
8. Negative control without a plasmid
9. Donor/rural/hospitalised/used antibiotics/ ESBL (types plasmids:-L/M, A/C)

10. Donor/urban/hospitalised/used antibiotics/ ESBL, CIP resistant (types plasmids:-L/M)
11. Control plasmids from *E. coli* V517

4.4 Patterns of *E. coli* isolates using Enterobacterial Repetitive Intergenic Consensus Sequence Polymerase Chain Reaction (ERIC-PCR)

ERIC- PCR The gel shows ERIC PCR patterns of *E. coli* isolates. Two 1000 base pair Smart Ladders were included on the gel as a marker. All the strains produced patterns of between 3-9 bands. Isolates 1-3 from rural children with diarrhoea and carrying a CTX-M had similar patterns. The second cluster consisted of isolate 3-6. Isolate 3 and 5 were from urban children that also carried a CTX-M gene in combination with a TEM-1 gene while the CTX-M carrying isolates numbers 4 and 6 were from rural children. These rural isolates were also resistant to ciprofloxacin. The third cluster consisted of isolates number 7-11. Isolates 7 9 and 10 were from urban children while isolate number 8 and 11 were from rural children. All the isolates in this cluster were resistant to Cephalosporin, Nalidixic acid, Trimethoprim, Sulphamethoxazole and Tetracycline. Isolate number 12 was from a rural child with severe diarrhoea and clustered poorly with all other strains. However, this isolate had a similar resistant pattern as isolate 7-11.

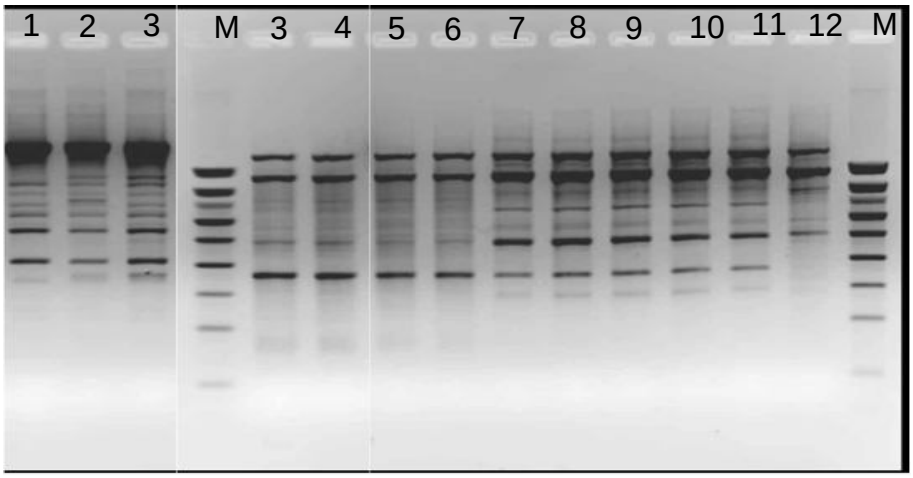

Figure 4.17: ERIC- PCR patterns of isolates from rural and urban children

M: 1000bp molecular weight marker.

Restriction polymorphisms of CTX-M genes

The identities of various digested genes are shown on each lane. These results confirm the existence of specific CTX-M genes among isolates from different sources. This data further confirms that *bla*CTX-M-3, -9, -14 and -15 are the most coming among the isolates analyzed. The CTX-M-9 and 15 were only detected in strains resistant to Ceftazidime while all the other genes were detected in strains resistant to other third generation Cephalosporims such as Cefotaxime and Ceftriaxone.

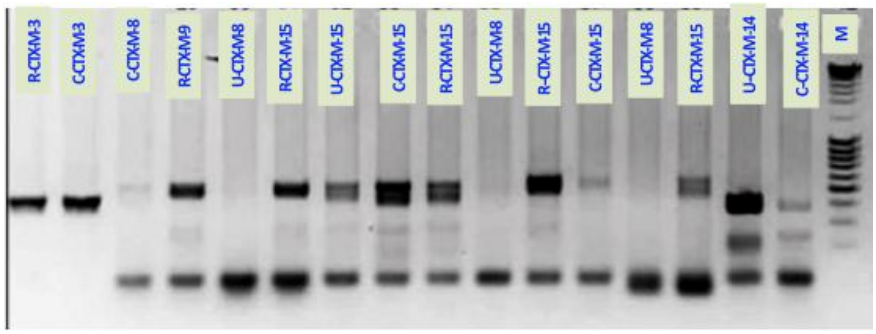

Figure 4. 18: Restriction polymorphisms of CTX-M genes from rural isolates (R), urban isolates (U) compared to those of known controls whose CTX-M genes have been sequenced and characterized (c).

CHAPTER FIVE: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Prevalence of antimicrobial resistance in *E. coli* isolates profiles in rural and urban children

While antimicrobials have proven invaluable in the management of bacterial infectious disease, resistance to these agents actually predates the introduction of the first true antibiotic (penicillin) into clinical usage, and resistance continues to compromise the use of old and new antimicrobial alike (Akortha and Egbule, 2008). Although amoxicillin-clavulanic acid, ceftazidime, Gentamicin, and ceftriaxone are not indicated to treat diarrhoea in children, the susceptibilities to these antibiotics in all isolates were tested since they could be empirically or incidentally used. Resistance in this study was noted in over 30% of all isolates Ampicillin, Sulfonamides, Tetracycline, Streptomycin and Amoxicillin-Clavulanic acid. Resistance to these antimicrobials has been documented in Kenya and the results observed in this study are comparable to the antibiotic resistance in those studies (Brooks *et al.,* 2003; Oundo *et al.,* 2008).

Resistance of antimicrobials Sulfonamides, Tetracycline, Chloramphenicol, Trimethoprim and Streptomycin have been reported from other developing countries but these resistances were lower than those reported in this study (Bartoloni *et al.,* 2006; Zaidi *et al.,* 2003). Resistance was lowest for Ciprofloxacin (4%), Nalidixic Acid (5%), Cefepime (5%), Ceftriaxone (7%) and

Ceftazidime (7%). Although β-lactam and quinolones are frequently used for the treatment of K. pneumonia and *E. coli* infections, ESBL-production and Quinolones resistance have unfortunately hampered their usage (Shams *et al.*, 2014)

The minimal inhibitory concentration values of these antimicrobials were within the resistance range to these older generation antimicrobials but 4th generation Cephalosporin such as FEP and to Fluoroquinolones such as CIP and to advanced classes of amino glycosides such as CN were low (Szmoalka and Nagy, 2013). In general, differences in prevalence of resistance to antimicrobials among strains from urban and those from rural settings were not statistically different (p=0.051 CI=1.41- 5.113, OR=2.68). Over 30% of strains from children living in rural and urban settings were resistant to AMC, SXT, TET, and S. This could be attributed to user related factors such as self-medication, non-compliance, and advertising pressures or mothers who bring children to health centers might seek antibiotics not just for the episode of illness, but stockpile the extra antibiotic for that occasion when reaching the clinic might not be so easy, a survival strategy where access is limited common in developing countries (Laxminarayan and Klugman, 2011). The difference between the prevalence resistance of isolates from peri-urban settings and those from rural settings to SXT, CRO, C, NA, and S was at least 5% with those from peri-urban settings recording higher values, this agrees with the findings in a rural set up in Western Kenya (Shapiro *et al.*, 2011) .

A past study by (Brooks *et al.,* 2003) conducted in Western Kenya showed that over 70% of patients with diarrhoea are likely to receive an antimicrobial drug to which the etiologic agent is not susceptible. The high resistance prevalence to SXT observed in this study probably point to misuse of Co-Trimoxazole in rural and urban settings. Although it is not clear if these antimicrobials are frequently used to treat diarrhoea, the use of these antimicrobials has been linked to increased resistance to sulphur-potentiated antimicrobials (Chiller *et al.,* 2009).

5.2 Resistance among *E. coli* isolates from inpatients and outpatients

Diarrhoeagenic *E. coli* (DEC) infections is indistinguishable from viral gastroenteritis, isolation and identification of DEC strains could allow caretakers to provide appropriate treatment for pathogen-specific illness. Oral rehydration therapy (ORT) in children with dehydrating forms of diarrhoea has reduced death rates worldwide. Oral Rehydration Therapy however does not shorten duration of illnesses and shedding, whereas antimicrobial therapy may be of value for some forms of DEC diarrhoea (Talbert *et al.,* 2013). Antimicrobial therapy may be indicated in children with DEC diarrhoea that is promptly identified and in children with persistent diarrhoea. This study showed that most DEC strains that cause diarrhoea in hospitalised children are resistant to TMP-SMX and ampicillin, drugs commonly used to treat pediatric diarrhoea. This resistant pattern is an emerging problem for DEC isolated from children in other developing countries (Putnam *et al.*, 2004) and for other entrobacteria worldwide (Faure, 2013). All isolates were sensitive to ciprofloxacin and other Quinolones are not approved for

children because of the risk of damage to immature joints (Bhattacharya and Sur, 2003), and most parenteral third-generation cephalosporins, for example, cefotaxime are administered only in a hospital setting.

5.3 Prevalence of multidrug resistance

Isolates were grouped according to their resistance combinations to major classes of antimicrobials and sampling settings, 52% of the isolates were resistant to more than three different classes of antimicrobials tested and were thus classified as multi-drug resistant (MDR). Contrary to related studies by (Fornasini *et al.*, 1992; Lietzau *et al.*, 2007) , the current study suggests a positive correlation between use of antibiotics and carriage of MDR strains. The strains from acute diarrhoea are more resistant than those from chronic cases. It would be expected that children who suffer acute episodes of diarrhoea are more likely to use antibiotics than those with mild diarrhoea that is not seen as potentially life-threatening (WHO, 2009). It is also possible that mothers are more likely to seek hospital treatment for children with acute diarrhoea than those with non-severe chronic diarrhoea (Keusch, 2006). Trends of antimicrobial resistant research in eastern Africa have shown gradual increase in the pattern from the mid-70s as observed in the current study (WHO, 2012). A relatively high antibiotic resistance as well as multidrug resistance was detected in the *E. coli* isolates. Membrane-active agents such as Kanamycin have lowest resistant potentials because they have no specific roots and are difficult to alter fundamental bacterial membrane composition by mutation (Smillic *et al.*, 2010). Except for aminoglycosides the resistance to protein inhibiting antibiotics

showed considerable showed considerable contribution to the MDR. Although aminoglycosides, macrolides and tetracycline exert their main mechanisms of action through inhibition of the protein synthesis, different mechanisms of resistance have been reported for each of these classes.

5.4 Resistant genes in *E. coli* isolates from children under five years

High use of antibiotics is one factor that has been shown to increase the risk of developing and acquiring ESBL for patients at hospitals and in the community. (Usha *et al*., 2010). A major finding in this study is the detection of *E. coli* isolates harbouring genes encoding SHV, TEM, CTX-M and OXA enzymes, which is the first documentation in the study site. The findings in this study indicate that CTX-M genes that confer an ESBL-phenotype are the most common among isolates resistant to cephalosporin. This finding is important because antimicrobial resistance has become one of the most serious public health concerns worldwide (Kang and Song, 2013). Occurrence of CTX-M-15 among strains resistant to ceftazidime has been reported in past studies that indicate that this enzyme is the most robust among the CTX-M-encoding genes which explains why they are widely diffused in clinically relevant Enterobacteriaceae representing one of most frequently encountered plasmid types (Lahlaoui *et al.,* 2014). *E. coli* is the most often responsible for producing CTX-M β-lactamases and seems to be a true community ESBL pathogen (Pitout and Laupland, 2008). These genes are also frequently borne on conjugative plasmids and are therefore likely to be disseminated in rural and urban settings and among strains from hospital and

community settings, familiar spread has been suggested to contribute to transmission of EBSL- producing in the community (Dalsgaard *et al.,* 2009). Identification of strains with combined resistance to β-lactams, Fluoroquinolones and Amino glycosides (especially in children without a history of antibiotic use or hospitalization) is worrying (Davies and Davies, 2010).

Resistance to AMP and S were the most transferrable phenotypes while determinants encoding resistance to C, CN, and CIP were the least transferrable, of the 120 isolates exhibiting resistance to AMP and S, resistance determinants to these antimicrobials were transferred in 86% of tests while transfer of C, C, CN and CIP occurred in 24% of 40 isolates exhibiting different combinations to these antimicrobials. All transconjugants resistant to CIP were also resistant to NA. Resistance to AMC was always transferred together with resistance to other cephalosporims. Most transconjugants resistant to CRO were also resistant to CAZ. Among the 40 isolates with combined resistance to AMP and other cephalosporims, the AMP resistance gene was conjugatively transferred independent of the cephalosporin in 29 isolates. Plasmids incL/M, HI2 and F-type plasmids were the most commonly implicated in transferrable resistance for ESBLs (Kiiru *et al.,* 2013; Wolters *et al.,* 2015). In 89% of 118 strains resistant to both SUL and TRIM, resistance to these antimicrobials were co-transferred but in some isolates carrying FIA, FIB and L/M plasmids transferred resistance to SUL occurred independently but not those conferring resistance to TRIM alone. One

isolate containing L/M transferred resistance to at least 10 antimicrobials en bloc (Carattoli, 2009).

Integrons had become an important horizontal gene transfer system resistance genes in clinical isolates. Isolates with the ESBL phenotype were also more likely to harbour integrons compared to those susceptible to advanced classes of β-lactams (OR: 8.14; CI: 3.61 to 18.37; p=0.0001). At least 50% of all the integrons detected in this study had Variable Cassette Region (VCR) of more than 1 kb except for 3 isolates that were not MDR. In these 3 isolates, the VCRs measured below 1 kb. Several studies have investigated the prevalence of integrons in clinical isolates of *E.coli.* These studies have established a strong association between the presence of integrons and antibiotic isolates which is in agreement with study (Boucher *et al.,* 2007; Cambray *et al.,* 2010).

Only class 1 integrons were detected in the isolates tested in the current study. These integrons were detected in strains from urban and rural backgrounds respectively. Isolates that were MDR were more likely to carry integrons. This study showed that majority of integrons has VCRs of > 4 Kb. The study therefore suggests that these elements are implicated in resistance to multiple antimicrobials. Limited studies have investigated integrons content in *E. coli* from food-animal sources in Kenya (Kikuvi *et al.,* 2007) and in Salmonella strains obtained from human sources (Onyango *et al.,* 2009).The wide dispersion of class 1 integrons is attributed to their incorporation into this widely distributed *Tn*21 transposons that

is in turn borne on broad-host-range plasmids (Hall, 2012). Bacteria are present inside and on the surfaces of the human body. It is therefore possible that a strong antimicrobial selection pressure may favour proliferation of strains bearing integrons (Beceiro *et al.,* 2013)

The unrecognized wide spread presence of integrons containing Gram negative bacteria, both within hospitals and in the community possess a serious threat of the spread of antibiotic resistance (Roy *et al.,* 2011)

5.5 Relationship Diversity between extended Spectrum Beta-lactamases diarrhoeagenic *E. coli* isolates

Of the ten isolates that were susceptible to all β-lactams, one tested positive for bla_{TEM} but the rest contained no *bla* gene. All isolates were resistant to Penicillins but not to Cephalosporin did not test positive for $bla_{\text{CTX-Ms}}$ but carried bla_{TEM}, bla_{SHV}, bla_{OXA} in different combinations. Strains exhibiting resistance to Cephalosporin but not FEP (66%) carried combinations of $bla_{CTX\text{-}M}$+$bla_{TEM,}$ while 75% of those resistant to Cephalosporin including FEP carried $bla_{\text{CTX-M}}$+$bla_{\text{OXA.}}$

This study also shows that the carriage of multiple *bla* genes complicates the phenotypic interpretation of the resistance phenotypes. This calls for hospital and community-based surveillance programs to monitor emergence and spread of isolates with expanded resistance to β-lactam antibiotics. Isolates with multiple combinations of *bla* genes and especially those carrying $bla_{CTX\text{-}M}$+bla_{OXA} and those with $bla_{\text{CTX-M-}}$+bla_{TEM} were resistant to a wider panel of β-lactams the results are in agreement with a recent study (Kiiru, 2011). Such combinations apparently

increase the spectrum of resistance to β-lactam antibiotics and inhibitor-based antimicrobials as observed in isolates carrying a combination of $bla_{CTX\text{-}M}$ with bla_{OXA} and/ or $bla_{\text{TEM-1}}$ (Bonnin, *et al.*, 2012).

A past study implicates OXA-30 in the resistance to β-lactamase inhibitors (CLSI, 2012; Karisik *et al.*, 2006). It is therefore possible that carriage of a combination of bla_{OXA} genes with other *bla* genes may confer resistance to AMC as observed in this study. This finding is particularly threatening when considering the explosive diffusion of the $bla_{\text{CTX-M 15}}$ gene that has been witnessed worldwide during the last decade (Kiiru *et al.*, 2013). In this collection of isolates, genes encoding resistance to important antimicrobials such as Cephalosporin, cephamycins Fluoroquinolones and Aminoglycosides are borne on narrow host-range plasmids such as those belonging to IncFII, IncH12. They are also borne on broad host-range plasmids such as those belonging to IncL/M (Kiiru *et al.*, 2013; Poirel *et al.*, 2012). IncL/M family is also responsible for the spread of the class D carbapenemase OXA-48 that has been identified in many clonally located geographical areas (Poirel *et al.*, 2012).

Dissemination of these markers is not confined to plasmids belonging to specific replicon types, further indicating high potential for spread to unrelated genera. The plasmid host range is known to be highly variable among plasmids and the term “narrow host range” and “broad host range” are used as qualitative indicators (De Solar *et al.*, 1996; Shapiro, 2010). These plasmids have diffused both in rural and

urban settings are also detected in children who do not have a recent history of hospitalization or antimicrobial use. Some of these plasmids were shown to mediate conjugal transfer of resistance to as many as ten antimicrobials (Suzuki *et al.,* 2010).

Past studies conducted locally have implicated plasmids in conjugal transfer of resistance markers Salmonella and *E. coli* (Cain *et al.,* 2010: Novais *et al.,* 2006: Womble and Rownd 1988). Inability to detect plasmids or replicon in Transconjugants may be due to lack of high sensitivity of the methods used in this study. Sometimes a plasmid is said to be "lost" when the progeny cells do not receive the plasmid. The loss of plasmids in a population is sometimes referred to as plasmid segregation (Norman *et al.,* 2009). It has also been suggested that the host may limit plasmid copies while small plasmids may integrate into chromosomes upon conjugation, therefore reducing chances of detection of plasmids using electrophoresis techniques (Naseer *et al.,* 2010). Conjugative plasmids can exhibit a broad or narrow host range. In case of a narrow host range, transfer is restricted to and between a small numbers of similar bacterial species (Smillic *et al.,* 2010).These observations may partially explain why some plasmids were detected using replicon typing but not using gel electrophoresis.

This study results show that resistance profiles of the isolates analyzed and their epidemiological characteristics do not determine genetic relationship based on ERIC- PCR patterns. These results further confirm the observations that *E. coli* is a

highly polymorphic and non-clonal. This is in agreement with observation by (Schioter *et al.*, 2000) that advent of molecular genetic tools and their application to microbial ecology has demonstrated that only a small proportion of natural diversity has been discovered. This further indicates that the resistance profiles are also gained from independent genetic mechanisms rather than through clonal expansions. The apparent similarities in genetic profiles among strains from different sources possibly indicate that similar genetic elements involved in resistance are implicated in the acquisition and dissemination of these resistances confirming that the emergence of antibiotic resistance is the consequence of a complex interactions of factors involved in the evolution and spread of resistance mechanisms (Lupo *et al.*, 2012; Normark and Normark, 2002).

5.6 Conclusions

This study provides data that indirectly link carriage of MDR strains to various socio-demographic factors and antibiotic use patterns. The following conclusions can be drawn from this study.

(a) Isolates recovered from children in the current study are resistant to first line of treatment for diarrhoea.

(b) Recent history of antimicrobial use and hospitalization is a serious predisposing factor to carriage of Multi Drug Resistant strains.

(c) Multi Drug Resistant strains are prevalent among isolates recovered from urban and rural children possibly because of similar antimicrobial-use patterns in the two settings.

(d) B-lactamases are significantly implicated in resistance to β-lactam antimicrobials. There is a need to slow down the rate of spread of ESBL-producers.

(e) Most resistances are transferrable via conjugation. This is likely to increase the prevalence of Multi Drug Resistant strains. Resistance profiles are gained from independent genetic mechanisms rather than through clonal expansions.

5.6 Recommendations

Based on the findings from this study, the following recommendations were made:

i. There is a need to revise the current treatment regimens in line with the data from this study.

ii. In order to effectively treat diarrhoea episodes caused by multi drug resistant strains, there is a need to implement effective infection control measures and surveillance programs to monitor emergence and spread of MDR strains.

iii. There is need to revise and implement proper antimicrobial use policies, affordable and safe oral antimicrobial drugs to treat Enterobacterial

infections in children while revising the existing empiric treatment regiments.

iv. Clinical laboratories to consider isolation, identification and detection beta-lactamase carriage genes from children with DEC diarrhoea.

v. Formal surveillance system in rural and urban set ups are necessary to monitor the genetic diversity of isolates.

5.7 Recommendation for Further Research

5.7.1 Operational Research

1. Policies on ensuring and monitoring patients' compliance with the recommended treatment and prescriptions.
2. Discouraging indiscriminate, inadequate and unduly prolonged use of antimicrobial agents and while selecting antibiotics for treatment**,** preferably acting specific narrow spectrum antimicrobial agents whenever possible.

5.7.2 Applied Research

1. Genetic studies to identify the genetic mechanisms allowing some pathogenic *E. coli* to retain a high potential for recombination.
2. Studies to investigate the ecological, socio-economical, and epidemiological basis of *E. coli* infection as an emerging pathogen in children.

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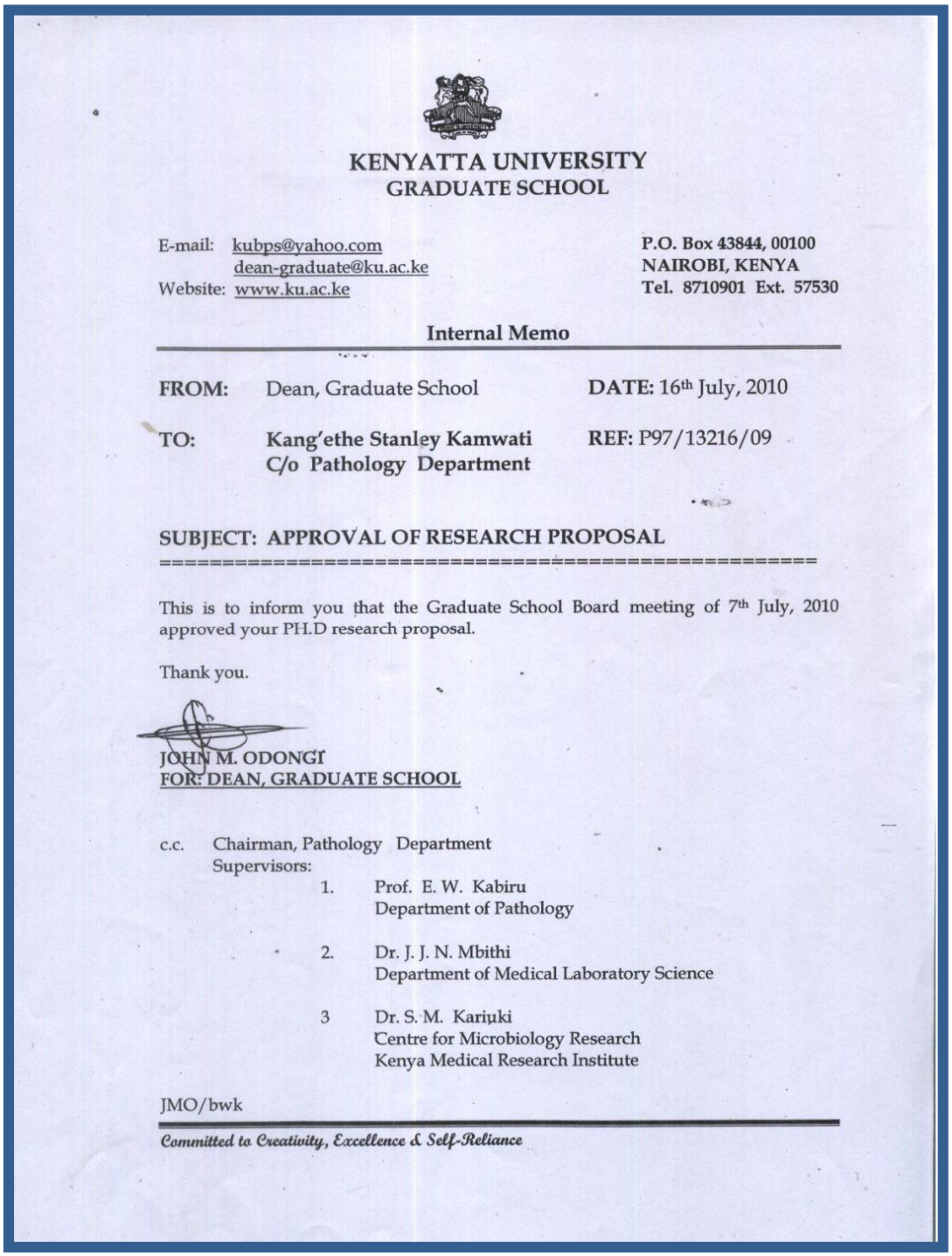
APPENDICES

Appendix I: Approval of Research proposal



KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: kubps@yahoo.com
dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 8710901 Ext. 57530

Internal Memo

FROM: Dean, Graduate School DATE: 16th July, 2010

TO: Kang'ethe Stanley Kamwati
C/o Pathology Department
REF: P97/13216/09

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

This is to inform you that the Graduate School Board meeting of 7th July, 2010 approved your PH.D research proposal.

Thank you.

JOHN M. ODONGI
FOR: DEAN, GRADUATE SCHOOL

c.c. Chairman, Pathology Department
Supervisors:
1. Prof. E. W. Kabiru
Department of Pathology
2. Dr. J. J. N. Mbithi
Department of Medical Laboratory Science
3 Dr. S. M. Kariuki
Centre for Microbiology Research
Kenya Medical Research Institute

JMO/bwk

Committed to Creativity, Excellence & Self-Reliance

Appendix II: Research Authorization

KENYATTA UNIVERSITY
GRADUATE SCHOOL
P.O. Box 43844,
NAIROBI
Tel. No. 8710901/9 Ext. 57530
E-mail: kubps@yahoo.com

Our Ref: P97/13216/09 **Date**: 16th July 2010
Your Ref:

The Permanent Secretary,
Ministry of Education,
P.O. Box 30040,
NAIROBI.

Dear Sir/Madam,

RE: RESEARCH AUTHORIZATION

I write to introduce **Mr. Kang'ethe Stanley Kamwati** who is a Postgraduate Student of this University. He is registered for a Ph.D degree programme in the Department of Pathology

Mr. Kang'ethe intends to conduct research for a Masters entitled, *"Determination of the Horizontal Genetic Transfer Resistance In E. Coli Isolated From Children Aged Below 5 Years With Diarrhoeal Diseases In Thika District Hospital, Kenya."*

Any assistance given to him will be highly appreciated.

Yours faithfully,

JOHN M. ODONGI
FOR: DEAN, GRADUATE SCHOOL

JMO/bwk

Appendix III: Research Authorization latter by National Council for Science and Technology (NCST)

REPUBLIC OF KENYA

NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

Telegrams: "SCIENCETECH", Nairobi
Telephone: 254-020-241349, 2213102
254-020-310571, 2213123,
Fax: 254-020-2213215, 318245, 318249
When replying please quote

P.O. Box 30623-00100
NAIROBI-KENYA
Website: www.ncst.go.ke

Our Ref: NCST/RRI/12/1/MED/215/4

Date: 18th October 2010

Mr. Stanley Kamwati Kang'ethe
Kenyatta University
P. O. Box 43844
NAIROBI

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on ***"Determination of the horizontal genetic transfer resistance in E. Coli isolated from children aged below 5 years with Diarrhoeal diseases in Thika District Hospital, Kenya"*** I am pleased to inform you that you have been authorized to undertake research in **Thika District Hospital, Thika District** for a period ending ***31st December 2011.***

You are advised to report to **the Medical Superintendent, Thika District Hospital** before embarking on the research project.

On completion of the research, you are expected to submit **two** copies of the research report/thesis to our office.

P. N. NYAKUNDI
FOR: SECRETARY/CEO

Copy to:

The Medical Superintendent
Thika District Hospital
P. O. Box 227 - 01000
THIKA

Appendix IV: Research Permit by NCST

PAGE 2

THIS IS TO CERTIFY THAT:

Prof./Dr./Mr./Mrs./Miss. STANLEY KANG'ETHE KAMWATI

of (Address) KENYATTA UNIVERSITY P.O. BOX 43844, NBI

has been permitted to conduct research in .....Location, THIKA .....District, CENTRAL .....Province,

on the topic Determination of the Horizontal Genetic transfer resistance in E. Coli isolated from Children Aged below 5 years with Diarrheal Diseases in Thika District Hospital, Kenya.

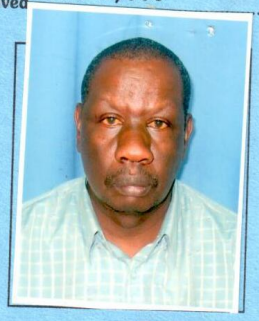
for a period ending 31ST DECEMBER, 20 11

PAGE 3

Research Permit No. NCST/RRI/12/1/MED/215

Date of issue 18/10/2010

Fee received SHS 2,000



Applicant's Signature

Secretary National Council for Science and Technology

CONDITIONS

1. You must report to the District Commissioner and the District Education Officer of the area before embarking on your research. Failure to do that may lead to the cancellation of your permit
2. Government Officers will not be interviewed with-out prior appointment.
3. No questionnaire will be used unless it has been approved.
4. Excavation, filming and collection of biological specimens are subject to further permission from the relevant Government Ministries.
5. You are required to submit at least two(2)/four(4) bound copies of your final report for Kenyans and non-Kenyans respectively.
6. The Government of Kenya reserves the right to modify the conditions of this permit including its cancellation without notice

GPK6055t3mt10/2009

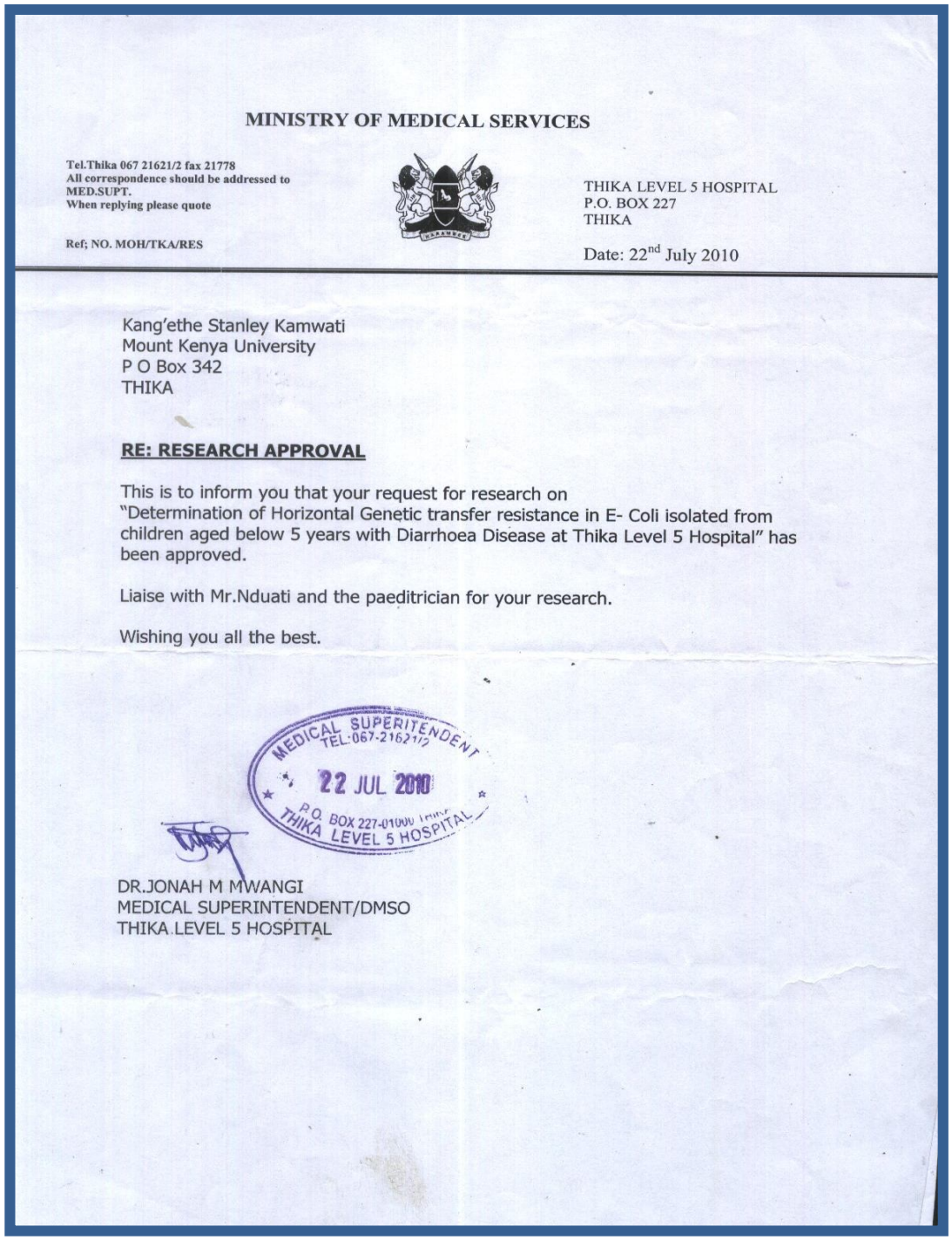
HARAMBEE

REPUBLIC OF KENYA

RESEARCH CLEARANCE PERMIT

(CONDITIONS— see back page)

Appendix V: Research approval from Ministry of Medical Services



MINISTRY OF MEDICAL SERVICES

Tel.Thika 067 21621/2 fax 21778
All correspondence should be addressed to MED.SUPT.
When replying please quote

Ref; NO. MOH/TKA/RES

THIKA LEVEL 5 HOSPITAL
P.O. BOX 227
THIKA

Date: 22nd July 2010

Kang'ethe Stanley Kamwati
Mount Kenya University
P O Box 342
THIKA

RE: RESEARCH APPROVAL

This is to inform you that your request for research on "Determination of Horizontal Genetic transfer resistance in E- Coli isolated from children aged below 5 years with Diarrhoea Disease at Thika Level 5 Hospital" has been approved.

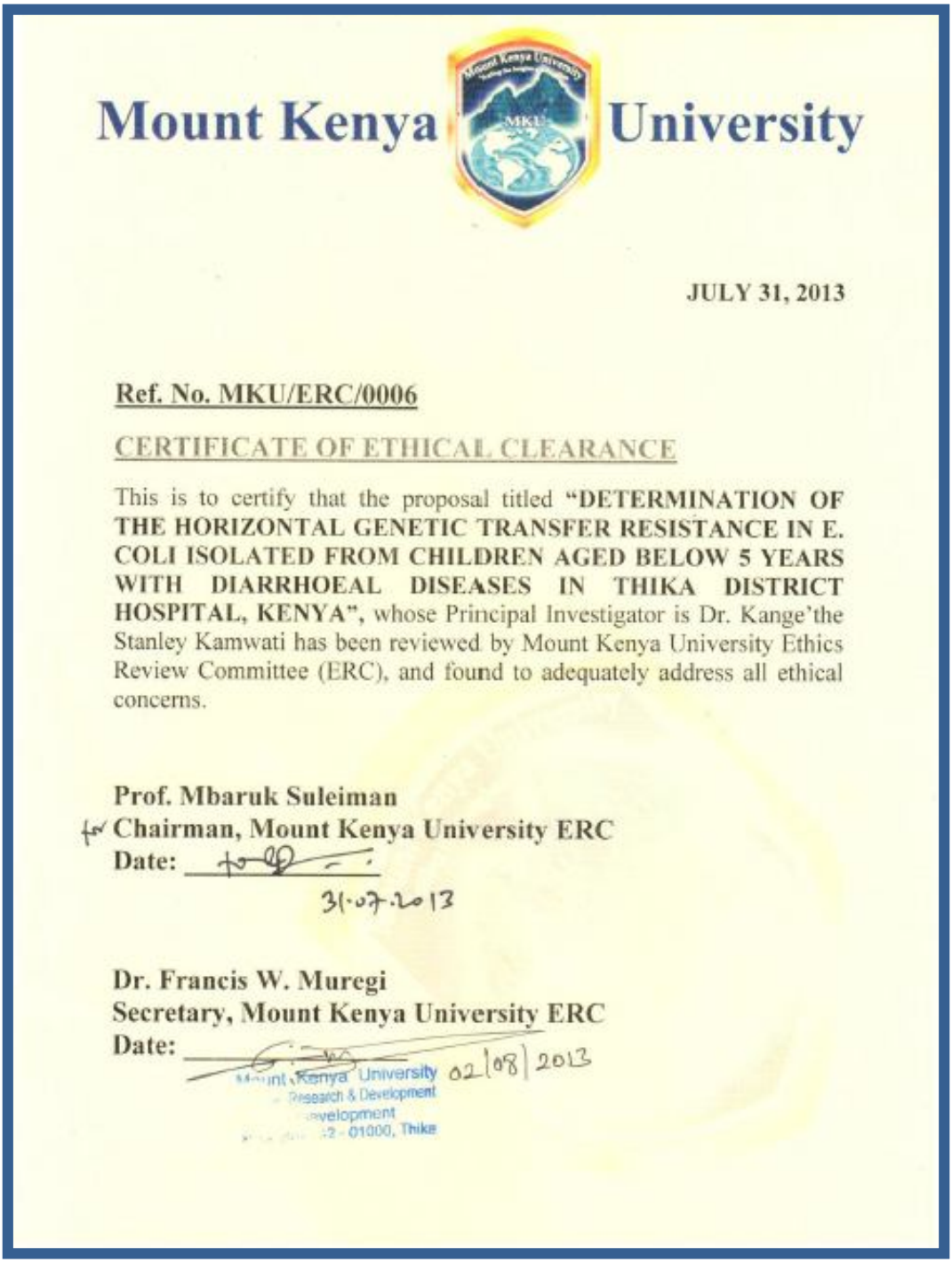
Liaise with Mr.Nduati and the paeditrician for your research.

Wishing you all the best.

MEDICAL SUPERITENDENT TEL:067-21621/2
22 JUL 2010
P.O. BOX 227-01000 THIKA
THIKA LEVEL 5 HOSPITAL

DR.JONAH M MWANGI
MEDICAL SUPERINTENDENT/DMSO
THIKA LEVEL 5 HOSPITAL

Appendix VI: Certificate of Ethical Clearance.



Mount Kenya University

JULY 31, 2013

Ref. No. MKU/ERC/0006

CERTIFICATE OF ETHICAL CLEARANCE

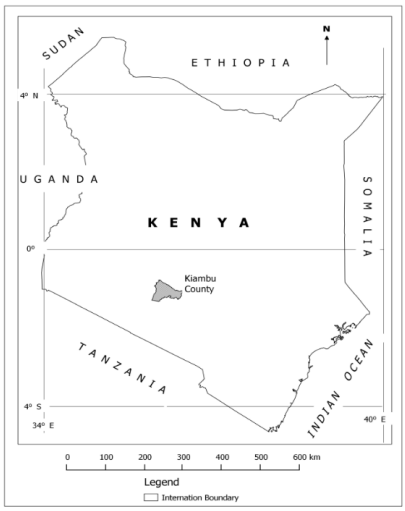
This is to certify that the proposal titled **"DETERMINATION OF THE HORIZONTAL GENETIC TRANSFER RESISTANCE IN E. COLI ISOLATED FROM CHILDREN AGED BELOW 5 YEARS WITH DIARRHOEAL DISEASES IN THIKA DISTRICT HOSPITAL, KENYA"**, whose Principal Investigator is Dr. Kange'the Stanley Kamwati has been reviewed by Mount Kenya University Ethics Review Committee (ERC), and found to adequately address all ethical concerns.

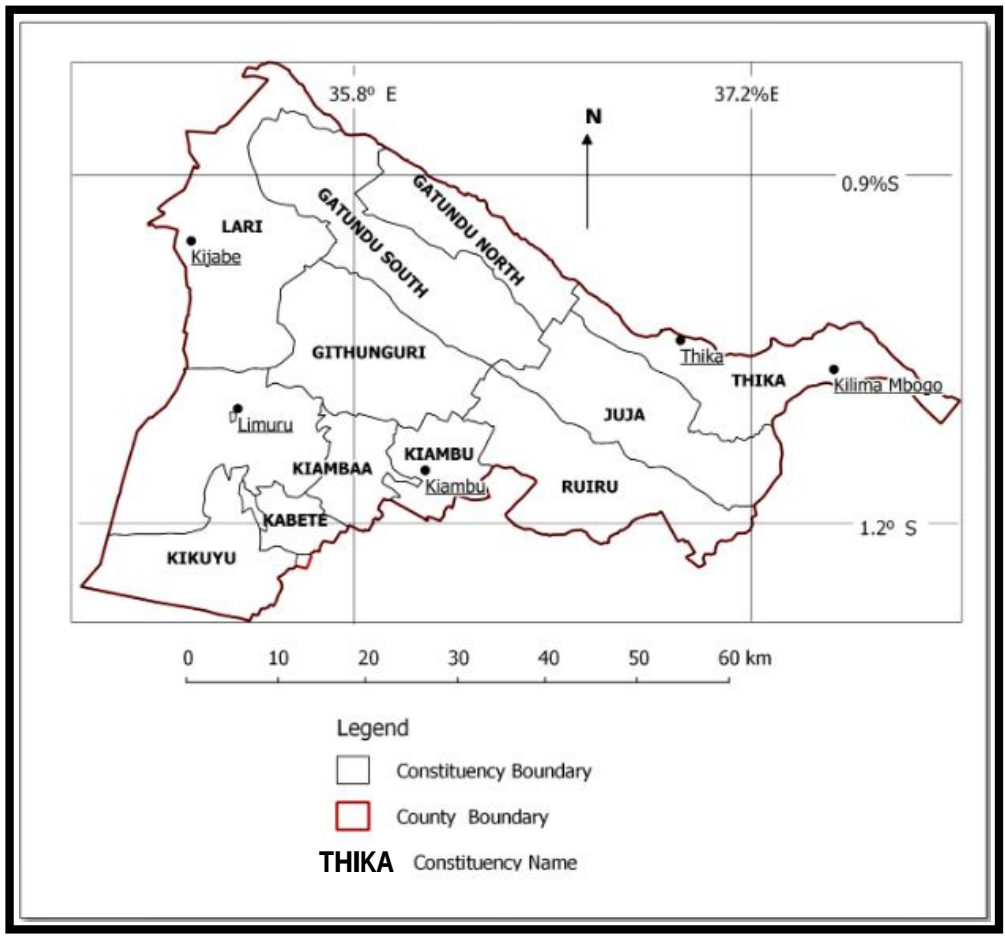
Prof. Mbaruk Suleiman
for Chairman, Mount Kenya University ERC
Date: 31.07.2013

Dr. Francis W. Muregi
Secretary, Mount Kenya University ERC
Date: 02/08/2013

Mount Kenya University
Research & Development
...velopment
...2 - 01000, Thika

Appendix VII: Map of Kenya Showing Position of Kiambu County (Inset)





Source: IEBC

Appendix VIII: Informed Consent Form

My name is _____ a Postgraduate student from Kenyatta University doing a Ph.D degree in medical microbiology. I am collecting stool specimen from children below five years.

Purpose of the study: to determine the genetic basis of resistance in *E. coli* isolated from children aged below 5 years who attend the Thika District Hospital with Diarrhoeal diseases. The study also seeks to establish the prevalence of and risk factors for faecal carriage of resistant *E. coli* strains.

Procedure to be followed: in this study you will give a stool sample for bacteriological examination and molecular analysis. As a participant in this study you will be interviewed and requested to complete a questionnaire.

Risks: The procedure for collection of the stool sample does not pose any risk to you

Benefits: Results useful for your treatment obtained from this study will be communicated to your doctor.

Confidentiality of the records: Information gathered from you will be coded for purposes of confidentiality and that your name will not be identified from these records. The code numbers will also be used in reports and publications.

Basis of participation: It is important for you to know that you have choice to decline from participating in this study. Should you have any question or

clarification to be made, you can ask the Principal Investigator, Kangethe Stanley Kamwati of Mobile No 0721218159.

Signature: I have read the above information and had an opportunity to ask questions and have been answered. I consent to my child taking part in the study. I fully understand there are no risks associated with the collection of the stool samples.

Signature _____ Date _____

(Parent/guardian)

I, the undersigned, have fully explained the relevant details of this study and the person authorized to consent for the patient.

Signature _____ Date _____

(Investigator)

Appendix IX: Questionnaire

Antimicrobial Resistance *Escherichia coli* Genes from Children Aged Below 5 Years Presenting With Diarrhoea at Thika Level Five Hospital, Kiambu County, Kenya

Patient's name……..… Guardian's Name ……..

Relationship…….. Date of birth……...

Age…….……. Sex……..…

Clinic Number…….

In/Out patient No ……. Date of collection ……..

Study No…….

Date of birth …….

Sex M ☐ F ☐

Specimen taken for microbiological survey

a) Stool ☐

Do you have any of the following symptoms? (Tick as appropriate)

Acute diarrhoea; (Less than two weeks) ☐

Chronic diarrhoea; (Over two weeks) ☐

Recurrent/intermittent diarrhoea ☐

Diarrhoeal episodes per day ☐

Vomiting, Abdominal swelling ☐

Others _____

Where do you live?

(Estate location) ☐

8. Has the child taken any antibiotics in the last three months?

Yes ☐ No ☐

9. Has the child been hospitalised in the previous 3 months

Yes ☐ No ☐

12. List ailments or conditions that the child suffer from if any

_____

_____

_____

Appendix X: Antibiotics used in the study and their abbreviations.

AK	Amikacin
AMC	Amoxicillin-Clavulanic
AMP	Ampicillin
FEB	Cefepime
C	Chloramphenicol
CAZ	Ceftazidime
CIP	Ciprofloxacin
CRO	Ceftriaxone
K	Kanamycin
NA	Nalidixic Acid
TET	Tetracylines
S	Streptomycin

Appendix XI: Antimicrobial Test Results

	Clinical data			AMC	CAZ	Sul	TRIM	AMP	CRO	CN	C	NA	CIP	K	S
	Antibiotic use	Residence	Hospitalised	(8, 16, 32)	(8, 16-32, 64)	(ND)	(8, 16)	(8, 16, 32)	(8, 16-32, 64)	(4, 8, 16)	(8, 16, 32)	(16, 32)	(1, 2, 4)	(16, 32, 64)	(ND)
1	Yes	Urban	Inpatient	2	4	24	12	8	0.04	0.02	0.02	0.2	0.01	2	24
2	No	Rural	Outpatient	2	6	48	32	48	0.04	2	0.02	0.2	0.01	2	32
3	Yes	Rural	Outpatient	4	6	128	128	8	16	0.02	6	0.02	0.01	2	16
4	Yes	Rural	Inpatient	8	6	24	16	4	2	0.02	8	0.02	0.01	2	12
5	No	Urban	Inpatient	16	64	128	128	> 256	64	12	4	2	0.02	2	64
6	No	Urban	Outpatient	2	4	128	64	32	32	0.02	8	0.2	1	4	8
7	No	Rural	Inpatient	2	4	128	256	8	2	0.02	6	0.02	0.01	4	12
8	Yes	Urban	Outpatient	4	6	4	8	4	0.2	1	2	0.02	0.01	4	8
10	No	Rural	Outpatient	4	4	32	16	8	2	2	6	0.02	0.01	4	12
11	No	Urban	Inpatient	4	8	128	64	32	16	0.02	6	4	0.02	4	16

12	Yes	Urban	Inpatient	4	6	128	128	32	4	0.02	2	0.02	0.01	4	12
13	Yes	Urban	Inpatient	6	8	128	64	128	64	2	6	1	0.01	4	8
14	Yes	Rural	Outpatient	8	4	6	8	4	0.2	1	1	1	0.01	4	16
15	Yes	Urban	Inpatient	8	4	64	32	32	8	0.02	32	0.02	0.01	4	16
16	Yes	Urban	Inpatient	12	128	16	8	> 256	128	6	8	2	1	4	16
17	Yes	Rural	Outpatient	16	24	128	128	> 256	48	4	6	0.02	0.01	4	32
18	Yes	Rural	Inpatient	24	8	256	256	32	6	1	32	1	1	4	16
19	No	Rural	Outpatient	2	4	4	8	2	0.2	1	6	1	0.01	6	32
20	Yes	Rural	Outpatient	4	4	128	128	32	32	2	2	1	1	6	16
21	Yes	Rural	Inpatient	4	4	256	128	32	32	2	8	0.2	1	6	16
22	No		Outpatient	6	8	256	256	256	64	2	4	0.2	0.01	6	16
23	Yes	Urban	Inpatient	8	4	32	16	48	32	0.02	4	0.02	0.01	6	12
24	Yes	Rural	Outpatient	8	8	256	256	32	16	2	4	1	0.01	6	16
25	Yes	Urban	Outpatient	128	64	128	128	> 256	128	6	8	8	2	6	16
26	No	Urban	Inpatient	2	6	16	8	2	0.2	0.02	0.02	1	0.01	8	12

27	No	Rural	Outpatient	2	4	32	16	8	0.002	1	0.02	0.02	0.01	8	32
28	No	Rural	Outpatient	2	4	64	32	48	32	0.02	4	0.2	1	8	8
29	No	Rural	Inpatient	4	4	12	16	32	4	0.02	6	2	0.01	8	8
30	No	Rural	Inpatient	4	8	256	256	128	64	2	6	2	0.02	8	24
31	No	Rural	Outpatient	6	4	64	128	64	32	2	6	2	0.02	8	32
32	No	Urban	Inpatient	8	4	64	64	48	16	2	6	1	0.01	8	24
33	No	Urban	Outpatient	4	6	128	256	64	16	2	4	1	0.02	12	16
34	No	Urban	Inpatient	16	6	128	48	32	8	0.02	48	32	4	16	24
35	Yes	Urban	Outpatient	12	24	128	128	> 256	48	24	16	24	8	32	48
36	No	Urban	Outpatient	128	64	256	128	> 256	128	16	12	32	4	32	64
37	Yes	Rural	Inpatient	12	24	128	128	> 256	128	32	32	32	8	48	64
38	Yes	Urban	Outpatient	16	24	128	128	> 256	48	12	12	1	0.1	48	256
39	No	Rural	Outpatient	48	6	256	128	128	16	2	16	28	6	48	32
40	Yes	Rural	Inpatient	128	128	256	128	> 256	128	16	32	2	4	48	64
41	No	Rural	Inpatient	8	8	128	64	48	16	2	8	2	0.02	64	64

42	No	Urban	Outpatient	32	6	32	16	16	8	1	24	2	1	64	256
43	No	Urban	Inpatient	32	6	64	48	32	4	0.02	16	2	1	64	128
44	Yes	Rural	Outpatient	32	4	128	128	8	6	2	64	1	1	64	64
45	Yes	Urban	Inpatient	128	24	128	48	> 256	64	16	32	24	8	64	128
46	Yes	Urban	Inpatient	12	64	128	128	> 256	128	64	24	8	2	128	>1024