ANTIMICROBIAL RESISTANCE PATTERNS AND GENETIC BASIS OF EXTENDED SPECTRUM β-LACTAMASES IN FAECAL *ESCHERICHIA COLI* ISOLATED FROM SEVERELY MALNOURISHED AND NON-MALNOURISHED CHILDREN ATTENDING MBAGATHI DISTRICT HOSPITAL, NAIROBI

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Antimicrobial Resistance Patterns and Genetic Basis of Extended

Spectrum β-Lactamases in Faecal *Escherichia coli* Isolated from

Severely Malnourished and Non-Malnourished Children Attending

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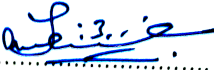
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DECLARATION

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

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DEDICATION

To my dear Parents

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LIST OF ABBREVIATIONS AND ACRONYMS

A/E	Attachment effacement
AMC	Amoxicillin/Clavulanic acid
AMP	Ampicillin
ATCC	American Type Culture Collection
bla	beta-lactamase gene
C	Chloramphenicol (C)
C.I	Confidence Interval
CAZ	Ceftazidime
CIP	Ciprofloxacin
CLSI	Clinical Laboratory Standards Institute
CMR	Centre for Microbiology Research
CMY	Cephamycinase Enzymes
CN	Gentamicin
CPD	Cefpodoxime
CSV	Coma Separated File
CTX	Cefotaxime
CTX-M	CefoTaXimases 'Munich'
DAP	Diaminopimelic acid
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphate
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
EDTA	Ethylene diamine-tetra-acetic acid
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ERC	Ethical Review Committee
ERIC	enterobacterial repetitive intergenic consensus
ESBL	Extended or Expanded Spectrum beta-Lactamase
et al.	"et alia" (Italian word referring to 'and others')
ETEC	Enterotoxigenic *Escherichia coli*
FEP	Cefepime
FOX	Cefoxitin
GARP	Global Antibiotic Resistance Partnership
h	Hour
H$_2$S	Hydrogen Sulphide
HUS	Hemorrhagic Uremic Syndrome
IncF	Incompatibility Group F
K. pneumoniae	*Klebsiella pneumonia*
kb	Kilobase
KEMRI	Kenya Medical Research Institute
KLU	*Kluyvera ascorbata*

LEE	Locus of enterocyte effacement
LIM	Lysine, Indole, Motility Agar
LPS	Lipopolysaccharides
M	Molar
MDR	Multi Drug Resistant
MgCl$_2$	Magnesium chloride
min	Minute
MLST	Multi-Locus Sequence Typing
MUAC	Middle/ Mid Upper Arm Circumference
NA	Nalidixic Acid
NAG	N-acetyl glucosamine
NAM	N-acetyl muramic acid
NDM	New Delhi metallo-beta-lactamase
O.R	Odds Ratio
OXA	Oxacillinase Enzymes
PBP	Penicillin Binding Proteins
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
QRDR	Quinolone Resistance Determinants
RAPD	Random amplified polymorphic DNA
REP	repetitive extragenic palindromic
RFLP	Restriction fragment length polymorphism
S	Streptomycin
SCC	Scientific Steering Committee
SHV	Sulfhydrl Variable Enzymes
spp.	Species
ST	Sequence Type
SXT	Sulfamethoxazole/Trimethoprim
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA
TCA	Ticarboxylic Acid Cycle
TEM	Temoneira Enzymes
TSI	Triple Sugar Iron Agar
TZP	Tazobactam/Piperacillin
U	Unit
UK	United Kingdom
USA	United States of America
UTI	Urinary Tract Infections
V	Volt
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate Agar

DEFINITION OF TERMS

Severely Malnourished: Children with a Mid Upper Arm Circumference (MUAC) of less than 110 millimeters [below −3 standard deviations or Z- scores] and/or bipedal edema. Referred to Cases in this study.

Extended Spectrum β-lactamase phenotype: Isolates of *E. coli* resistant to ceftazidime, cefpodoxime and cefotaxime were considered presumptive extended-spectrum-β-lactamase (ESBL) producing phenotypes, if they showed synergy to clavulanic acid.

ABSTRACT

Severely malnourished children have increased risk of severe infections and are therefore more likely to use antimicrobial agents. This could in turn present a strong selection pressure for the emergence and spread of multidrug resistant (MDR) strains.

In this case-control, hospital-based study, 109 *E. coli* isolates were obtained from both severely malnourished and non-malnourished children. Higher resistances were observed in severely malnourished children to cefpodoxime, cefepime, ceftazidime, cefotaxime, amoxicilin/clavulanic acid, ciprofloxacin, nalidixic acid, chloramphenicol, and gentamicin than in non-malnourished children. Severely malnourished children were 8.2 times more likely to carry ESBL-producers than non-malnourished children (P < 0.0001, O.R - 8.2254, C.I - 3.6374 to 18.6003). bla_{CTX-M} was present in 25 (49%) and 6 (75%) among Cases and Controls respectively. Among the 31 CTX-M producers, 30 (97%) carried $bla_{CTX-M-15}$. Also $bla_{CTX-M-15}$ occurred in combination with bla_{TEM-1} and bla_{OXA-1} in 10 (32 %) isolates. Other *bla* alleles detected alongside $bla_{CTX-M-15}$ but rarely reported globally were bla_{TEM-33} 1(3%) and $bla_{TEM-158}$ 1(3%).

ST131 clones were present in 12 (39%) of the CTX-M-15 isolates. This study also identified a novel ST4481 clone and has been deposited in the Warwick database.

This study reports the first occurrence of CTX-M-15-producing *E. coli* strains from Mbagathi District Hospital belonging to ST131, ST405, ST44, ST205, ST1642, ST1722, ST617, ST38, ST1675, ST648 and ST167, ST940 and a newly submitted ST4481. ST4481 has not been associated with CTX-M-15 enzymes anywhere else in the world.

This study provides proof that ST131 strains with CTX-M-15 enzymes are indeed widespread. Isolates from non-malnourished children only fit into few ST-clones (ST405, ST940 and ST131) while ambiguity was observed in ST clones from malnourished children.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

1.1.1 *Escherichia coli* as a model for antimicrobial resistance

Escherichia coli (E. coli) are model indicator organisms in antimicrobial resistance in community and hospital-based infections. *E. coli* belong to family *Enterobacteriaceae* and the genus *Escherichia* (Nataro & Kaper, 1998). *E. coli* are prevalent facultative anaerobes of the human gut and also colonize the intestines of majority of animal species and are therefore suitable indicators of fecal contamination of drinking water and food. The bacteria typically colonize the infant gastrointestinal tract within hours after birth (Welch, 2006). Transmission mode of *E. coli* is via the faecal-oral route or via direct (person-person) transmission (Nataro & Kaper, 1998).

1.1.2 *E. coli* as an etiologic agent of diarrhoea

E. coli are one of the leading causes of acute diarrhoea in developing countries in children under 5 years of age with significant morbidity and mortality (Qadri, Svennerholm, Faruque, & Sack, 2005). *E. coli* may act as reservoirs for resistance genes and this become more problematic if the resistance genes are horizontally transferable to other pathogens (Pfeifer, Cullik, & Witte, 2010). *E. coli* commonly isolated from diarrhoeal patients belong to five different groups namely: enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), and enteroinvasive *E. coli* (EIEC) (Nataro & Kaper, 1998). EAEC can colonize the mucosa of both the small and large intestine and can lead to mild inflammation in the colon (Okhuysen & Dupont, 2010). EAEC is characterized by its 'stacked brick' adhesion when cultured with Hep-2 cells (Flores & Okhuysen, 2008). EPEC are capable of producing attaching and effacing (A/E) lesions (Nataro & Kaper, 1998). A/E strains use a pool

of locus of enterocyte effacement (LEE)-encoded and non-LEE-encoded effector proteins to subvert and modulate cellular and barrier properties of the host (Ochoa & Contreras, 2011). EHEC are responsible for major outbreaks of bloody diarrhoea and haemolytic uremic syndrome (HUS) throughout the world. EHEC has two major virulence strategies: production of Shiga toxin (Stx) and formation of attaching and effacing (AE) lesions on enterocytes (Fasano, 2001; Nataro & Kaper, 1998). The EIECs are invasive *E. coli* that are nearly genetically, biochemically, and clinically and pathologically identical to *Shigella* spp. (Nataro & Kaper, 1998). Like *Shigella*, they cause an epithelial invasion of the large bowel leading to inflammation and ulceration of the mucosa (Croxen & Finlay, 2010). Human ETEC infections are contracted by consumption or use of contaminated food and water and presents as a sudden onset of secretory diarrhoea that is usually self-limiting but can lead to dehydration due to loss of fluid and electrolytes (Qadri *et al.,* 2005). The major features of the five *E. coli* pathovars are summarised in **Table 1-1** below.

Table 1-1: Major characteristics of the different *E. coli* pathovars

Pathovar	Populations at risk	Disease	Transmission route	Main virulence factors
EIEC	Children and Adults in developing countries, foodborne outbreaks in developed countries	Acute dysentery	Faecal- oral	1. *ipa*H encoding the invasive antigen H 2. Type III secretion system with virulence factors *IpaA, IpaB, IpaC and IpgD*
EAEC	Children and adults worldwide	Persistent watery, mucoid, bloody or non-bloody diarrhoea with low-grade fever & little or no vomiting	Faecal-oral	1. Aggregative adherence fimbriae (AAF) 2. Toxin *astA*
EHEC	Children and adults worldwide	Watery diarrhoea bloody diarrhea. Little or no fever. Dysentery, haemorrhagic colitis (HC) and haemolytic-uremic syndrome (HUS)	Faecal- oral	1. Shiga toxins (*Stx*1 and *Stx*2). 2. Intimin
EPEC	Mainly infants and children in developing countries	Profuse watery diarrhoea, commonly with vomiting and low-grade fever	Faecal- oral	1. Intimin (eae); in both typical and atypical EPEC 2. Bundle-forming pili in typical EPEC isolates*
ETEC	Children in developing countries and travellers,	Watery diarrhoea, without blood, fever and vomiting in minority of Cases	Faecal- oral	1.Colonization factors. 2. Toxins, LT and ST (in forms of STp or STh)

Adapted from (Croxen & Finlay, 2010; Nataro & Kaper, 1998; Qadri *et al.,* 2005). **LT**- heat labile, **STp**- heat stable toxin from porcine origin, **STh**- heat stable toxin of human origin typical EPEC have *bfp* (bundle forming pilus) and *eae* (intimin) and atypical EPEC have *eae* (intimin) and *stx* (shiga toxin gene).

1.1.3 Link between persistent enteric infections and malnutrition

Prolonged enteric infections can cause malnutrition due to protein and nutrient loss or due to impaired absorptive function. These effects are additionally influenced by

intestinal host-pathogen interactions such as host-pathogen-flora mucosal interactions or nutrient competition that increase the chances of invasive infections and sepsis (Guerrant, Oriá, Moore, OB, & AM, 2008). Severely malnourished children are more likely to have other complications such as diarrhoea, pneumonia and bacteraemia (Talbert *et al.,* 2012). Such conditions may warrant the use of antimicrobials. Severely malnourished children have greater risk and a predisposition to longer duration of severe diarrhoea. Furthermore, severe acute malnutrition accompanied by diarrhoea, may also predispose a child to inappropriate use of antimicrobials further raising chances of colonization with MDR strains (Galetto-lacour *et al.,* 2013; Opondo *et al.,* 2011; WHO, 2005). It is therefore important to assess the difference in resistance phenotypes encountered among strains recovered from severely malnourished children and among healthy ones.

1.1.4 Antimicrobials used to manage *E. coli* infections

The basics of treatment of *E. coli* depend on the site of infections and are mostly managed using broad-spectrum antimicrobials whose mechanisms of action is summarised in **Table 1-2**.

Table 1-2: Mechanisms of action of antimicrobial agents

Mechanism	Antimicrobial(s)
Interference with cell wall synthesis	β-lactams: penicillins, cephalosporins, carbapenems, monobactams. glycopeptides: vancomycin, teicoplanin
Protein synthesis inhibition	
• Bind to 50S ribosomal subunit	Macrolides, chloramphenicol, clindamycin.
• Bind to 30S ribosomal subunit	aminoglycosides, tetracyclines.
Interference with nucleic acid synthesis	
• Inhibit DNA synthesis	Quinolones.
• Inhibit RNA synthesis	Rifampin
Inhibition of metabolic pathway	Sulfonamides, folic acid analogues
Disruption of bacterial membrane structure	Polymyxins, daptomycin.

Adapted from (McDermott, Walker, & White, 2003).

According to prescription trends in two hospitals in Ethiopia, quinolones and β-lactams are the most used antimicrobials in hospitalised patients (Banja W. D, 2009). Apart from human medicine, antimicrobials are also used in veterinary medicine and aquaculture to enhance productivity and to boost survival of the flock or herds. Exposure to antimicrobials may have an impact in microbial ecosystems of humans, animals and the environment, which may lead to the development of antimicrobial resistance (GARP, 2011; WHO, 2012).

1.1.5 Antibiotic resistance as a public health threat

Antimicrobial resistance among *Escherichia coli* is of increasing global concern (Peirano, 2010). Increasing antimicrobial resistance limits treatment options, raises health care costs, and increases the number; severity and duration of infections. Strains causing life-threatening infections are becoming resistant to multiple antimicrobials. Enzyme-mediated β-lactams, fluoroquinolones and aminoglycosides are of great clinical and epidemiologic importance because these agents are used for treatment of most serious infections especially when such infections involve septicaemia and bloody diarrhoea (Maltha *et al.,* 2014).

Resistance to β-lactams is mainly as a result of production of extended-spectrum ß-lactamases (ESBLs). ESBLs are enzymes capable of hydrolysing penicillin's, broad-spectrum cephalosporin's and monobactams, and are generally derived from parent Temoneira (TEM-1/2) and Sulfhydrl Variable (SHV-1)-type enzymesas a result of point mutations in the original enzymes (Rupp & Fey, 2003). Recently, Cefotaximases (CTX-M enzymes) have replaced SHV and TEM enzymes as the prevalent type of ESBLs, principally in community-acquired infections caused by *E.coli* (Falagas & Karageorgopoulos, 2009). The CTX-M-type ESBLs now exceed over 160 enzymes (http://www.lahey.org/Studies/other.asp), in which they are divided into five different groups based on amino acid identities: group CTX-M-1 includes CTX-M-1, -3, -10, -12, -15, -28, -30 and FEC-1; group CTX-M-2 includes CTX-M-2, -4, -5, -6, -7, -20, and Toho-1; group CTX-M-8 includes only CTX-M-8; group CTX-M-9 includes CTX-M-9, -13, -14, -16, -17, -19, -21, -24, -27, and Toho-

2, and finally; group CTX-M-25 includes CTX-M-25 and CTX-M-26 (J. D D Pitout, Nordmann, Laupland, & Poirel, 2005). CTX-M β-lactamases possess strong hydrolytic activity against cefotaxime. Bacterial strains that have CTX-M enzymes usually show MIC (minimum inhibitory concentration) levels against cefotaxime in the resistant range (>64 μg/ml), but MIC levels against ceftazidime in the susceptible range (2 to 8 μg/ml). However, it has been reported that some CTX-M-positive isolates, especially those harbouring $bla_{CTX-M-15}$ have the ability to hydrolyze ceftazidime, and therefore are resistant to a wider panel of cephalosporins (Paterson & Bonomo, 2005). It has also been shown that cefepime is efficiently hydrolysed by the CTX-M enzymes, for which MIC levels are higher than for other ESBL-producing bacteria.

Isolates producing CTX-M enzymes have been reported worldwide especially for CTX-M-15, CTX-M-14, CTX-M-3, CTX-M-2, and CTX-M-9. In many countries, the CTX-M-15 has become the most prevalent enzyme among *E. coli* strains during the last couple of years. In Africa reports from South Africa, Central African Republic, Tanzania, Tunisia, Egypt, Algeria, Nigeria and Kenya indicate that these enzymes are now spread across most continents (Kiiru *et al.,* 2011; Ndugulile, Jureen, Harthug, Urassa, & Langeland, 2005; Ogbolu, Daini, Ogunledun, Alli, & Webber, 2011; Peirano, van Greune, & Pitout, 2011; Touati *et al.,* 2006; Vlieghe, Phoba, Tamfun, & Jacobs, 2009). In 2011, a local study reported the occurrence of $bla_{CTX-M-15}$-producing ST131 in faecal and urine samples of patients in Kenya but reports of such strains from faecal samples are rare (Kiiru *et al.,* 2011). In this past study, the CTX-M-15-producing isolates were resistant to most cephalosporins, but not to piperacillin/tazobactam or cefoxitin (Kiiru *et al.,* 2011).

1.1.6 Sequence Type 131 with CTX-M-15 enzymes are globally distributed

CTX-M-15-producing *E. coli* strains, especially those belonging to sequence type 131 (ST131) are of great clinical and epidemiological importance because these strains normally harbour a panel for virulence and resistance genes on transferable plasmids (Woodford *et al.,* 2009). A high prevalence of this clone (~30%–60%) has

also been implicated in fluoroquinolone resistance in *E. coli*. Additionally, majority of ST131 strains also harbour a variety of β-lactamase genes encoding CTX-Ms, TEM, OXA, SHV and CMY β-lactamases (Peirano & Pitout, 2010). *E. coli* strains of ST131 have emerged rapidly and disseminated globally in hospitals and the community settings, causing life threatening MDR infections typically associated with frequent recurrences and that are hard to treat (Johnson *et al.,* 2013; Peirano *et al.,* 2014). Sequence type 131 *E. coli* isolates fall under two categories: fluoroquinolone resistant isolates and those resistant to both ciprofloxacin and aminoglycosides. These MDR strains are becoming increasingly prevalent, resulting in some patients progressing to severe illness or death despite receiving conventional empirical or prophylactic therapy.

Due to their high MDR phenotypes and co-resistance to fluoroquinolones and aminoglycosides, treatment of invasive ST131 may require the use of carbapenems, one of the last lines of treatment options against Gram-negative bacteria (Paterson & Bonomo, 2005).

1.2 Statement of the Problem

Malnutrition is a challenge the urban-poor population living in informal settlements and slums face. To manage the high risk of mortality, children who are malnourished may need antimicrobials to treat co-morbidities such as bacteraemia, urinary tract infections, diarrhoea, and pneumonia. Recently, there has been reports of emergence and spread of Multi-Drug Resistant (MDR) *E. coli* strains that are resistant to important classes of antimicrobials such as ciprofloxacin and amino glycosides. These two classes of antimicrobials are important "chemotherapeutic replacer agents" that are recommended for alternative treatment of infections caused by ESBL-producers that have now become widespread in patients across all ages. Some of these MDR strains that exhibit co-resistant to ciprofloxacin and aminoglycosides belong to sequence type 131 (ST131), a clone that poses a real challenge for clinicians due to limitation of alternative antimicrobials that can be used to manage associated infections. The differences in resistances, the prevalence of ESBL colonization and associated *bla* genes in severely malnourished children and non-

malnourished children is still not known. Additionally, little data exist on existence of fecal ST131 and diversity of other clonal complexes among ESBLs from children under 5 years.

1.3 Justification of the Study

Severely malnourished children have been reported to be more vulnerable to MDR *E. coli* strains than healthy populations due to their age and due to that fact that such children are more likely to be treated using antimicrobials. The data generated in this study on resistance profiles among isolates recovered from severely malnourished and from non-nourished children will aid in better management of enteric illnesses associated with such strains, especially the MDR ESBLs. The data will also find use among clinicians seeking to make data-informed prescriptions for malnourished children.

1.4 Research questions

- Are *E. coli* strains from faecal samples of children with severe malnourishment more resistant to antimicrobials compared to those recovered from non-malnourished children?
- What are the common resistance phenotypes of *E. coli* observed among strains exhibiting resistance to ß-lactams (ESBL phenotype) and those susceptible to this class of antibiotics in these two study populations?
- What is the genetic relatedness and diversity of the sequence types (ST-types) among *E. coli* isolates recovered from the two children populations?

1.6 Objectives

1.6.1 General objective

To compare resistance (phenotype and genotypes) in *E. coli* isolated from severely malnourished and non-malnourished children below 5 years, all attending Mbagathi District Hospital.

1.6.2 Specific objectives

1. To determine antimicrobial susceptibility patterns of *E. coli* from severely malnourished and non-malnourished children.
2. To determine the most common resistance phenotypes among strains exhibiting ESBL in severely malnourished and non-malnourished children.

To establish the phylogenetic relatedness among strains showing resistance to broad-spectrum β-lactams.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Classification of *E. coli*

Escherichia coli are classified into; Domain: Bacteria, Kingdom: Bacteria, Phylum: Proteobacteria, Class: Gamma Proteobacteria, Order: Enterobacteriales, Family: *Enterobacteriaceae*, Genus: Escherichia Species: *Escherichia coli* (Welch, 2006). They are identified from other *Enterobacteriacea* by their ability to utilise particular carbohydrates. *E. coli* are lactose fermenters, indole positive, do not utilise citrate, do not liberate H_2S from triple sugar-iron agar or lysine iron agar, are methyl red-positive, urea negative, are motile and deaminate lysine (Welch, 2006).

2.2 *E. coli* and Infections

There are several toxin-producing strains of *E. coli* causing diarrhea. Some of the non-pathogenic *E. coli* strains can cause disease if they spread outside the intestines, in immune-suppressed or a healthy person. Most common extra-intestinal infections caused by *E. coli* are community onset Urinary Tract Infections (UTI's) (Kariuki *et al.,* 2007). Other types of infections caused by this species include are meningitis, septicemia, pneumonia, intra-abdominal sepsis and gynecological infections (Bertin, Girardeau, Martin, & Darfeuille-michaud, 2000).

Most gut illnesses associated with *E. coli* do not require antimicrobials. However, the use of antimicrobials is warranted in severely malnourished children to treat co-morbidities that include diarrhea, pneumonia and bacteremia (Schlaudecker *et al.,* 2011; A. Talbert *et al.,* 2012).

Antimicrobial options in treatment of these infections are; trimethoprim, cefuroxime, ciprofloxacin, amoxicilin/clavulanic acid and amoxicillins (Iannotti, Trehan, Clitheroe, & Manary, 2014; WHO, 2013).

2.3 Antimicrobial resistance in *E. coli*

Ineffectiveness of antibiotics is as a result of selective pressure brought about by increased use and misuse of antibiotics (Frost, 2010). β-lactams, fluoroquinolones and aminoglycosides remain active against a significant proportion of *E. coli* strains causing nosocomial infections in Kenya (Brooks *et al.,* 2006; A. W. Talbert, Mwaniki, Mwarumba, Newton, & Berkley, 2010). However, reports of multidrug resistant *E. coli* clone ST131 are changing the existing strategies for chemotherapy. Some of the strains belonging to ST131 clone carry plasmid-borne *aac(6')-lb-cr*, and *bla$_{CTX-M-15}$* (Clermont *et al.,* 2008). The *bla$_{CTX-M-15}$* genes confer resistance to third generation cephalosporin's, while *aac(6')-lb-cr* confer low-level ciprofloxacin resistance and aminoglycosides gentamicin resistance. For such strains, carbapenems remain the only plausible alternative active against strains, antimicrobials that are more expensive and toxic (Rawat & Nair, 2010).

Screening strains for resistance types is increasingly important for recognizing multi-drug resistant clones and assessing the risk of potential outbreaks causing sporadic infections in community and hospital settings. Epidemiological screening is imperative for effective surveillance of hyper-endemic ST131 strains that are multidrug resistant (Johnson *et al.,* 2013).

2.4 Relationship between severe malnourishment and antimicrobial use

The bidirectional association between diarrhea and malnourishment has been documented (Nel, 2010). One approach is, malnutrition after diarrheal illness arises from anorexia, diminished absorptive function, and mucosal damage as well as nutrient exhaustion associated with each episode of diarrhea. The other is, malnutrition increases the severity and duration of diarrhea making diarrhea the leading co-morbidity in severely malnourished children (Ferdous *et al.,* 2013; Moore, 2011; Roy *et al.,* 2011). **Figure 2-1** highlights the possible targets for intervention for breaking the malnutrition-diarrhea cycle, one of which is the use of antimicrobials (Guerrant *et al.,* 2008).

Enteric infections, even those caused by *E. coli* lead to intestinal inflammation and damage. These, in turn, can cause serious nutrient mal-absorption. Moreover, impaired innate and acquired mucosal defenses may, lead to severe intensity of infections and rises chances of invasive illnesses. Apart from diarrhea, severely malnourished children often have other complications such as, pneumonia, urinary tract infections and bacteremia (Okomo *et al.,* 2011; Roy *et al.,* 2011), which may warrant the use of antimicrobials to boost survival.
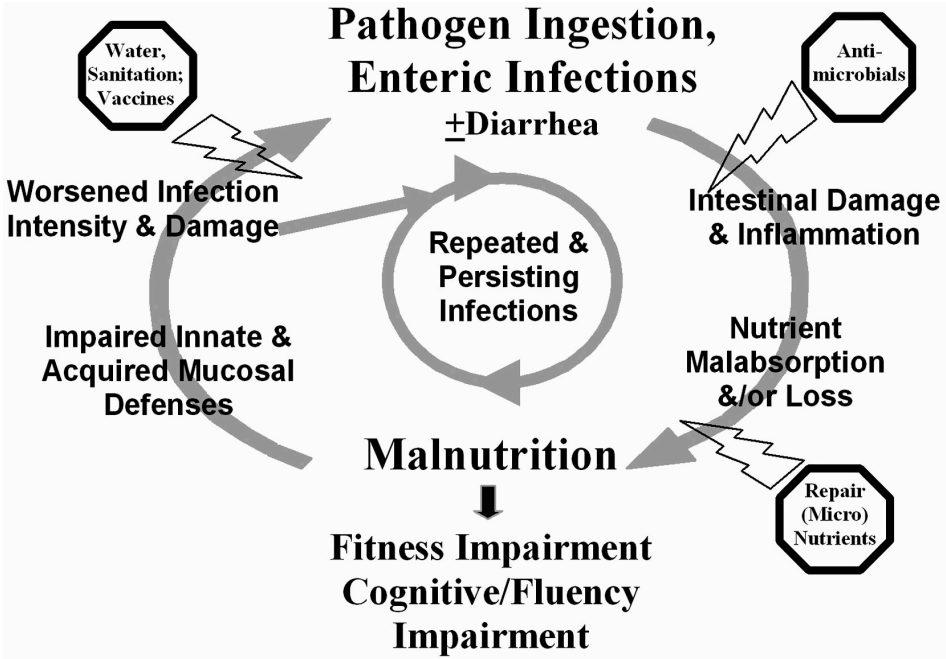
Figure 2-1: Gut-trophic interventions to breaking the vicious cycle between malnutrition and diarrhea by repairing the intestinal mucosa

2.5 ß-lactams, mode of action and basis of resistance

The cell wall of *Enterobacteriacea* consists of an inner cytoplasmic membrane and outer membrane consisting of lipopolysaccharides (LPS) and lipoproteins. LPS consists of lipid A, core polysaccharide, and O antigen. The periplasm is a space between the inner cytoplasmic membrane and outer lipid membrane. This space contains a fish-like network of peptidoglycan chains. A large layer of peptidoglycan

is found in Gram-positive bacteria; Gram-negative bacteria have a much thinner layer of peptidoglycan surrounded by a lipid bilayer outer membrane. The individual peptidoglycan cell-wall units are produced inside the cell, the final cross-linking is catalyzed outside the cytoplasmic membrane by a group of membrane-anchored bacterial enzymes known as the cell-wall transpeptidases (Welch, 2006).

Peptidoglycan is an important component of the bacterial cell wall. It's a protectant from osmotic rupture, determines cell shape, and is integral to cell growth and division. The peptidoglycan is composed of a basic repeating unit of an alternating chains of disaccharide N-acetylglucosamine (NAG) and N-acetylmuramicacid (NAM) linked by β-(1,4)-glycoside units. An amino-acid chain composed of four amino acids usually replaces the carboxyl group of muramic acid. The most common are L-alanine, D-alanine, D-glutamic acid, D-glutamine and L-lysine ordiaminopimelic acid (DAP) (Welch, 2006).

β-lactam antibiotics act on bacteria by inhibiting the bacterial enzymes, transpeptidases and carboxypeptidases, located in the cytoplasmic membrane which catalyses synthesis of the cross-linked peptidoglycan. These enzymes are commonly called penicillin-binding-proteins (PBPs) (McDermott *et al.,* 2003).

In the cross-linking reaction in Gram-negative bacteria, a peptide bond is formed between the D-alanine on one chain and the free amino end of a diamino pimelic acid on the other chain causing the elimination of the terminal D-alanine and designated the transpeptidase reaction. The β-lactam antibiotics are analogues of the terminal amino acid (D-alanyl-D-alanine) residues on the precursor NAM/NAG-peptide subunits of the peptidoglycan layer. In the presence of the β-lactam antibiotics, the transpeptidases and carboxypeptidases react with acyl-D-alanyl-D-alanine to form a lethal serine-ester-linked acyl (penicilloyl, cephalosporoyl) enzyme complex. The β-lactam enzyme complex found in the periplasmic space is very stable, and blocks the normal transpeptidation reaction. This result disrupts the synthesis of the cell wall and makes the growing bacteria highly susceptible to cell lysis and death (McDermott *et al.,* 2003) **Figure2-2**.
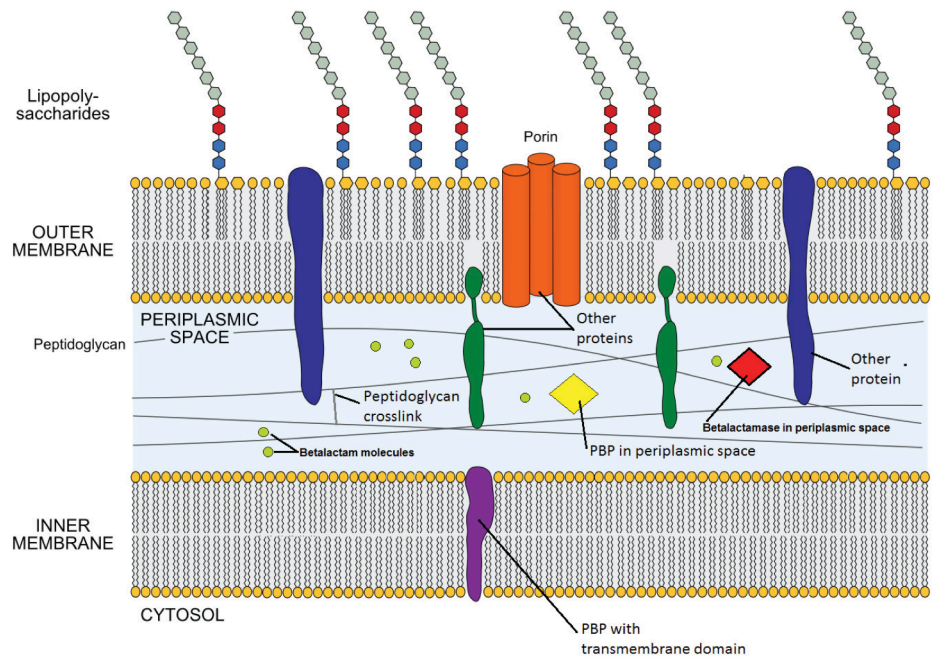
Figure 2-2: The gram-negative cell wall with location of two different penicillin-binding proteins, one beta-lactamase and beta-lactams © Creative Commons

The β-lactam antibiotics can be divided into six different groups, the penicillins, cephalosporins, carbapenems, cephamycins, monobactams, and β-lactamase inhibitors.β-lactam antibiotics contain a β-lactam ring which is a hetero-atomic ring structure consisting of three carbon atoms and one nitrogen atom. The β-lactam ring of natural or semi-synthetic penicillins is fused with a thiazolidine ring. In cephalosporins, the β-lactam ring is merged with adihydrothiazine ring (Amyes, 2010) **Figure 2-3**.
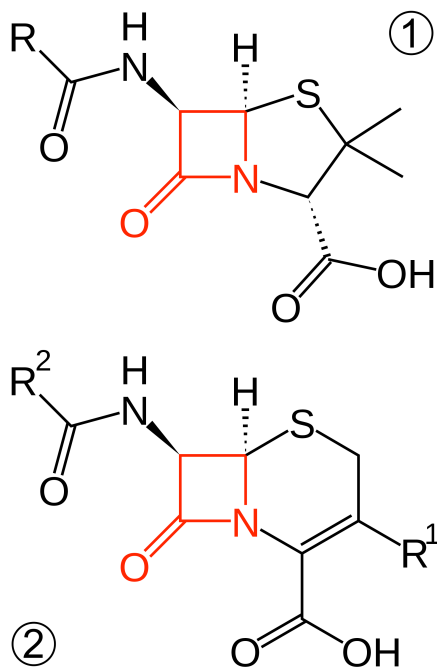
Figure 2-3: β-lactam ring (in red) in penicillins (1) and cephalosporins (2).

One of the mechanisms of resistance towards β–lactams is production of β-lactamases that hydrolyze the β-lactam ring (2-Azetidinone) and render the antibiotic inactive before it reaches the penicillin-binding protein (PBP) target mainly mediated by (Temoniera-1) TEM-1 and sulfhydryl variable -1 (SHV-1) enzymes (McDermott *et al.,* 2003). It has been shown that the amino acid substitutions or the re-arrangements of the omega loop and probably of the β-3 strand of parent TEM-1 and SHV-1enzymes result in the enlargement and altered shape of the active site resulting in broader enzyme-substrate interactions of large oxyimino substituents of the extended-spectrum cephalosporins e.g ceftazidime (Palzkill, Le, Venkatachalam, La Rocco, & Ocera, 2006). Moreover, resistance to ceftazidime and cefotaxime is a characteristic of CTX-M enzymes with an ESBL phenotype (Gutkind, Di Conza, Power, & Radice, 2013).

2.6 The CTX-Ms are heavily implicated in the ESBL-phenotype

CTX-M derived from "CefoTaXimase Munich" family constitutes a complex and non-homogeneous group of enzymes (Gutkind *et al.,* 2013). Since the initial identification of CTX-M enzymes, at least five genetically distinct groups based on their amino acid identities and alignment of the amino acid sequence have been identified from isolates around the world, including 164 representatives, both plasmid-borne and chromosome-encoded enzymes (http://www.lahey.org/Studies/other.asp).

CTX-M group 1 consist of; CTX-M-1, -3, -10, -12, -15, -28 and -30, and FEC-1); CTX-M group 2 comprising CTX-M-2, -4, -5, -6, -7 and -20, and Toho-1; CTX-M group 8 with CTX-M-8 only; CTX-M group 9 comprising CTX-M-9, -13, -14, -16, -17, -19, -21, -24 and -27, and Toho-2; and the CTX-M group 25 consisting CTX-M-25 and -26 (Cantón, González-Alba, & Galán, 2012; D'Andrea, Arena, Pallecchi, & Rossolini, 2013).

CTX-M group 1 enzymes display a typical "cefotaximase" phenotype, with efficient hydrolysis towards amino-penicillins, first- and second-generation cephalosporins (not cephamycins), and some oxyimino-cephalosporins such as cefotaxime and ceftriaxone, with ceftazidime, ceftizoxime and imipenem the most stable tested antibiotics (Bonnet, 2004). CTX-M-3 is found all over the world including many countries in Europe, Asia, Pacific region and Africa. Other prevalent CTX-M-1-related β-lactamases include CTX-M-1, CTX-M-32, and CTX-M-15 (also known as UOE- 1), which has been described in many European, Asian, African, America and Australia (Albrechtova *et al.,* 2012; Peirano & Pitout, 2010). CTX-M-1, CTX-M-3 and CTX-M-15 have been associated with well-described outbreaks in several locations (D'Andrea *et al.,* 2013; Ibrahimagić, Bedenić, Kamberović, & Uzunović, 2015). Other members of the CTX-M-1 cluster include CTX-M-10-12, -22, -23, -28-30, -32-34, -36, -37, -42, -52-55, -57, -58, -60-62, -64, -66, -68, -69, -71-73, -79, -80, -82, -88 and, KLUC-2 (D'Andrea *et al.,* 2013; Lahlaoui, Ben Haj Khalifa, & Ben Moussa, 2014).

CTX-M-2 was the first reported member of CTX-M group 2 of related enzymes, and was present in isolates as early as the late 1980s. CTX-M-2 enzymes were first detected cefotaxime-resistant Salmonella serovars from different pediatric hospitals in Argentina that were cefotaxime resistant but susceptible to inhibitors. CTX-M-2 is considered one of the most prevalent ESBLs in South America especially in Uruguay, Peru, Bolivia, and Paraguay and Argentina (Cruz *et al.,* 2013). Other CTX-M- 2 derived ESBLs include CTX-M-44 (formerly known as TOHO- 1), CTX-M-4, CTX-M-5, CTX-M-6 and CTX-M- 31. Additional plasmid-borne members of the CTX-M-2 group that are less common include: CTX-M-7, -20, -35, -43, -56, -59, -74, 76-77 (Lahlaoui *et al.,* 2014).

CTX-M group 8 has a few representatives and CTX-M-8 was the first to be isolated in three Amp-C-producing (resistant to cefoxitin) *Enterobacteriacea* (*E. cloacae, E. aerogenes and C. amalonaticus*) isolated in Brazil. Other members of the group include CTX-M-40 and CTX-M-41 (Lahlaoui *et al.,* 2014).

CTX-M-9 was the first reported enzyme of CTX-M-9 group 9 and was detected in *E. coli* isolated in 1996 in Spain. The most widely disseminated member of this group is CTX- M-14, which has been found in isolates collected from Europe, America, Asia and Africa (Coque *et al.,* 2008; Slama *et al.,* 2007). Additional plasmidic members of the CTX-M-14 group include CTX-M-13, -16-19, -21, -24, -27, -38, -46-51, -65, -67, -81, and -83-87 (Lahlaoui *et al.,* 2014). CTX-M-25 and CTX-M-26 were the first enzymes described in *E. coli* from CTX-M group 25. This group of β-lactamases contains six members including CTX-M-25, CTX-M-26, CTX-M-39, - 89, -91, and CTX-M-78 (Cantón *et al.,* 2012; Lahlaoui *et al.,* 2014). To date, CTX-M-15 and CTX-M-14 enzymes are the most predominant types of ESBLs with CTX-M-15 showing global distribution while the latter being prevalent in Spain (Lahlaoui *et al.,* 2014).

2.7 CTX-M-15 enzymes, link to ST131 and Epidemiology

The *bla*CTX-M-15 gene is one of the most important CTX-M ESBL enzymes among *Enterobacteriacea* because they are the most widespread and have been reported globally (Baroud, Araj, & Matar, 2011). These enzymes have been detected in both

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clinical and community isolates and in all continents including Africa (Cantón *et al.,* 2012; Tansarli, Poulikakos, Kapaskelis, & Falagas, 2014). CTX-M-15 enzymes strongly hydrolyze expanded-spectrum cephalosporins such as; cefotaxime, ceftriaxone, ceftazidime, and cefepime (Cantón *et al.,* 2012). In Kenya, CTX-M-15 enzymes have been described in *E. coli* from dogs and in human urinary tract infections (Albrechtova *et al.,* 2012; Kiiru *et al.,* 2011).

The genes encoding these enzymes are normally disseminated via plasmids belonging to incompatibility group F (IncF), that also harbour transposons and Insertion Sequences. Plasmids backbones of many IncF harbour other genes encoding resistance to other classes of antimicrobials such as *bla*$_{OXA-1}$ (encoding resistance to oxacillin), *aac6'-lb-cr* (encoding resistance to ciprofloxacin and gentamicin), *tet*A (encoding resistance to tetracyclines), *bla*$_{TEM-1}$ (encoding resistance to 1st generation cephalosporins), and *cat*B4 (encoding resistance to chloramphenicol), (Smet *et al.,* 2010). IncF plasmids are conjugative plasmids, highly transmissible, most prevalent in *E. coli* and *K. pneumoniae* carrying ESBL genes and also genes that confer resistance to tetracyclines, aminoglycosides and fluoroquinolones (Rafaï *et al.,* 2015). IncF plasmids are considered pandemic, as they have been detected in different countries and in bacteria of different origins and sources (Carattoli, 2009).

The global pandemic *E. coli* ST131 clone is largely responsible for the dissemination of the CTX-M-15 enzymes together with an aminoglycoside-modifying enzyme, *aac(6')-lb-cr*, that has the ability to resist both ciprofloxacin and gentamicin rendering infections related to this clone difficult to treat and manage (Totsika *et al.,* 2011). However, there are some ST131 strains that do not bear *bla*$_{CTX-M-15}$ gene but instead have Quinolone Resistance Determinants (QRDR) genes that include (*qnrA, qnrB, qnrC, qnrD qnrS* and *qepA*) genes (Arias, Seral, Gude, & Castillo, 2010; E. Ruiz, Sa, Arlet, Torres, & Bioquı, 2012). Therefore, resistance to fluoroquinolones is a better marker for ST131 than to ß-lactams and aminoglycosides (Marie-Hélène Nicolas-Chanoine, Bertrand, & Madec, 2014).

Little data exist on the presence of ST131 strains in Africa and especially those exhibiting co-resistance to ß-lactams, fluoroquinolones and aminoglycosides. In recent study conducted in South Africa, 45% of ESBL isolates were ST131 and carried either $bla_{CTX-M-14}$ or $bla_{CTX-M-15}$ genes (Peirano *et al.,* 2011). Another study conducted in the Central African Republic, showed that 50% of CTX-M-15-producing *E. coli* were ST131 (Rogers, Sidjabat, & Paterson, 2010). A high percentage of CTX-M-15 enzymes of ST131 (100%) have also been described in a small number of travel-related ESBL-producing *E. coli* infections from Africa (Johann D D Pitout, Campbell, Church, Gregson, & Laupland, 2009). Up to now, little data exists to show what *E. coli* sequence types harbour the $bla_{CTX-M-15}$ in Kenya. However, a recent study using a limited number of human isolates showed that this ST131 may be significantly implicated in the dissemination of these genes among human isolates (Kiiru, Kariuki, Goddeeris, & Butaye, 2012) while another study showed that dogs and their owners may harbour the same $bla_{CTX-M-15}$ carrying strains with their owners (Albrechtova *et al.,* 2012). Spread of ST131 clones with CTX-M-15 enzymes would implicate to limited treatment options and longer hospital days (Emary *et al.,* 2012; Iannotti *et al.,* 2014).

2.8 Multi-locus sequence typing

Genetic fingerprinting is a technique used to determine genetic relatedness of bacterial of the same species from the same source to study diversity and dynamics of microbial communities. This makes it easier to follow successional population changes, which is an important goal in microbial ecology. There are gel-based methods of determining relatedness of bacterial isolates namely; Restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC) and Pulsed Field Gel Electrophoresis (PFGE).

Multi-locus sequence typing (MLST) is a better tool for determination of genetic relatedness based on minute changes in housekeeping genes. MLST has now been developed for more than 48 microbial taxa and the data is available in public

databases (http://pubmlst.org/databases3.shtml). These allows researchers to determine MLST of their study isolates and deposit new profiles in this data base (Pérez-losada, Cabezas, Castro-nallar, & Crandall, 2013).

MLST characterizes relatedness of bacteria by comparing the sequences of selected housekeeping genes (Aanensen & Spratt, 2005; Jolley, Chan, & Maiden, 2004). Highly conserved housekeeping genes are rarely mutated and therefore, single nucleotide polymorphism on such a gene is of immense evolutionary significance. Typing of *E. coli* is widely based on the Achtman system (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/) which targets the alleles of seven housekeeping genes including *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gryB* (DNA gyrase), *icd* (isocitrate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate synthetase) and *recA* (ATP/GTP binding motif) (Jolley *et al.,* 2004; Wirth *et al.,* 2006) as shown in **Table 2-2.**

Table 2-1: Housekeeping gene function in the two MLST schemes for *E. coli*

Warwick database (*E. coli* 1)		Pasteur database (*E. coli* 2)	
Gene	Function	Gene	Function
Adk (adenylate kinase)	Provide ADP required for oxidative and substrate chain phosphorylation	*din*B (DNA polymerase)	Response to DNA damage
*fum*C(fumurate hydratase)	Catalytic activity of Tricarboxylic acid cycle in the fumurate metabolic process	*icd*A (isocitrate dehydrogenase)	TCA cycle
*gyr*B (DNA gyrase)	DNA replication and transcription	*pab*B (p-aminobezoate synthase)	Biosynthesis of folate
icd (isocitrate/isopropylmalate dehydrogenase)	TCA cycle	*pol*B (polymerase polII)	DNA replication
mdh (malate dehydrogenase)	TCA cycle	*put*P (proline permease)	Proline uptake
*pur*A (adenylosuccinate dehydrogenase)	Purine biosynthesis	*typ*A (tryptophan synthase sub-unit A)	Tryptophan biosynthesis
*rec*A (ATP/GTP binding motif)	DNA repair/ Recombination	*typ*B (tryptophan synthase sub-unit B)	Tryptophan biosynthesis
		*uid*A (beta-glucuronidase)	Hydrolysis of β-D-glucoronic acid

The sequence types obtained from MLST could further be grouped into clonal complexes. A clonal complex is a set of sequence types that are believed to be from a common founding genotype. Therefore, the study of sequence types and clonal complexes using MLST is important in characterizing the strains. Particularly, MLST is a gold standard for identification and delineation of the ST131 pandemic clone. There are two main databases for *E. coli*: the Pasteur and the Warwick databases. Up to now, little has been done to determine the MLST types implicated in MDR phenotype in *E. coli* implicated in various infections in Kenya especially those with the CTX-M-15.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study design

This was a hospital-based case-control study. *E. coli* isolates were obtained from two categories of participants; severely malnourished children (Cases) and non-malnourished children, (Controls) **Figure 3-1**.

Figure 3-1: Conceptual diagram of study design
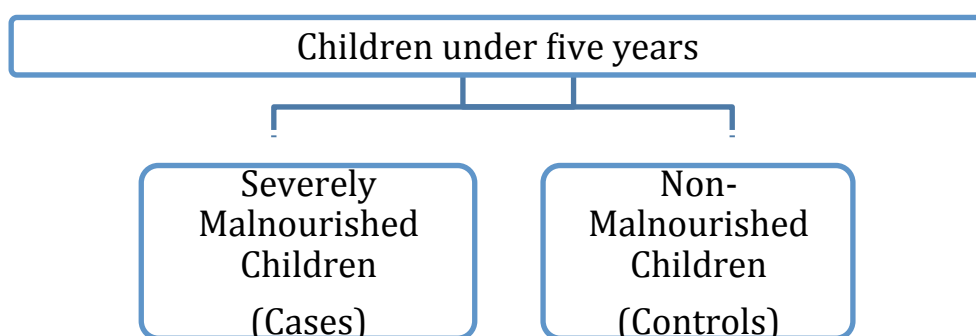
3.2 Study site

The samples were obtained from children seeking treatment at Mbagathi District Hospital. This health facility is situated near Kibera, the largest slum in Kenya that is a home to almost 800, 000 people. The hospital is located 1°18'33"S 36°48'12"E.

The choice of this hospital was based on the findings of a past study that found that in general, children treated here, especially those with diarrhoea, often suffer various degrees of malnourishment (Daboné, Delisle, & Receveur, 2011; Mohiddin, Phelps, & Walters, 2012).

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3.3 Study population

The study population comprised of children below five years, attending Mbagathi District Hospital.

3.3.1 Inclusion criteria for Cases (severely malnourished)

- Children aged below 60 months. Verification for age was done using Child Welfare Clinic records.

- Children with severely malnourishment, i.e. with mid upper arm circumference (MUAC) of less than 11.5cm.

- Children meeting the above criteria whose parents signed an informed consent were included in the study.

3.3.2 Inclusion criteria for Controls (not-malnourished)

- Children below the age of 60 months, with a MUAC of more than 11.5cm

- Children who meet above criteria and whose parents signed an informed consent allowing their children to be recruited

3.3.3 Exclusion criteria

- Children over age of 60 months and those who did not meet other criteria for inclusion.

- Children who meet the criteria but whose parents did not give informed consent to participate in the study.

3.4 Sampling

3.4.1 Sample size determination

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

Where n is the sample size,

z = Z statistic for a level of confidence at 95%. Z value is 1.96

p = 17% is the prevalence of diarrhoea among children under five years as reported in the Kenya Demographic Health Survey, 2010.

d = precision

Adapted from (Daniel, 1999)

$$n = \frac{1.96^2 \times 0.17\ (0.83)}{0.05^2}$$

Minimum sample size was 217 children.

N = 217 children. The number was rounded off to 218 to allow inclusion of an even number of Cases and Controls i.e. 109 severely malnourished children and 109 non-malnourished children.

3.4.2 Sampling strategy

The ages of potential recruits (children aged 2- 60 months, attending Mbagathi District Hospital for management and treatment of enteric infections), were first verified using Child Welfare Clinic records. The children were then anthropometrically graded for malnutrition based on the Mid Upper Arm Circumference (MUAC). Severely malnourishment was defined as three standard deviations from the mean (-3 SD) i.e. <11.0cm for children aged below 6 months and <11.5cm for children aged 7-60 months. Children who had diarrhoea but were not malnourished with a MUAC of greater than 11.5cm were categorised as Controls.

3.5 Specimen handling and processing

3.5.1 Specimen collection

Mothers or Guardians were requested to allow their children to join the study after careful explanation of the study's objectives. The Guardian/Parent filled the consent form, agreeing or declining to participate in the study on behalf of the child, (**Appendix 1-4**). Stool samples (or anal swab samples where stool samples were not available) were obtained from the study participants. Only one sample was collected from each study participant. Stool samples were obtained in sterile, wide-mouthed, screw cap containers.

3.5.2. Swab specimen collection and shipment procedure

Rectal swabs were obtained aseptically when the child was in supine position. Each swab was labelled with a unique participant number before specimen collection. Sterile cotton tipped swab was shallowly inserted into the anal canal (maximum of an inch) before withdrawal. The rectal swab was then returned into its plastic casing with the Amies transport media (Oxoid, Basingstoke, UK). This was meant to sustain microorganisms in a viable but a slow state of growth during transportation to the KEMRI-CMR laboratory. All faecal specimens were transported inside cool boxes at 4^0C.

3.5.3 Initial bacterial identification

At the CMR laboratory, specimens were enriched aerobically at 37^0C for between 18 and 24 hrs in Buffered Peptone Water (Oxoid, Basingstoke, UK). Inocula were then spread on MacConkey agar and Xylose Lysine Deoxycholate (XLD) agar (Oxoid, Basingstoke, UK) plates and incubated overnight at 37^0C. One lactose-fermenting colony (pink on MacConkey and yellow on XLD) were picked from each plate and biochemically typed using Triple Sugar Ion Agar (TSI), Lysine Indole Motility (LIM), Urea and Simmon Citrate agar. All media were obtained from Oxoid, UK (**Plate 3-1).**

Plate 3-1: Biochemical identification of *E. coli*

TSI, LIA, Urea, SIM, and Simmon Citrate agars for the biochemical identification of *E. coli*

Key:

    A. Triple Sugar Iron Agar showing Acid butt and Acid slant with gas production. Sugars fermented are Lactose, Sucrose and Maltose.

    B. Lysine Iron Agar indicating positive reaction for lysine and acid production in the butt and acid production in the slant.

    C. Urea indicating negative reaction

    D. Sulphur Indole Motility (SIM) media indicating negative results for sulphur, Positve for indole demonstrated by the pink ring and positive for motility demonstrated by change in turbidity.

    E. Simmon Citrate media indicative of a negative reaction**.**

3.5.4 General Antimicrobial Susceptibility testing

Antimicrobial susceptibility tests for pure and well-characterised isolates were performed using Kirby–Bauer disc diffusion technique with antibiotic discs (Oxoid, Basingstoke, UK) on Mueller Hinton agar (Oxoid, Basingstoke, UK) using an antimicrobial arrangement pattern illustrated in **Appendix 5**.

The first plate comprised of ampicillin (AMP, 10 µg), Cefpodoxime (CPD, 30 µg), Cefotaxime (CTX, 30 µg) and ceftazidime (CAZ, 30 µg), cefepime (FEP, 30 µg), Cefoxitin (FOX, 30 µg) and AMC containing amoxicillin 20 µg and clavulanic acid 10 µg placed at the centre

The second plate had discs containing gentamicin (CN, 5 µg), streptomycin (S, 25 µg), ciprofloxacin (CIP, 30µg), nalidixic acid (NA, 30 µg), chloramphenicol (C, 30µg), sulfamethoxazole/trimethoprim (SXT 23.75/1.25µg) and tazobactam/Piperacillin (TZP) in potency ratio of 100/10 µg placed at the centre.

E. coli ATCC 25922 was included as a control strain on each test occasion. Susceptibility tests were interpreted using the Clinical and Laboratory Standards Institute (CLSI, 2015) guidelines. Strains with intermediate zone diameters were regarded as resistant as detailed in a past study (Kiiru *et al.,* 2012) **Table 3-1**.

Table 3-1: Categories of antimicrobials used in the study and their respective drug category

Plate 1- ESBL Plate		Plate 2	
Antimicrobial	*Class*	*Antimicrobial*	*Class*
Ampicillin (AMP)	Penicillin	Gentamicin (CN)	Aminoglycosides
Cefpodoxime (CPD)	3rd Generation Cephalosporin	Streptomycin (S)	Aminoglycosides
Cefotaxime (CTX)	3rd Generation Cephalosporin	Ciprofloxacin (CIP)	Fluoroquinolone
Ceftazidime (CAZ)	3rd Generation Cephalosporin	Nalidixic Acid (NA)	Quinolone
Cefepime (FEP)	4th Generation Cephalosporin	Chloramphenicol (C)	Chloramphenicol
Cefoxitin (FOX)	Cephamycin	Sulfamethoxazole/Trimethoprim (SXT)	Folate and dihydofolatebiosynthesis inhibitor
Amoxicillin/Clavulanic acid (AMC)	β-lactam/β-lactamase inhibitor combination	Tazobactam/Piperacillin (TZP)	β-lactam/β-lactamase inhibitor combination

Adapted from Kiiru *et al.,* 2011

3.5.5 Confirmation of ESBLs using disc diffusion test

Phenotypic ESBL detection was performed using disk diffusion test using CLSI 2015 guidelines. Only isolates showing synergy zones between amoxicillin/clavulanic and one or more-third generation cephalosporins were picked as ESBL producers.
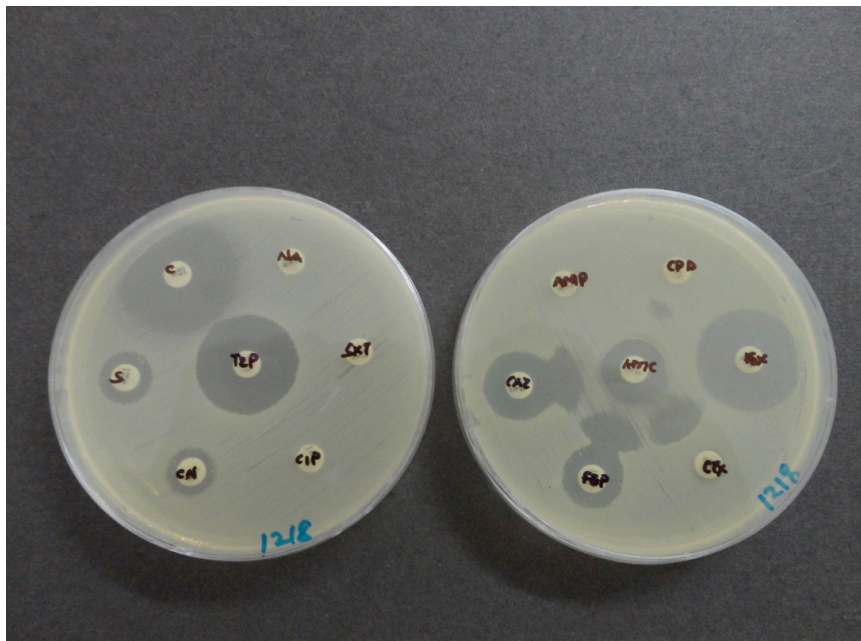
Plate 3-2: Phenotypic detection of ESBL producer in an 'in-house' *E. coli* strain

A disc containing amoxicillin-clavulanate (20+10 µg) [AMC] was placed the center of the plate and, at a distance of 25-30 mm from the center. Around AMC were standard 30 µg discs of ceftazidime, ceftriaxone, cefotaxime, cefepime while cefpodoxime and ampicillin discs had a drug potency of 10µg. Enlargement or distortion of the inhibition zones to form a keyhole appearance/ghost inhibition zone between the discs of cephalosporins and the amoxicillin/clavulanate disc was interpreted to indicate production of an ESBL enzyme, as illustrated in **Plate 3-2**. Isolates were then categorized either as Non-ESBL or ESBL based on their antibiograms. Isolates of *E. coli* resistant to ceftazidime, cefpodoxime, cefotaxime

were considered presumptive extended-spectrum-lactamase (ESBL) producing phenotypes if they showed synergy to clavulanic acid. Susceptibility profiles for all isolates were determined using the Laboratory Standards Institute guidelines (CLSI, 2013).

3.6 DNA extraction

DNA extraction was done using the boiling method in sterile distilled water. In brief, a pea-sized inoculum of pure colonies of the isolates was added to a tube containing 1ml of sterile distilled water and lysis done by incubation at 95^0C for 10 min. The lysates were centrifuged on a table-top centrifuge for 10 min at 14000 rpm. The supernatant was used as the template in PCR analysis.

3.6.1 Detection of *bla$_{TEM}$*, *bla$_{SHV}$*, *bla$_{CTX-M}$* and *bla$_{CMY}$* genes by PCR

Isolates that demonstrated the ESBL-phenotypes were screened for *bla$_{TEM}$*, *bla$_{SHV}$*, and *bla$_{CTX-M}$* genes. The presence of the β-lactamase genes was screened by PCR using primers listed in **Appendix 6.** *bla$_{TEM}$*, *bla$_{SHV}$* and *bla$_{CTX-M}$* genes were detected using a PCR procedure previously described by Kiiru *et al.,*(2012). Briefly, the gene of interest was amplified in a total reaction volume of 25μl containing 10pmol each of primer, 20mM of each dNTPs, 10mM Tris-HCl (pH 8.8), 25mM MgCl$_2$, and 1.25U Taq DNA Polymerase (ThermoFischer Scientific, Rockford, IL, USA). At least 2-5μL of template DNA was added to 23 μL of master mixture. The reaction mixture was placed in MJ-mini Bio-Rad thermal cycler (Bio-Rad, USA). The PCR amplification cycle was performed with cycling conditions consisting of an initial denaturation step at 95^0C for 5 min, followed by 35 cycles of 94^0C for 30 sec, annealing temperatures were selected based on the gene of interest for 1 min (55^0C for *bla$_{TEM}$* or 50^0C for *bla$_{SHV}$* and 60^0C for *bla$_{CTX-M}$*) and an extension temperature of 72^0C for 1min 30 sec. The final extension step was set at 72^0C for 10min. The presence and sizes of amplicons were determined on 1.2 % agarose gels (Invitrogen Life Technologies, Paisley, UK), using GeneRuler 100 bp DNA Ladder Plus (Fermentas Sweden, Helsingborg, Sweden) as a size marker (Hansen *et al.,*

2012).After electrophoresis DNA fragments was visualized by Bio-Rad Gel documentation system (Bio-Rad, USA).

3.7 Whole genome sequencing

Due to their clinical and epidemiologic importance, all isolates carrying the CTX-M enzymes by PCR were selected for full genome sequencingusing illumina next generation sequencing strategies. *E.coli* K12 was used as a positive control and data quality control was done by post-trimming quality plots.

3.8 Multi-locus sequence types of *bla*$_{CTX-M}$ isolates

Genetic relatedness of CTX-M positive isolates was determined based on the MLST technique, using *adk*, *fim*C, *gry*B, *icd*, *mdh*, *pur*A and *rec*A housekeeping genes of the Warwick system (Wirth *et al.,* 2006). After sequencing the PCR products, Velvet files were created in Fasta format. Different contigs were combined from Kmer files and bioinformatics commands used to analyse for MLST. The command line used was "`bsub.py 1 mlst 'get_sequence_type –s "Escherichia coli 1" –c –y *.fa`"

The results were then relayed in comma separated file format (csv) compatible with Microsoft Excel.

3.9 Construction of Phylogenetic tree

A bioinformatics script was used to interrogate for MLST types from whole genome data of each isolate that was sequenced and a phylogenetic tree generated in a "phylip" format constructed using the "neighbour-joining method". The Tree was generated using the Kimura 2-parameter substitution model and branches were evaluated using the bootstrapping method with 1000 replications. Branch values below 70% were regarded as non-significant. Phylogenetic analyses were performed using the Mega6 software (Tamura *et al.,* 2011).

3.10 Data management

Data collection included compiling records of children data and recording of Inhibition zones against various antimicrobials and interpretation of such zones to Sensitive, Intermediate or Resistant categories. These data was recorded in Microsoft excel. Data was kept confidential by use of unique identifiers instead of patient names in a password-protected database.

3.11 Data Analysis

Descriptive statistics were used for computing frequencies and proportions of phenotypic resistance, and susceptibility to various antimicrobials and for the presence of resistance genes among isolates from different categories of participants. Susceptibility data was analysed using WHONET 5.6 software and further processed on Microsoft Excel. Differences in ESBL carriage and differences in resistance between Malnourished and Non-Malnourished strains were determined using 2-tailed Fisher's exact test or the Chi-square tests (X^2) with GraphPad Prism$^©$ software. Tests values were set at 95% confidence intervals (CI) and a p value of greater than 0.05 was not considered significant.

3.12 Ethical considerations

Ethical consideration was sought from The Kenya Medical Research Institute (KEMRI) Ethical Review Committee (SCC No 2382). Informed consent was obtained from the parent or guardian and information kept confidential (**Appendix 7 and 8**).

3.13 Dissemination of study findings

Study findings were communicated to clinicians at Mbagathi District Hospital to support diagnosis and prescription of the study participants. Study findings were also presented in The Mount Kenya University Scientific conference that occurred in 18th August 2013 at Safari Park Hotel. Selected data were also published in *The Chronicles of Young Scientist Journal* (Njoroge, Kiiru, & Kikuvi, 2014) **Appendix 9**.

CHAPTER FOUR

4.0 RESULTS

A total of 218 non-duplicate *E. coli* comprising 109 isolates from severely malnourished children (Cases) and 109 isolates from the non-malnourished children (Controls). Both groups had similar gender distribution patterns with the number of females slightly higher than that of males **(Figure 4-1).**
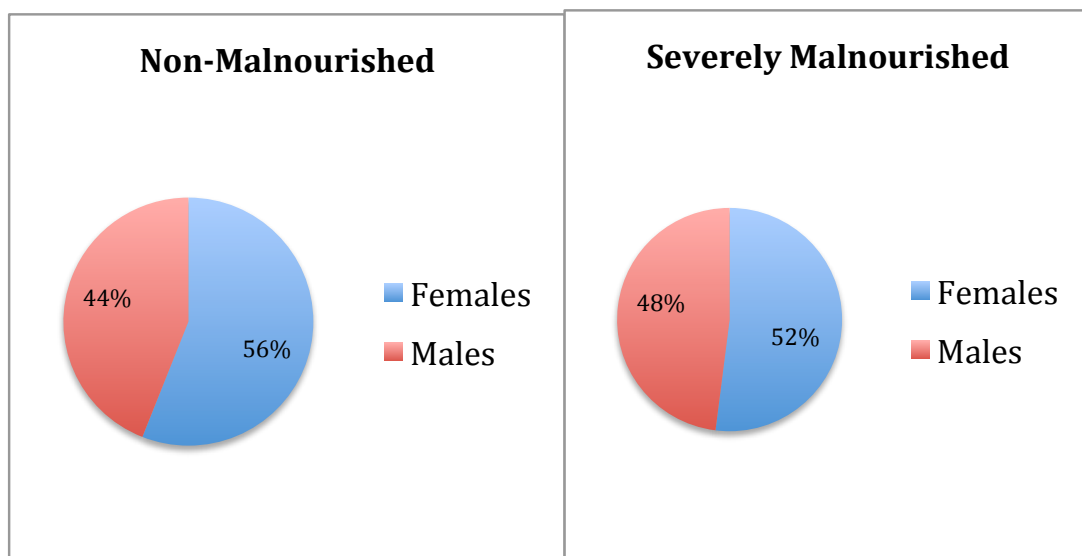
Figure 4-1: Gender distribution patterns of non-malnourished and severely malnourished children attending Mbagathi District Hospital

4.1 Antimicrobial susceptibility patterns

Low resistance levels were observed for piperacillin-tazobactam (TZP) at 6% isolates from Controls and 2% among those from Cases, **Figure 4-2**. Similar low resistances of between 14-18% were also observed among isolates from both patient categories against cefoxitin (FOX). In contrast, high resistance for ampicillin (AMP) was also observed in isolates from Cases (68%) and among Controls (63%). Similar high resistances were observed for sulfamethoxazole/trimethoprim (SXT) among isolates from Cases (63%) and Controls (65%). Resistance to; amoxicillin-

clavulanic acid (AMC), cefpodoxime (CPD), cefoxitin (CTX), cefepime (FEP), ceftazidime (CAZ), ciprofloxacin (CIP), nalidixic acid (NA), streptomycin (S) and gentamicin (CN) was significantly higher among isolates from Cases (above 25%) than those from Controls (below 6%). Inhibitions zones for all the *E. coli* isolates obtained from this study alongside their interpretations are detailed in **Appendix 10** for Controls and **Appendix 11** for Cases.
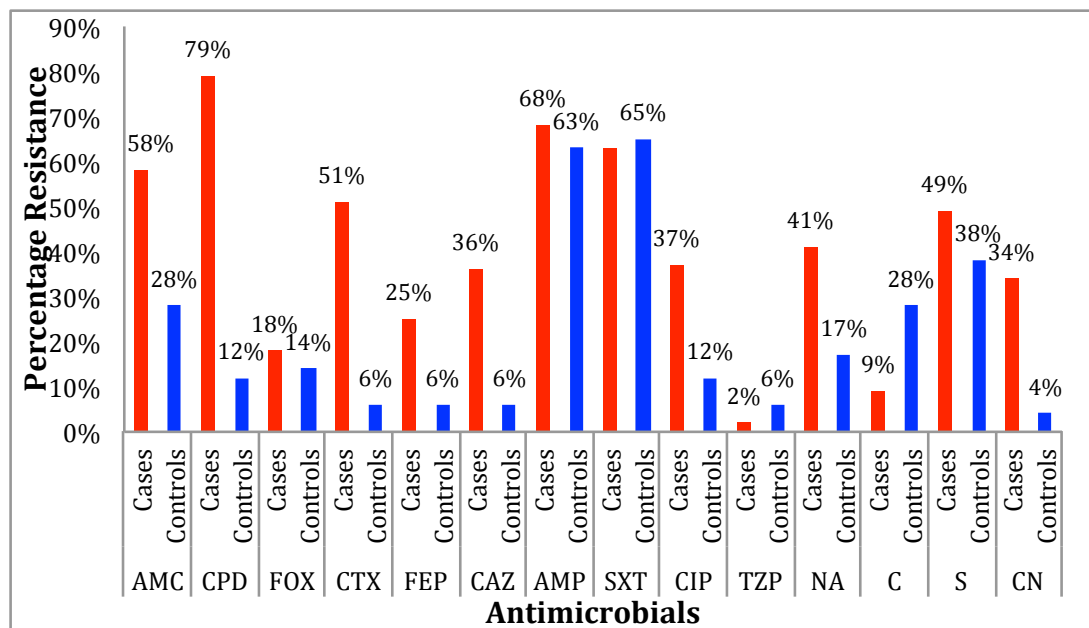
Figure 4-2: Percentage resistances of isolates from non-malnourished and severely malnourished children attending Mbagathi District Hospital

Key: Cases-Malnourished, Controls-Non-Malnourished, AMC:amoxicillin-clavulanic acid, CPD:cefpodoxime, FOX:cefoxitin, CTX:cefotaxime, FEP:cefepime, CAZ:ceftazidime, AMP:ampicillin, SXT:sulfamethoxazole-trimethoprim, CIP:ciprofloxacin, TZP:tazobactam-piperacillin, NA:nalidixic acid, C:chloramphenicol, S:streptomycin, CN:gentamicin

In general, resistances to most classes of antimicrobials were significantly higher among isolates from Cases than those from Controls but no statistically significant differences were observed in these two categories for cefoxitin (p-0.4609, OR-1.408, CI-0.6788-2.921), ampicillin (p-0.5686, OR-1.226, CI-0.7062-2.146),

sulfomethoxazole/trimethoprim (*p*- 0.8877, O.R- 0.9232, C,I -0.5305-1.607) and
tazobactam/piperacillin (*p*- 0.1706, O.R- 0.3178, C.I -0.6266-1.611), **See Table 4-1**.

Table 4-1: Comparison of antibiograms for all *E. coli* isolates from non-malnourished and severely malnourished children attending Mbagathi District Hospital

Drug Name	Patient Category	Resistant	Susceptible	*P*. Value	O.R	C.I
AMC	Cases (n=106)	62	44	<0.0001*	3.71	2.10 - 6.60
	Controls (n=109)	30	79			
CPD	Cases (n=109)	48	61	<0.0001*	5.81	2.91 - 11.61
	Controls (n=108)	13	96			
FOX	Cases (n=109)	20	89	0.4609	1.41	0.68 - 2.92
	Controls (n=109)	15	94			
CTX	Cases (n=109)	56	53	<0.0001*	15.4	6.56 - 36.14
	Controls (n=109)	7	102			
FEP	Cases (n=109)	27	82	<0.0001*	5.6	2.21 - 14.21
	Controls (n=108)	6	102			
CAZ	Cases (n=106)	38	68	<0.0001*	9.59	3.85 - 23.93
	Controls (n=109)	6	103			
AMP	Cases (n=109)	74	35	0.5686	1.23	0.71 - 2.15
	Controls (n=109)	69	40			
SXT	Cases (n=109)	69	40	0.8877	0.92	0.53 - 1.61
	Controls (n=109)	71	38			
CIP	Cases (n=109)	40	69	<0.0001*	4.69	2.29 - 9.58
	Controls (n=109)	12	97			
TZP	Cases (n=109)	2	107	0.1706	0.32	0.06 - 1.61
	Controls (n=108)	6	102			
NA	Cases (n=109)	45	64	<0.0001*	3.56	1.89 - 6.70
	Controls (n=109)	18	91			
C	Cases (n=108)	10	98	0.0004*	0.26	0.12 - 0.56
	Controls (n=109)	31	78			
S	Cases (n=109)	53	46	0.0263	1.88	1.08 - 3.28
	Controls (n=108)	41	67			
CN	Cases (n=109)	37	72	<0.0001*	13.23	4.52-38.77
	Controls (n=107)	4	103			

The table represents statistical analyses of the resistances profiles between the two categories of isolates

Key: AMC:amoxicillin-clavulanic acid, CPD:cefpodoxime, FOX:cefoxitin, CTX:cefotaxime, FEP:cefepime, CAZ:ceftazidime, AMP:ampicillin, SXT:sulfamethoxazole-trimethoprim, CIP:ciprofloxacin, TZP:tazobactam-piperacillin, NA:nalidixic acid, C:chloramphenicol, S:streptomycin, CN:gentamicin.
*- statistically significant,
CI- 95% confidence interval,

O.R: odds ratio.

Higher resistances to all classes of antimicrobials (above 53%) were observed in ESBL than non-ESBL isolates recovered from malnourished children (Cases) **Figure 4-3**.
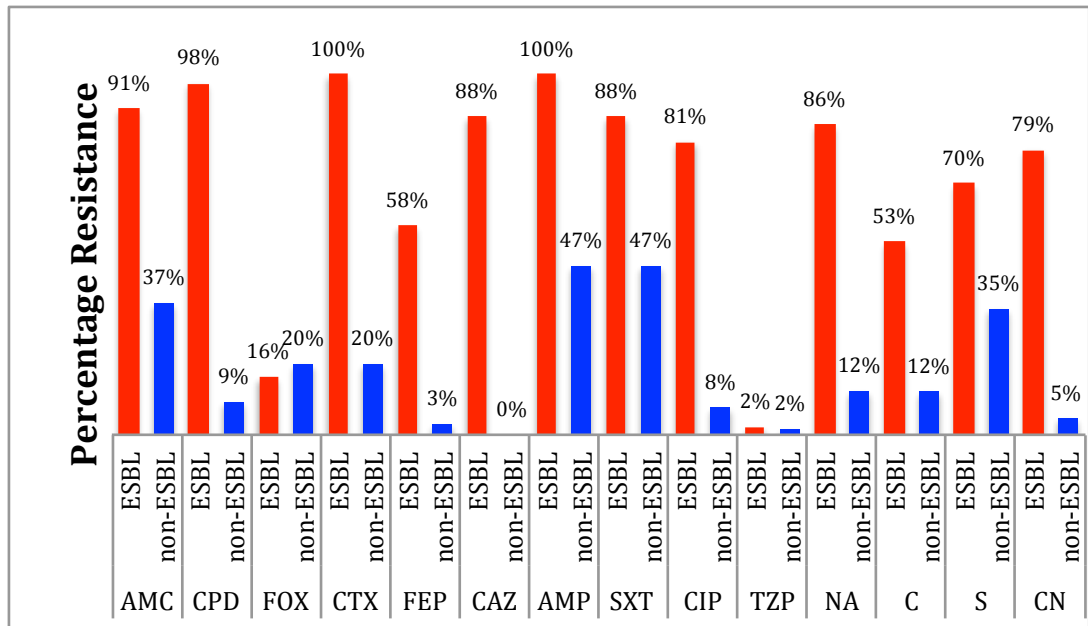
Figure 4-3: Resistance patterns in ESBL and non-ESBL isolates from Severely Malnourished Children

Key: AMC:amoxicillin-clavulanic acid, CPD:cefpodoxime, FOX:cefoxitin, CTX:cefotaxime, FEP:cefepime, CAZ:ceftazidime, AMP:ampicillin, SXT:sulfamethoxazole-trimethoprim, CIP:ciprofloxacin, TZP:tazobactam-piperacillin, NA:nalidixic acid, C:chloramphenicol, S:streptomycin, CN:gentamicin

Similarly, ESBL isolates from the control group recorded higher resistance values (above 25%) against all antimicrobials compared to non-ESBL strains isolated from the same group, **Figure 4-4**.
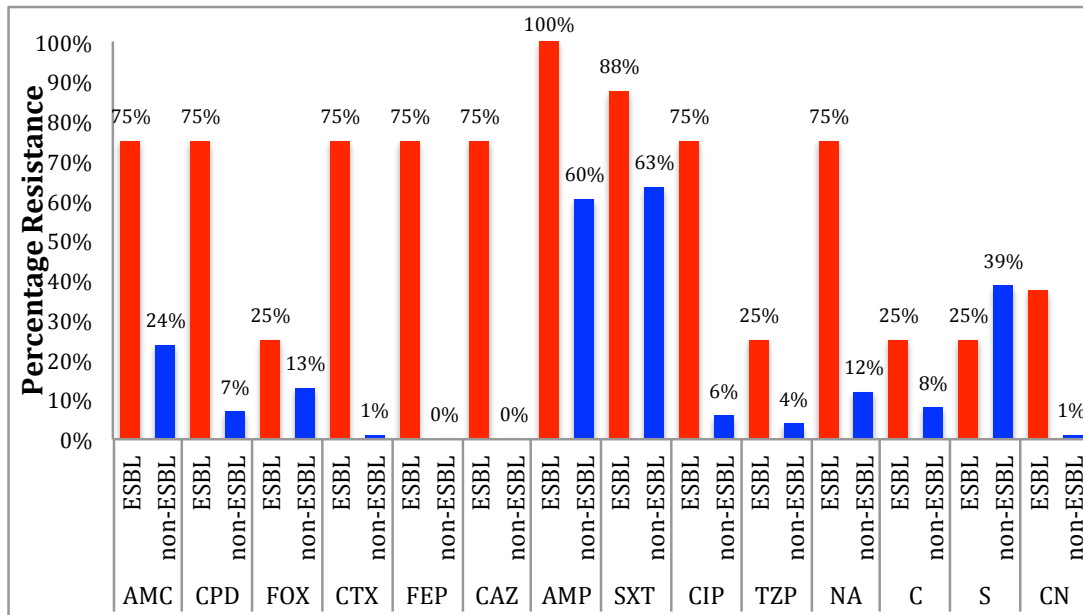
Figure 4-4: Resistance patterns in ESBL and non-ESBL isolates from non-Malnourished children

Carriage of ESBL-producers was also more common among strains obtained from children with malnutrition (39%) than from non-malnourished children (7%) (*P*<0.0001, OR 8.2254, CI: 3.637 − 18.60). ESBL-producers were also more likely to exhibit resistance to other classes of antimicrobials than non-ESBL strains. ESBL isolates from malnourished children were in particular more resistant to cefotaxime (p=0.02, OR=33.46, CI=1.44-778), to streptomycin (p=0.04, OR=6.92, CI=1.23-39) and towards gentamicin (p=0.03, OR=6.30, CI=1.26-31.5) than those from control group. There was however no significant differences in antimicrobial resistance profiles among isolates obtained from Cases and Controls to the rest of the antimicrobials (**Table 4-2**).

Table 4-2: ESBL isolates obtained from malnourished children compared to those from non-malnourished children attending Mbagathi District Hospital

Drug Name	Patient Category (n)	Resistant	Susceptible	P. Value	O.R	C.I
AMC	Cases (43)	39	4	0.23	3.25	0.49 to 21.80
	Controls (8)	6	2			
CPD	Cases (43)	42	1	0.06	14	1.10 to 179
	Controls (n=8)	6	2			
FOX	Cases (n=43)	7	36	0.62	0.58	0.10 to 3.51
	Controls (n=8)	2	6			
CTX	Cases (n=43)	43	0	0.02*	33.46	1.44 to 778
	Controls (n=8)	6	2			
FEP	Cases (n=43)	25	18	0.46	0.46	0.08 to 2.56
	Controls (n=8)	6	2			
CAZ	Cases (n=43)	38	5	0.3	2.53	0.40 to 16.1
	Controls (n=8)	6	2			
AMP	Cases (n=43)	43	0	-	-	-
	Controls (n=8)	8	0			
SXT	Cases (n=42)	38	4	1	1.35	0.13 to 14.0
	Controls (n=8)	7	1			
CIP	Cases (n=42)	35	7	0.62	1.67	0.27 to 10.0
	Controls (n=8)	6	2			
TZP	Cases (n=43)	1	42	0.06	0.07	0.01 to 0.91
	Controls (n=8)	2	6			
NA	Cases (n=43)	37	6	0.6	2.05	0.33 to 12. 7
	Controls (n=8)	6	2			
C	Cases (n=43)	23	20	0.24	3.45	0.62 to 19. 1
	Controls (n=8)	2	6			
S	Cases (n=43)	30	13	0.04*	6.92	1.23 to 39.0
	Controls (n=8)	2	6			
CN	Cases (n=43)	34	9	0.03*	6.3	1.26 to 31.5
	Controls (n=8)	3	5			

*- Statistically significant

Non-ESBL isolates from malnourished children were also more likely to exhibit higher resistances compared to non-ESBL strains obtained from Controls. In this regard, non-ESBL strains from the Cases were more likely to be resistant to cefotaxime compared to those from Controls (p=<0.0001, OR=24.53, CI=3.121-192.7), and to sulfamethoxazole-trimethoprim (p=<0.04, OR=0.5121, CI=0.2725-0.9621), **Table 4-3**.

Table 4-3: Non-ESBL isolates obtained from malnourished children compared to those from non-malnourished children attending Mbagathi District Hospital

Drug Name	Patient Category (n)	Resistant	Susceptible	P. Value	O.R	C.I
AMC	Cases (n=66)	16	50	0.13	0.55	0.28 to 1.11
	Controls (n=101)	37	64			
CPD	Cases (n=66)	6	60	0.77	1.34	0.43 to 4.20
	Controls (n=101)	7	94			
FOX	Cases (n=66)	13	53	0.28	1.66	0.72 to 3.85
	Controls (n=101)	13	88			
CTX	Cases (n=66)	13	53	0.000*	24.53	3.12 to 192
	Controls (n=101)	1	100			
FEP	Cases (n=66)	2	64	0.15	7.87	0.37-166
	Controls (n=101)	0	101			
CAZ	Cases (n=66)	0	66	-	-	-
	Controls (n=101)	0	101			
AMP	Cases (n=66)	31	35	0.06	0.53	0.28 to 1.00
	Controls (n=101)	62	39			
SXT	Cases (n=66)	31	35	0.04*	0.51	0.27 to 0.96
	Controls (n=101)	64	37			
CIP	Cases (n=66)	5	61	0.75	1.3	0.40 to 4.44
	Controls (n=101)	6	95			
TZP	Cases (n=66)	1	65	0.65	0.37	0.04 to 3.42
	Controls (n=101)	4	97			
NA	Cases (n=66)	8	58	1	1.02	0.39 to 2. 65
	Controls (n=101)	12	89			
C	Cases (n=66)	8	58	0.42	1.6	0.57 to 4. 51
	Controls (n=101)	8	93			
S	Cases (n=66)	23	43	0.74	0.85	0.45 to 1.62
	Controls (n=101)	39	62			
CN	Cases (n=66)	3	63	0.3	4.76	0.48 to 46.8
	Controls (n=101)	1	100			

*- Statistically significant

4.2 PCR results for selected *bla* genes

Isolates exhibiting resistance to one or more third generation cephalosporin were screened for carriage of various *bla* genes using PCR methods. In total, 43 isolates from severely malnourished children and 8 from the non-malnourished group were selected for further analysis. The results of a few representative isolates are shown in **Plate 4-1.**
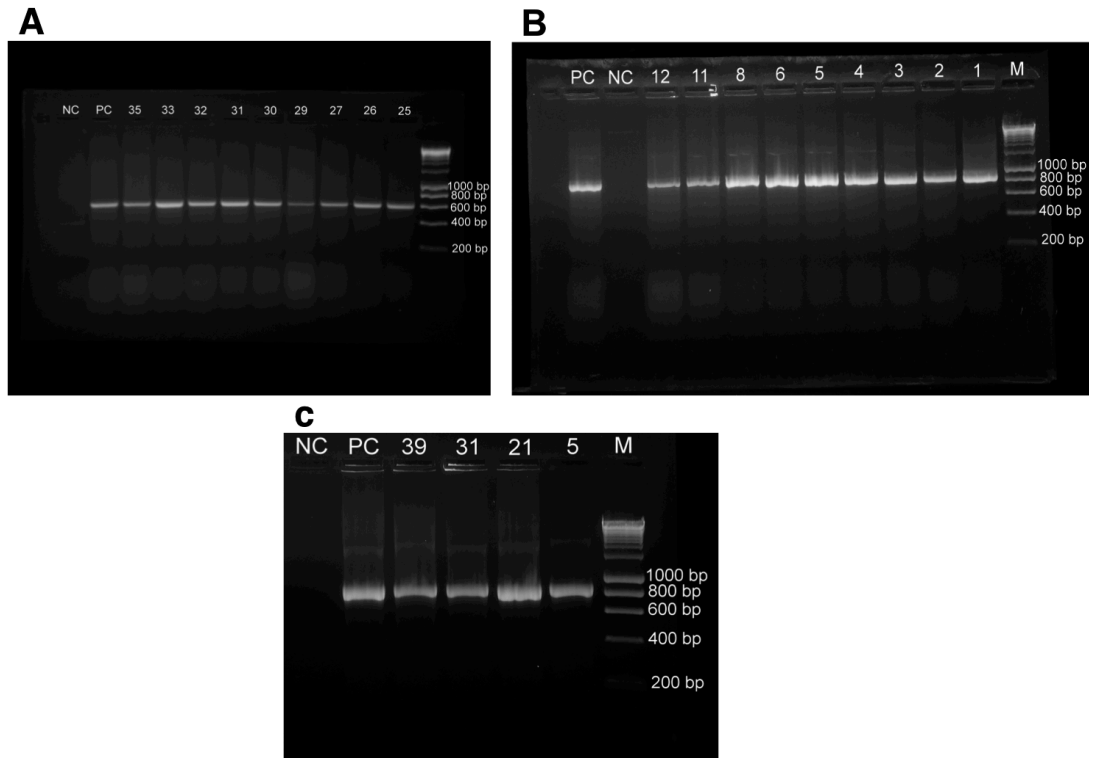
Plate 4-1: Electrophoresis gel results for selected genes in this study

Key

A: *bla*CTX-M (593 bp)

B: *bla*SHV gene (845 bp)

C: *bla*TEM gene (840 bp)

NC- negative control

PC- positive control

M- Molecular size marker/DNA ladder

Numbers at the top repesent random DNA numbers of isolates

*bla*TEM gene was the most prevalent (86%) among Cases and Controls 7(88%). *bla*CTX-M was present in 25 isolates from Cases while six isolates from Controls

carried this gene. The prevalence of bla_{SHV} was at 19% among Cases and 13% in Controls. **Table 4-4**.

Table 4-4: Distribution of various *bla* genes among isolates from non-malnourished and severely malnourished children attending Mbagathi District Hospital

bla gene	Patient Category	Positive	Negative	*P*. value	O.R	C.I
TEM	Cases	37	6	1	0.88	0.09 – 8.50
	Controls	7	1			
SHV	Cases	8	35	1	0.625	0.67 – 5.82
	Controls	1	7			
CTX-M	Cases	25	18	0.45	2.16	0.39 – 11.96
	Controls	6	2			

In this study, co-existence of multiple *bla* genes in the same isolate was observed in a number of isolates. A combination of bla_{TEM}+bla_{CTX-M} was found to be the most common among Controls (62%) than in Cases (19%), **Table 4-5.**

Table 4-5: Co-carriage of *bla* resistance genes among non-malnourished and severely malnourished children attending Mbagathi District Hospital

	CTX-M+ TEM+ SHV	SHV only	CTX-M only	TEM + SHV	TEM only	TEM +CTX-M
Cases (n=43)	0	4 (9%)	17 (40%)	4 (9%)	10 (23%)	8 (19%)
Controls (n=8)	0	1 (12%)	1 (12%)	0	1 (12%)	5 (62%)
Total (n=51)	0	5 (10%)	18 (35%)	4 (8%)	11 (41%)	13 (25%)

4.3 Diversity of CTX-Ms among ESBL producers

Of the 51 isolates that exhibited an ESBL phenotype, 43 were from Cases and 8 were from Controls. Twenty-five (49%) of these isolates from Cases and 6 (75%) from Controls were positive for the bla_{CTX-M} gene. Based on sequencing data, 30 (99%) of the 31 isolates that bore this gene carried the $bla_{CTX-M-15}$ while a single isolate

harbored carried a gene that had a 99% identity to the poorly described bla_{KLUA-9} and $bla_{CTX-M-20}$ (GENBANK accession numbers CAJ31225 and CAC95175) and shared 78% nucleotide identity to $bla_{CTX-M-15}$. Additionally, $bla_{KLUA-9-like}$ has three amino-acid substitutions at position 225, 270 and 290 from bla_{KLUA-9} CAJ31225 see **Plate 4-2 and Table 4-6**.

```
sample_0965_tag16    MMTQSIRRSMLTVMATLPLLFSSATLHAQANSVQQQLEALEKSSGGRLGVALINTADNSQ 60
blaCTX-M-20          MMTQSIRRSMLTVMATLPLLFSSATLHAQANSVQQQLEALEKSSGGRLGVALINTADNSQ 60
blaKLUA-9            MMTQSIRRSMLTVMATLPLLFSSATLHAQANSVQQQLEALEKSSGGRLGVALINTADNSQ 60
                     ***********************************************************

sample_0965_tag16    ILYRADERFAMCSTSKVMAAAAVLKQSESDKHLLNQRVEIKKSDLVNYNPIAEKHVNGTM 120
blaCTX-M-20          ILYRADERFAMCSTSKVMAAAAVLKQSESDKHLLNQRVEIKKSDLVNYNPIAEKHVNGTM 120
blaKLUA-9            ILYRADERFAMCSTSKVMAAAAVLKQSESDKHLLNQRVEIKKSDLVNYNPIAEKHVNGTM 120
                     ***********************************************************

sample_0965_tag16    TLAELGAAALQYSDNTAMNKLIAHLGGPDKVTAFARSLGDETFRLDRTEPTLNTAIPGDP 180
blaCTX-M-20          TLAELGAAALQYSDNTAMNKLIAHLGGPDKVTAFARSLGDETFRLDRTEPTLNTAIPGDP 180
blaKLUA-9            TLAELGAAALQYSDNTAMNKLIAHLGGPDKVTAFARSLGDETFRLDRTEPTLNTAIPGDP 180
                     ***********************************************************

sample_0965_tag16    RDTTTPLAMAQTLKNLTLGKALAETQRAQLVTWLKGNTTGSASIRAGLPKSWVVGDKTGS 240
blaCTX-M-20          RDTTTPLAMAQTLKNLTLGKALAETQRAQLVTWLKGNTTGSASIRAGLPKSWVVGDKTGS 240
blaKLUA-9            RDTTTPLAMAQTLKNLTLGKALAETQRAQLVTWLKGNTTGSASIQAGLPKSWVVGDKTGS 240
                     ********************************************:***************

sample_0965_tag16    GDYGTTNDIAIIWPENHAPLVLVTYFTQPEQKAESRRDVLAAAAKIVTHSF 291
blaCTX-M-20          GDYGTTNDIAVIWPENHAPLVLVTYFTQPEQKAESRRDFLAAAAKIVTHGF 291
blaKLUA-9            GDYGTTNDIAIIWPENHAPLVLVTYFTQPDQKAESRRDVLAAAAKIVTHGF 291
                     **********:*******************:*******.*********.*
```

Plate 4-2: Clustawl alignment of $bla_{KLU-LIKE}$ gene from this study as sample_0965_tag16

Key: Asterisks denote amino acid similarity while colons and periods represent amino acid substitutions; numbers at the end are amino acid positons.

Table 4-6: Amino-acid mutations in $bla_{KLU-9-LIKE}$ gene obtained f rom plate 4-2

Amino-acid mutation location (1-291)	$bla_{KLUA-9-like}$ This study	bla_{KLUA-9} CAJ31225	$bla_{CTX-M-20}$ CAC95175
225	R	Q	R
251	I	I	V
270	E	D	E
279	V	V	F
290	S	G	G

R-arginine, Q-glutamine, I-isoleucine, V-valine, E-glu tamic acid, D-aspartic acid, F-phenylalanine, S-serine, G-glycine, all representing non-synonymous substitutions from their respective nucleotide sequences.

4.4 Combination of *bla* genes among isolates exhibiting resistance to cephalosporins

$bla_{CTX-M-15}$ gene occurred in combination with bla_{TEM-1} and bla_{OXA-1} in 10 (32%) of the isolates. Other *bla* genes found in combination with this gene included bla_{TEM-33} (3%) and $bla_{TEM-158}$ (3%), **Table 4-7**.

Table 4-7: Combination of *bla* genes present

Susceptibility profile	*bla* genes combinations	Isolates n=31
AMC, CPD, CTX, CAZ, AMP, SXT, CIP, NA, C, S, CN	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{OXA-1}	10 (32%)
AMC, CPD, CTX, CAZ, AMP	bla_{OXA-1}, $bla_{CTX-M-15}$	9 (29%)
AMC, CPD, CTX, FEP, CAZ, AMP, SXT, TZP, C, S, CN	$bla_{CTX-M-15}$	2 (6%)
AMC, CPD, CTX, FEP, CAZ, AMP, SXT, CIP, NA, C, S, CN	$bla_{TEM-158}$, $bla_{CTX-M-15}$	1 (3%)
AMP, SXT, CN	bla_{KLUA-9} like'	1 (3%)
AMC, CPD, CTX, FEP, CAZ, AMP, SXT, CIP, NA, C	bla_{TEM-33}, $bla_{CTX-M-15}$, bla_{OXA-1}	1 (3%)
AMC, CPD, FOX, CTX, FEP, CAZ, AMP, SXT, CIP, TZP, NA, C, S, CN	$bla_{CTX-M-15}$, bla_{OXA-1}, bla_{TEM-1}, bla_{AmpC}	7 (23%)

Key: AMC:amoxicillin-clavulanic acid, CPD:cefpodoxime, FOX:cefoxitin, CTX:cefotaxime, FEP:cefepime, CAZ:ceftazidime, AMP:ampicillin, SXT:sulfamethoxazole-trimethoprim, CIP:ciprofloxacin, TZP:tazobactam-piperacillin, NA:nalidixic acid, C:chloramphenicol, S:streptomycin, CN:gentamicin.

4.5 Multi-locus sequence types

Thirty-one bla_{CTX-M}-carrying strains were investigated for clonal relatedness by interrogating their whole genome data for specific MLST sequences.

Our analysis identified a novel Sequence Type, ST4481 whose sequence data was deposited in the Warwick database

(http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/GetTableInfo_html).

The most distinguishing resistances profiles among ST131 strains were resistant towards cephalosporins, quinolones and aminoglycosides. It was however noted that resistance phenotypes alone were a reliable marker that could be used to infer the ST-type of any of the isolates analyzed.

A detail of MLST types observed in this collection is summarized in **Table 4-8**.

Table 4-8: Resistance phenotypes and their respective ST

Isolate number	Patient Category	Resistance Phenotype	MLST type	adk	fumC	gyrB	Icd	mdh	purA	recA
						Housekeeping genes allele profile				
1204	Case	AMC, CPD, CTX, FEP, CAZ, AMP, SXT, CIP, NA, C, S, CN	38	4	26	2	25	5	5	19
963	Case	AMC, CPD, CTX, FEP, CAZ, AMP, SXT, CIP, NA, C, S	44	10	11	4	8	8	8	7
968	Case	CPD, CTX, CAZ, AMP, SXT, CIP, NA, C, S	44	10	11	4	8	8	8	7
1000	Case	AMC, CPD, CTX, FEP, CAZ, AMP, CIP, NA, S, CN	131	53	40	47	13	36	28	29
1218	Case	AMC, CPD, CTX, FEP, CAZ, AMP, SXT, CIP, NA, S, CN	131	53	40	47	13	36	28	29
1203	Case	AMC, CPD, CTX, FEP, CAZ, AMP, SXT, CIP, NA, C, CN	131	53	40	47	13	36	28	29
965	Case	AMC, CPD, CTX, CAZ, AMP, SXT, NA, C, CN	131	53	40	47	13	36	28	29
1758	Case	AMC, CPD, CTX, FEP, CAZ, AMP, SXT, CIP, NA, C, S, CN	131	53	40	47	13	36	28	29
765	Case	AMC, CPD, CTX, FEP, CAZ, AMP, SXT, TZP, C, S, CN	131	53	40	47	13	36	28	29
999	Case	AMC, CPD, CTX, FEP, CAZ, AMP, CIP, NA, C, S, CN	131	53	40	47	13	36	28	29
1759	Case	AMC, CPD, CTX, FEP, CAZ, AMP, SXT, CIP, NA, S, CN	131	53	40	47	13	36	28	29
883	Case	AMC, CPD, CTX, FEP, CAZ, AMP, SXT, NA, CN	131	53	40	47	13	36	28	29
768	Case	AMC, CPD, CTX, FEP, CAZ, AMP, SXT, CIP, NA, C, S, CN	131	53	40	47	13	36	28	29
C111	Control	AMC, CPD, CTX, FEP, CAZ, AMP, SXT, CIP, NA	131	53	40	47	13	36	28	29
992	Case	AMC, CPD, CTX, CAZ, AMP, SXT, CIP, NA, S, CN	131	53	40	47	13	36	28	29
910	Case	AMC, CPD, CTX, CAZ, AMP, SXT, NA, S	167	10	11	4	8	8	13	2
790	Case	AMC, CPD, FOX, CTX, FEP, CAZ, AMP, SXT, CIP, NA, C, S, CN	205	6	23	15	16	9	8	7
783	Case	AMC, CPD, FOX, CTX, FEP, CAZ, AMP, SXT, CIP, NA, C, S, CN	205	6	23	15	16	9	8	7
C44	Control	AMC, CPD, FOX, CTX, FEP, CAZ, AMP, SXT, CIP, TZP, NA, C, S, CN	405	35	37	29	25	4	5	73
898	Case	AMC, CPD, FOX, CTX, FEP, CAZ, AMP, CIP, NA, S, CN	405	35	37	29	25	4	5	73
C30	Control	AMC, CPD, FOX, CTX, FEP, CAZ, AMP, SXT, CIP, TZP, NA, C, S, CN	405	35	37	29	25	4	5	73
1744	Case	CPD, CTX, AMP, SXT, CIP, NA, CN	617	10	11	4	8	8	13	73
1214	Case	AMC, CPD, FOX, CTX, CAZ, AMP, SXT, CIP, NA, C, S, CN	648	92	4	87	96	70	58	2
C49	Control	AMC, CPD, CTX, FEP, CAZ, AMP, SXT, CIP, NA, C	940	6	6	22	16	11	1	7
716	Case	AMC, CPD, CTX, CAZ, AMP, SXT, CIP, NA, C, S, CN	1642	6	4	5	18	11	8	6
766	Case	AMC, CPD, FOX, CTX, FEP, CAZ, AMP, SXT, CIP, NA, C, S, CN	1675	1	107	7	238	7	3	7
938	Case	AMC, CPD, CTX, CAZ, AMP, SXT, NA, C, S, CN	1722	101	4	97	29	70	158	2
772	Case	AMC, CPD, CTX, FEP, CAZ, AMP, SXT, CN	4481	6	95	4	18	11	26	14

Key

Apart from the sample number and MLST type column, the numbers in this table represent the position of the amino-acid substitution in each house keeping gene sequenced for determination of the MLST. AMP-Ampicillin, CPD -Cefpodoxime , CTX- Cefotaxime, CAZ- Ceftazidime, FEP- Cefepime, FOX- Cefoxitin, AMC-Amoxicillin/Clavulanic acid, CN- Gentamicin, S- Streptomycin, CIP- Ciprofloxacin, NA- Nalidixic Acid, C- Chloramphenicol (C), SXT-Sulfamethoxazole/trimethoprim, TZP- Tazobactam/Piperacillin.

Majority of the isolates analyzed belonged to ST131 (41%), ST405 (10%), ST44 (7%), and ST205 (7%). All other ST-types occurred at a prevalence of 3%. It is worthy to mention that ST940, ST1642, ST1675 are rarely reported from other studies. Majority of the isolates from Controls only fit into few ST-types (ST405, ST940 and ST131) **Table 4.9**.

Table 4-9: Distribution of CTX-M MLST types

MLST type	Frequency	Percentage	Cases	Controls
ST 131 (n=29)	12	41%	11(92%)	1(8%)
ST 405 (n=29)	3	10%	1(33%)	2(67%)
ST 44 (n=29)	2	7%	2(100%)	0
ST 205 (n=29)	2	7%	2(100%)	0
ST 1642 (n=29)	1	3%	1 (100%)	0
ST 1722 (n=29)	1	3%	1 (100%)	0
ST 617 (n=29)	1	3%	1 (100%)	0
ST 4481 (n=29)	1	3%	1 (100%)	0
ST 38 (n=29)	1	3%	1 (100%)	0
ST 1675 (n=29)	1	3%	1 (100%)	0
ST 648 (n=29)	1	3%	1 (100%)	0
ST 167 (n=29)	1	3%	1 (100%)	0
ST 940 (n=29)	1	3%	0	1(100%)

ST405 was isolated more in CTX-M-15 isolates from Controls than from Cases. ST131 was the most prevalent ST type.

4.6 Phylogenetic analysis of isolates carrying CTX-M genes

Concatenated MLST genes were used to generate a phylogenetic tree. ST44 and ST167 are closely related to ST131while ST38 and ST405 group shown to cluster furthest from these ST-types, **Figure 4-5**.
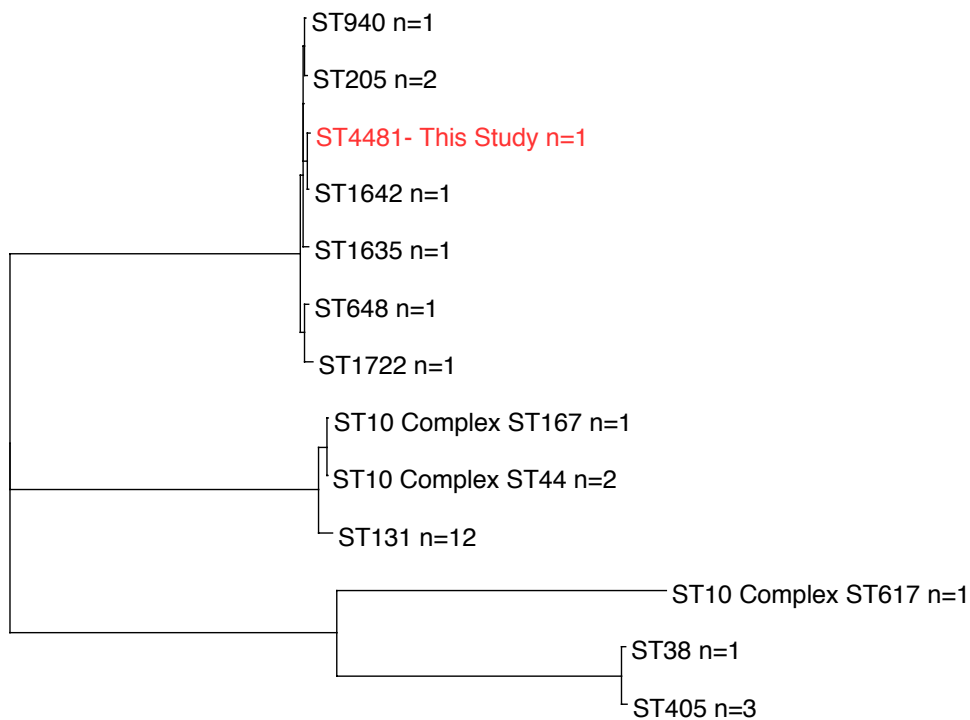
Figure 4-5: Phylogenetic unrooted tree based on MLST genetic polymorphism

Phylogenetic analysis of the CTX-M-15-positive *E. coli* isolates (n=31). Neighbour-Joining tree constructed from the concatenated sequences of the seven MLST genes. Multiple sequence alignments were carried out with SeaView (Gouy, Guindon, & Gascuel, 2010). The phylogenetic tree ware resampled with 1000 bootstrap replications to ensure the robustness of the data. The phylogenetic analyses were displayed with the FigTree program (**http://tree.bio.ed.ac.uk/software/figtree/**).
Tip labels consist of ST complexes and their respective ST from the isolates; the new ST deposited in the Warwick database from this study is labeled in red.

4.7 Carriage of other resistance genes in CTX-M type ESBL isolates

Besides β-lactam resistance, phenotypic resistance to other classes of antibiotics is of great concern. Resistance genes encoding cross-resistance to various antimicrobials were investigated and shown in **Appendix 12**.

CHAPTER FIVE

5.0 DISCUSSION

Although past studies in Kenya have reported on resistance trends among hospitalized and non-hospitalised as well as environmental strains, none of the previous studies had at the time of writing this thesis investigated resistance prevalence among isolates obtained from severely malnourished children. This study compared antimicrobial susceptibility profiles among *E. coli* isolates from malnourished children against those obtained from children who were not malnourished. Girls were more than boys, which is a phenomenon representative of the Kenyan population as reported by the Kenya Demographic Health Survey 2010, (Figure 4-1). High resistances (above 63%) were observed towards ampicillin and sulfamethoxazole/trimethoprim among isolates obtained from both Cases and Controls (Figure 4-2). These results are similar to those published in two related studies conducted in Kenya that found that clinical *E. coli* obtained in Kenya were more resistant to ampicillin (65.9%) and sulfamethoxazole/trimethoprim (68.3%) than isolates from developed countries such as Japan (Bii *et al.,* 2005). Another study found that isolates from food handlers working in nine luxurious tourist hotels in Nairobi were also resistant to ampicillin 46.2% and sulfamethoxazole/trimethroprim 51.3% (Onyango, Mbithi, & Ng'ayo, 2009). These findings, like those from the current study imply that treatment failure may occur if ampicillins and sulfamethoxazole/trimethroprim (co-trimoxazole) are used to manage infections in the absence of culture and susceptibility testing. This is an important finding because β-lactams and co-trimoxazole are an integral part of empiric treatment regimens for different kinds of infections in Kenya (Berkley *et al.,* 2005; Jones & Berkley, 2014). Our study shows that isolates from malnourished children (Cases) are more likely to be MDR than those obtained from the control group (Table 4-1, Figure 4-3, Figure 4-4 and Table 4-2). These findings are similar to a related study done in Niger on severely malnourished children attending a pediatric re-nutrition

center that found that MDR strains are prevalent in this group and that ESBL carriage rate was as high as 31% at admission (Hugede, Janssens, Woerther, & Raskine, 2011)..

Cephalosporins, aminoglycosides and quinolones are often recommended as alternative replacer antimicrobials for management of infections caused by β-lactamase producers. However, this study found that resistances to cephalosporins, quinolones, aminoglycosides and other classes of antimicrobials were higher among isolates obtained from severely malnourished children than those from non-malnourished children (Table 4-1). β-lactamase inhibitors such as clavulanic acid and tazobactam together with penicillins have been recommended as effective agents against ESBL-producers. However, this study found 25% of all ESBL isolates and five percent of non-ESBL isolates from severely malnourished children were resistant to tazobactam as a β-lactamase inhibitor (Figure 4-4). If such isolates are also resistant to cephalosporins, aminoglycosides and quinolones, the only plausible treatment option would be carbapenems. However, the use of carbapenems is not a tenable option in developing countries because they may be expensive to majority of the urban poor.

This study showed that isolates from severely malnourished children were more likely to exhibit co-resistance to multiple classes of antimicrobials such as quinolones and aminoglycosides than those from control strains. In contrast, results from a related study in The Gambia found that *E. coli* obtained from blood and urine specimens from severely malnourished children were significantly susceptible to ciprofloxacin, cefuroxime, cefotaxime and gentamicin (Okomo *et al.,* 2011). The differences in resistances in these two studies may partially indicate a difference in antimicrobial selection pressures in the two countries. Resistance to third-generation cephalosporins would increase the cost of treatment using more expensive antimicrobials. The patients infected with such highly resistant strains are more likely to have recurring infections and may therefore end up spending more days in hospitals. In general, the spread of such highly resistant strains may lead to higher

mortality among patients in developing countries where patients may not afford more effective but fairly expensive antimicrobials.

While it is not clear what factors are driving resistance trends observed locally, such as resistance to sulfamethoxazole/trimethoprim in non-ESBL isolates from non-malnourished children (Table 4-3), various studies have documented antibiotic misuse in Kenya [western Kenya] (Shapiro *et al.,* 2001). In the Shapiro *et al.* study, high resistances were recorded for penicillin, ampicillin, sulfamethoxazole/trimethoprim, tetracycline, nalidixic acid, metronidazole, and gentamicin among isolates obtained from children with gastroenteritis, diarrhea, or dysentery before culture and sensitivity results were done in various clinics in Asembo. The study by Shapiro *et al.* also found that at least 12% of the participant had taken more than one antimicrobial agents before seeking treatment (Shapiro *et al.,* 2001). Over the counter prescription could be one of the factors high numbers of isolates resistant to ampicilin and sulfamethoxazole/trimethoprim were observed in non-malnourished children (Table 4-3).

This study also showed that malnourished children are more likely to harbor ESBL-producers (39%) compared to control children (7%). Our results are close to the findings of a related study conducted in Niger that documented a 31% carriage of EBL-producers among malnourished children (Hugede *et al.,* 2011). A higher proportion of ESBL isolates from severely malnourished children were more likely to be resistant to injectable aminoglycosides such as gentamicin and streptomycin than those from non-malnourished children (Figure 4-3 and Figure 4-4). This means that severely malnourished are more exposed to these injectable antimicrobials than non-malnourished children and indication that these antimicrobials are more likely prescribed by a clinician. A related study has found that use and misuse of third-generation oral-cephalosporin is a risk factor for colonization with ESBL isolates among malnourished children (Birgy *et al.,* 2012). Taken together, these results may point to an emerging challenge of managing enteric infections among malnourished

children that often require chemotherapeutic interventions than non-malnourished children (Galetto-lacour *et al.,* 2013; Iannotti *et al.,* 2014).

Similarly, resistances among non-ESBL isolates from severely malnourished children were also likely to be more resistant to different classes of antimicrobials including fluoroquinolones and aminoglycosides compared with isolates from control children. Similar results (high resistances among non-ESBL isolates to fluoroquinolones, ampicillin, amoxicillin/clavulanic acid, nitrofurantoin and cefoxitin) have been reported in a related study conducted in Kenya on urine specimens (Kariuki *et al.,* 2007). A substantial percentage of non-ESBLs are highly MDR for they possess aminoglycoside modifying enzymes in transmissible plasmids making isolates resistant to fluoroquiolones and aminoglycosides (Robicsek, Jacoby, & Hooper, 2006). Additionally, majority (87%) of *qnr* (quinolones resistance) genes are physically linked to either integron-associated insertion sequences IS*CR*1 or the IS*26* making them easily transmissible among Gram-negative bacteria (Kiiru, Butaye, Goddeeris, & Kariuki, 2013). It is therefore likely that use of effective antimicrobials may only be one strategy of dealing with the spread of these highly MDR strains but infection control and proper hygiene in hospitals and the community remains the most appropriate measures of controlling the spread of these strains.

This study found that majority of ESBL-producers, regardless of the source, were more likely to exhibit co-resistance to multiple antimicrobials such as fluoroquinolones, aminoglycosides sulfamethoxazole/trimethoprim combinations and 3rd generation cephalosporins compared to non-ESBL isolates. These findings are similar to those reported in a study that showed that ESBLs-producers accounted for majority of MDR isolates (95.0%) compared to 53.0% among non-producers among *Klebsiella* strains from Ethiopia (Seid & Asrat, 2005).

The choice of drugs for the treatment of ESBL-producing bacteria is limited to carbapenems, for example imipenem, or, alternatively, fluoroquinolones and aminoglycosides, which may be used if these antibiotics show *in vitro* activity

(Paterson & Bonomo, 2005). However, co-resistance of 3rd generation cephalosporins with aminoglycosides and/or fluoroquinolones among ESBL-producers from this study is worrisome because use of any of these classes of antimicrobials can lead to treatment failure if empirically prescribed for Gram-negative infection causing strains in the absence of culture and susceptibility testing. Therefore, third-generation cephalosporins should be avoided for treatment of serious infections caused by ESBL-producers. In that case, carbapenems, (e.g. imipenem and ertapenem) that are widely recognized as the first-choice drugs against ESBL-producers can be used (Johann D D Pitout & Laupland, 2008). Increased use of carbapenems also may further drive antimicrobial resistance, resulting in even more limitation of therapeutic options unless new antimicrobial agents are developed (Allison & Campos, 2011). Reports of carbapenem resistance in *Enterobacteriacea* from hospital settings in Kenya include; NDM-1 among *Klebsiella pneumoniae* and *Acinetobacter baumannii* and VIM-2 enzymes among *Pseudomonas aeruginosa* (Johann D D Pitout *et al.,* 2008; Poirel, Revathi, Bernabeu, & Nordmann, 2011; Revathi, Siu, Lu, & Huang, 2013).

This study revealed that bla_{TEM} was the most common ß-lactamase among ESBL-producers and the second most common ESBL gene was bla_{CTX-M} (Table 4-4). TEM and CTX-M enzyme combination was the most common among ESBL isolates (Table 4-5). Further analysis showed that $bla_{CTX-M-15}$ was the most common gene among CTX-M positive isolates. These results are similar to those reported in a study conducted in United Arab Emirates that showed that a total of 14 (12.1%) and 29 (24.6%) *Salmonella* isolates were CTX-M-15 ESBL producers and TEM producers, respectively (Rotimi, Jamal, Pal, Sovenned, & Albert, 2008). The most plausible explanation on these findings is that, in most instances, CTX-M-15 enzymes are found alongside TEM-1 enzymes in a common plasmid and dissemination of these plasmids can be passed conjugatively among *Enterobacteriacea.* This therefore makes CTX-M-15 the most common prevalent ESBL enzymes globally. Similar to our findings, many previous studies have found that most isolates bearing $bla_{CTX-M-15}$ are frequently resistant to fluoroquinolones, aminoglycosides and

sulfamethoxazole/trimethoprim among other antimicrobials (Holt *et al.,* 2011; Montesinos, Rodriguez-villalobos, & Bogaerts, 2010; Woodford *et al.,* 2009). Co-resistance to both fluoroquinolones, aminoglycosides is a result of aminoglycoside modifying enzyme carried in the same plasmid with integrons additionally having genes encoding and sulfamethoxazole/trimethoprim resistance and other antimicrobials (Wang *et al.,* 2013).

In the current study, $bla_{CTX-M-15}$+bla_{TEM-1}+bla_{OXA-1} was found to be the most prevalent *bla* combinations (Table 4-7). Carriage of these combinations of *bla* genes partially explains why a significant number of CTX-M-ESBL isolates from this study (83%) were resistant to a wide range of β-lactams. Similar gene combinations have been reported in a related study that found that these three genes are normally borne on a conjugative IncFII plasmids (Carattoli, 2009). The co-production of ESBLs with inhibitor-resistant β-lactamases such as OXA-1 renders these strains resistant to commonly used β-lactamase inhibitors like clavulanic acid (Rogers *et al.,* 2010). Related studies show that $bla_{CTX-M-15}$ is normally borne on IncFII plasmids that also carry other genes such as bla_{OXA-1}, bla_{TEM-1}, *tetA*, and *aac(6')-lb-cr* that confer resistance to ß-lactams ($bla_{TEM-1)}$, inhibitors such as clavulanic acid (bla_{OXA-1}), tetracylines (*tetA*) and (fluoro)quinolones/aminoglycosides (*aac(6')-lb-cr)* (Rafaï *et al.,* 2015). It is also worth noting that $bla_{CTX-M-15}$ confers resistance to majority of ß-lactams including caftazidime (Cantón *et al.,* 2012) and may exist in close proximity to bla_{OXA-1}, bla_{TEM-1} and *aac(6')-lb-cr*. These genes are borne on integrons that are in turn borne on epidemic narrow host-range, self-conjugative IncFII plasmids (Carattoli, 2009). While majority of CTX-M ß-lactamase have a good hydrolytic activity against 3rd generation cephalosporins, their activity towards ceftazidime remains minimal (Cantón & Coque, 2006). It is therefore clear that patients infected with isolates carrying the $bla_{CTX-M-15}$ in combination with other resistance observed in our study may not be treated using majority of cephalosporins, tetracycline, gentamicin and ciprofloxacin and even ß-lactamase inhibitors such as clavulanic acid. Such strains are therefore likely to cause high morbidity and mortality

especially to young children and carbapenems remain the only option to manage infections caused by such strains.

The wide dissemination of $bla_{CTX-M-15}$ across almost all continents is normally attributed to global dissemination of *E. coli* strains belonging to the ST131 clone (Marie-Hélène Nicolas-Chanoine *et al.,* 2014). ST131 strains are so far the most versatile *E. coli* MDR strains capable of mounting resistances to majority of antimicrobials with highly homogeneous virulence genotypes and pathotypes (Marie-Hélène Nicolas-Chanoine *et al.,* 2014). In this study, the carriage of ST131 in CTX-M-ESBL isolates was at a prevalence of (38%) among Cases and at (3%) among Controls. A majority of these isolates carried a combination of $bla_{CTX-M-15}$+bla_{OXA-1}+bla_{TEM-1}+*aac(6')-lb-cr* further indicating that this clone is responsible for a significant proportion of high resistances observed in this study. An increase of prevalence of these ST131 isolates and an increase of infections caused by such strains can lead to an increase in use of carbapenem as an alternative antimicrobial further leading to emergence of even more resistant strains. While no carbapenem-resistant *E. coli* has been reported locally, carbapenem-resistant *Pseudomonas* and *Klebsiella* have been reported from tertiary hospitals in Kenya (Johann D D Pitout *et al.,* 2008; Poirel *et al.,* 2011). Therefore, the possibility of emergency of carbapenem-resistant *E. coli* in Kenya in the near future is high. Such strains will only shrink the already few treatment options even further.

This study also reported $bla_{TEM-158}$ among an ESBL strain from a severely malnourished child. This gene encodes a complex mutant TEM variant (CMT), which degrades most β-lactams including clavulanic acid. This gene has also been described in a recent study in Kenya and Tunisia but is rarely reported in other countries (Alibi, Ferjani, & Boukadida, 2015; Kiiru *et al.,* 2012). Additionally, bla_{TEM-33} encoding an inhibitor resistant TEM (IRT) was also detected in 3% of isolates carrying a bla_{CTX-M} gene. This gene has reduced affinity for β-lactamase inhibitors and confers resistance to clavulanic acid and sulbactam (Morosini, Martin, Maza, Pedrosa, & Cantón, 2008). Previous studies show that like the $bla_{TEM-158}$, this

gene is also borne on incFII plasmids (Smet *et al.,* 2010). Although isolates with a combination of $bla_{TEM33}+bla_{CTX-M-15}$ have been reported in Belgium (Smet *et al.,* 2010), it is important to note that this is the first study in Africa to report carriage of a combination of either bla_{TEM-33} or $bla_{TEM-158}$ with $bla_{CTX-M-15}$ in the same isolate. A possible explanation why our collection of isolates tested positive for rare resistance genes is the over-use and possibly misuse of amoxicillin-clavulanic acid in this country, than in other parts of Africa. The report of carriage of bla_{OXA-1}, $bla_{TEM-158}$ and bla_{TEM-33} that encode resistance to inhibitors could partially explain why resistance to amoxicillin-clavulanic acid was relatively high in this study. Dissemination of these enzymes in *Enterobactreaceae* populations would mean that amoxicilin/clavulanic acid would not be a reliable antimicrobial in the management of infections caused by these strains.

This study also reported occurrence of bla_{KLU-9}-like gene in one isolate (Table 4-6 and Table 4-7). The bla_{KLU-9} gene was first described in clinical *Kluyvera ascorbata* isolate and hydrolyses cefotaxime better than ceftazidime (Rodríguez *et al.,* 2007). I postulate that the mutation from the original bla_{KLU-9} makes this gene unique and has never been described before but it is likely to have similar attributes to bla_{KLU-9} (GENBANK's accession number CAJ31225).

This study found that isolates carrying $bla_{CTXM-15}$ belonged to different ST-types. This study also found that majority (41%) of $bla_{CTXM-15}$ positive isolates belonged to the now pandemic MDR clone ST131 (Table 4-8 and Table 4-9). Similar results have been reported in Nigeria where ST131 were found to account for 35.7 % among CTX-M- producers (Aibinu, Odugbemi, Koenig, & Ghebremedhin, 2012). Existence of ST131 bearing $bla_{CTX-M-15}$ means that Kenya is among the countries experiencing a global epidemic of such strains. In most studies, the ST131 are normally isolated from urine and only a few studies have reported these isolates from fecal samples (Marie-Helene Nicolas-Chanoine *et al.,* 2008). Since majority of our ST131 strains were also resistant to other antimicrobials such as aminoglycosides, fluoroquinolones

and β-lactam/β-lactamase inhibitors, treatment of infections arising from such strains may require use of alternative antimicrobials such as carbapenems.

Phylogenetic clustering of an un-rooted neighbour-joining tree for all CTX-M positive isolates from this study showed relatedness of the strains among each other (Figure 4-5). Although majority of studies implicate dissemination of $bla_{CTX-M-15}$ to ST-131, our study revealed that this gene was also carried among strains belonging to ST405, ST44, ST205, ST1642, ST1722, ST617, ST4481, ST38, ST1675, ST648, ST167 and ST940. While most studies have not found the $bla_{CTX-M-15}$ in most of these clones, this gene has been reported among strains belonging to ST405 in Tanzania, Tunisia, Egypt (Ben *et al.,* 2011; Fam *et al.,* 2011; Mshana *et al.,* 2011). The ST405 has also been reported to carry NDM-4 enzymes in Cameroon (Dortet, Poirel, Anguel, & Nordmann, 2013). ST44 has not yet been documented in Africa before but has been reported in isolates implicated in community-onset infectionsin patients from Columbia (S. J. Ruiz *et al.,* 2011). ST205 has been found in South Africa in fluoroquinolones resistant isolates with CMY-2 enzymes responsible for plasmid mediated cephamycin resistance (Aizawa *et al.,* 2014). The robustness of whole genome sequencing has aided the screening of resistance genes in a greater propensity than any other method and this could be the reason for a varying repertoire of resistance genes in our Kenyan isolates.

Whole genome sequencing revealed that most (41%) of the ST131 isolates from this study harbored $bla_{CTX-M-15}$ conferring resistance to 3rd generation cephalosporins, *tetB* conferring to resistance to tetracycline, *mphA* conferring resistance to macrolides *strB/strA* conferring resistance to streptomycin, *sul*2 conferring resistance to sulphonamide, bla_{TEM-1} conferring resistance to ampicillin, *aac-(6')-1b-cr* conferring resistance to aminoglycoside and ciprofloxacin, bla_{OXA-1} conferring resistance to oxacillin and clavulanic acid and *dfr* genes conferring resistance to trimethoprim resistance. These results clearly identify ST131 strains as an important reservoir for genes conferring resistance to multiple antimicrobials. The $bla_{CTX-M-15}$, *aac-(6')-1b-cr*, $bla_{OXA-1-like}$ gene combinations have been found in ST131 isolates in

Kenya among isolate from dog faecal samples (Albrechtova *et al.,* 2012). In this study the ST205 was shown to harbour a combination of $bla_{CTX-M-15}$, bla_{TEM-1}, *tetB*, *dfrA1*, *ereB*, *catA1*, *strA*, *strB*, s*ul1*, *dfrA17*, *sul2*, *mphA*, *tetC* and *aac(6')-Ib-cr* genes that are responsible for 3rd generation cephalosporins, ampicillin, tetracycline, erythromycin, chloramphenicol, streptomycin, sulphonamide, trimethoprim, macrolide, aminoglycoside and ciprofloxacin resistance. ST205 harboured $bla_{CTX-M-15}$, bla_{OXA-1}, *catB3*, *arr3*, *dfrA27* and/or *aadA16*, *aac(6')-Ib-cr, qepA* and *oqxAB*, genes conferring resistance to β-lactams, chloramphenicol, rifampicin, trimethoprim, streptomycin, gentamicin, and quinolones respectively as reported in a related study conducted in river water and clinical samples in Mexico (E. Ruiz *et al.,* 2012). Interestingly, *qepA* and *oqxAB* genes responsible for high-level quinolone resistance are absent in Kenyan ST205 isolates but present in the Mexican isolates. The ST167 was found to harbour $bla_{CTX-M-15}$, bla_{OXA-1}, *aac(3)-II, aac(6')-lb-cr, strA, strB, sul2* , and bla_{TEM-1} resistance genes as reported in a related study in Tunisia (Ben *et al.,* 2011). ST167 is also among the prevalent ST-complexes in ESBL *E. coli* isolates from Spanish hospital with no description of their resistance genes combination (Oteo *et al.,* 2008). This ST-type was found to be the second most prevalent group (28.6%) clonal group after ST131 in Nigeria and carried a combination of $bla_{CTX-M-15}$, *aac-(6')-lb-cr, qnrA1* resistance genes (Aibinu *et al.,* 2012). The ST648 reported in this study carried *mphA, sul1, dfrA17, strB, strA, catA1, aac-(6')-lb-cr*, bla_{OXA-1} $bla_{CTX-M-15}$, bla_{TEM-1}, *sul2*, *tetB*, and bla_{AmpC} genes encoding resistance to macrolide, sulphonamide, trimethoprim, chloramphenicol, aminoglycoside, ciprofloxacin, cephalosporins, tetracycline and cephamycins. The ST648 has been reported in Tanzania (Mshana *et al.,* 2011) and was found at a prevelence of 14% compared to a 51.4% prevalence of ST131 in all ESBL-producing *E. coli* isolates in Nepal (Sherchan *et al.,* 2015). A ST648 hospital isolate from USA had $bla_{CTX-M-15}$, *aac-(6')-lb-cr* and bla_{OXA-1} but no other resistance genes (Zong & Yu, 2010). It is also worth to mention that ST648 strains isolated in Britain have been shown to carry bla_{NDM-5} that confers resistance to carbapenems (Hornsey, Phee, & Wareham, 2011). Other ST reported in this study included ST1642 that has been reported in Japan

(Harada, Nakai, & Kataoka, 2012). ST1675 and ST940 has been found in wastewater treatment plants at Besancon City in France (Bréchet *et al.,* 2014). The ST38 strains reported in this study have also been isolated from pus swabs in Tanzania with $bla_{CTX-M-15}$+bla_{TEM-1} combinations (Mshana *et al.,* 2011). It is clear that although a high MDR phenotype was particularly associated with ST131 strains, other strains ST-types are implicated in high resistances and their emergence and spread should be monitored.

CTX-M type ESBL isolates from severely malnourished children had varying ST's (ST131, ST44, ST1642, ST1722, ST617, ST4481, ST205, ST38, ST1675, ST648 and ST167) compared to non-malnourished children whose ST clones were ST940, ST405 and ST131. The newly submitted ST4481 from our study has not been associated with drug-resistance in any part of the world as before this study. This therefore is the first report of ST940, ST1642, ST1675 (in Africa) and ST4481 (in the world) strains associated with CTX-M-15 enzymes. Since most of ESBL strains belonging to any of these ST-types were highly multidrug resistant, only a few treatment options remain for management of infections among patients, especially among malnourished children. Emergence of these highly resistant strains in hospital and community settings would also pose a serious challenge for infection control and antibiotic therapy in the future with over-reliance of carbapenems.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMENDATIONS

6.1 Conclusion

1. Girls were more than boys among severely malnourished and non-malnourished children in terms of gender distribution. Isolates from severely malnourished children (Cases) were more resistant to antimicrobials than non-malnourished children (Controls). These isolates are in particular more resistant to ß-lactamase inhibitors, multiple generations of cephalosporins, (fluoro)quinolones, aminoglycosides and chloramphenicol. This means that treatment options for severely malnourished children are more limited compared to non-malnourished children.

2. The study established that ESBL colonisation is 8.2 times higher in severely malnourished children compared to non-malnourished children. ESBL isolates, regardless of source, showed co-resistance to cephalosporins, fluoroquinolones, and aminoglycosides. This means that carbapenems are the only plausible alternatives to treat ESBL infections but affordability and availability of these antimicrobials especially to children from poor families is a challenge. CTX-M enzymes were the most prevalent ß-lactamases among ESBL isolates and majority of these isolates bore the $bla_{CTX-M-15}$ that has a better hydrolytic activity to many classes of ß-lactams than other related enzymes. They also harboured other resistance genes rendering these isolates MDR. Therefore, treatment with aminoglycosides, quinolones and ß-lactam/ß-lactamases inhibitor combinations is highly likely to lead to treatment failure.

3. Some of the ESBL producers were phylogenetically related. This study reports isolation of ST131 from faecal samples and also showed that majority of these strains are CTX-M-producers. The study also implicated strains belonging to ST44, ST940, ST1642, ST1675, ST1722 and ST 4481, in the spread of $bla_{CTX-M-15}$ enzymes in Kenya. This study does not only confirm ST131 as the principal

disseminators of the EBSL phenotype in Kenya but also shows that bla_{CTX-Ms} are also present in many other ST-types. This observation may be interpreted to mean that ST131 still remains the most prevalent clone in the dissemination of $bla_{CTX-M-15}$ but other clones also harbour this gene. These results further suggest that conjugative plasmids are likely spreading among different *E. coli* clones and ST-types and therefore, there is a high probability of an increase of MDR strains in future. Occurrence of bla_{TEM-33} or $bla_{TEM-158}$ with $bla_{CTX-M-15}$ suggests that these rare genes are also present in a proportion of ESBL *E. coli* isolates. Identification of a putative $bla_{KLU-'LIKE'}$ gene affirms that β-lactamases may be constantly evolving to continuingly add to a growing list enzymes conferring resistance to 3rd generation cephalosporins. Association of ST4481 with $bla_{CTX-M-15}$ suggest that dissemination of CTX-M-15 enzymes can also occur across other sequence types.

6.2 Recommendation

1. Treatment of severely malnourished children should only be guided by culture and antimicrobial susceptibility profiles and existing empiric treatment regimens should be used with caution especially among severely malnourished children.

2. Empiric treatment guidelines for severely malnourished children should be reviewed because ESBLs tend to more prevalent in this vulnerable group. This is particularly important because majority of ESBL-producers were also resistant to other classes of antimicrobials. The use of clavulanic acid as an empiric treatment regime should be reconsidered because high resistance to this antibiotic was observed and in most Cases, this resistance was associated with carriage of bla_{OXA-1}, bla_{TEM-33} or$bla_{TEM-158}$

3. In order to identify important clones associated with high MDR phenotypes, more and larger studies on circulating ST clonal complexes in *E. coli* especially those that are ESBL-producers should be carried out in future. It is important to put measures in place that will reduce or eliminate high

prevalence of malnourishment among children because this condition was also found to be an important risk factor for colonization with highly resistant ESBL isolates. I also recommend that such children should receive a combination of supplements (vitamin A, Zinc), probiotics (specifically *Lactobacillus rhamnosus* GG (LGG), *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) and oral rehydration solutions (ORS) that can mitigate diarrheal and enteric infections.

4. More studies to determine the ST clonal complexes in *E. coli* with their respective ESBLs should be undertaken in Kenya

6.3 Study limitations

1. The study was nested in a bigger clinical control trial and therefore it was at times difficult to obtain some details of study participant's such as past treatment history.

2. Stool samples were not always available and in such Cases, rectal swabs were obtained. I assume that the prevalence of the organism of interest in both specimens did not differ significantly.

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APPENDICES

Appendix 1   Patient Written consent form

Informed consent for the participants (Filled by Mothers/Guardian of children below 5 years**)**

Title of Research: Comparison of Phenotypic and Genotypic patterns of broad-spectrum β-lactam resistance in faecal *E. coli* isolated from severely malnourished and well not malnourished children attending Mbagathi District Hospital, Nairobi.

Investigators: Samuel Mwangi Njoroge: Jomo Kenyatta University of Agriculture and Technology, John Kiiru: KEMRI-CMR, Gideon Kikuvi: JKUAT

Purpose of the Study

The aim of this study is to compare drugs that work best in severely malnourished and non-malnourished children having diarrhoea. Since severely malnourished children have a higher risk of being put on drugs and I want to evaluate how commonly prescribed drugs given to severely malnourished children predispose them to drug resistance with treatment failure as the outcome. Non-malnourished children will be used as Controls. This study will not involve following-up your child. Your child will only give one sample/specimen. Study will take 12 months to complete.

Procedures

Study will involve obtaining a stool sample from your child. If the stool sample cannot be obtained from your child, anal swab will be the preferred substitute. Anal swab will be obtained without contamination when the child will be lying on his/her back with legs raised. A sterile cotton tipped swab will then be inserted an inch into the anus. The principal investigator will do swab collection procedures. The principal investigator will obtain anal swab in your presence. Specimen/Sample will be transported to KEMRI-CMR. *E. coli* and other bacteria will be isolated. This study will only focus on *E. coli* drug susceptibility profiling.

Benefits

Free laboratory expenses incurred during your child's specimen processing. This will include free culture of bacteria other than *E. coli* and expeditious reporting of any outbreak status disease causing agents that will be encountered to the hospital administration.

Risks/Discomforts

This study involves risk to injury only if the child is not calm and/or sample is collected while the child is not laying down when collecting an anal swab, but presenting the prospect of direct health benefits. No risk involved in obtaining a stool sample. Treatment will not be delayed waiting laboratory results.

Withdrawing Participation

If you feel like your child cannot carry on with the study you are free to stop he's/her participation. You have a choice to do so and I will still appreciate your willingness to participate in the study. Withdrawing from the study will not lead to denial of standard care. Data obtained from your child after withdrawing participation will not be used.

Confidentiality

The information I get from your child is purposely for research and will not be revealed to anybody. Your child's information will be confidential. This will be achieved by use of serial numbers to code your child's names. Names will not be used as identities.

Contact Information

If you have any questions regarding this study, please contact Samuel Mwangi, on phone number 0724842893. Questions about your participation rights should be directed to the Secretary KEMRI/ National Ethical Review Committee, P. O. BOX 54840-00200, Nairobi; Telephone number: 020-272 2541; 0722 205901, 0733 400003

Declaration

Having read and understood the purpose of the study, I willingly accept my child to take part in it.

Signature …………………….. **Date** ………………………

Note: By putting your signature, you are agreeing that: You have read this consent form and have been given the opportunity to ask questions

• You have known the risks and they have been explained to your satisfaction

• You understand Jomo Kenyatta University and KEMRI has no policy or plan to pay for any injuries you might receive as a result of participating in this research protocol

Your participation in this research is given at your free will

Witness

Signature.................................. Date....................................

The participant received a copy ☐

Appendix 2   **Consent to store and export bacteria isolates**

Title: Comparison of Phenotypic and Genotypic patterns of broad-spectrum β-lactam resistance in faecal *E. coli* isolated from severely malnourished and not malnourished children attending Mbagathi District Hospital, Nairobi.

Storage

E. coli isolates from this study will be stored in Tryptic soy broth with 15% glycerol at -80°C for future studies relevant to improved child health such as pathogenesis of *E. coli*, Host-bacteria interactions and antimicrobial resistance.

Reasons

- For comparison with other phenotypic multi-drug resistant *E. coli* isolates from other regions of the world.

- For sequencing of resistance genes present in E. coli isolates in the two groups.

- To further detect mobile genetic elements of drug resistance genes

Exportation of Isolates to another country

Isolates may be transported to the United Kingdom and Taiwan for further molecular testing in two institutions; National Taiwan University college of Medicine and Wellcome Trust Sanger Institute.
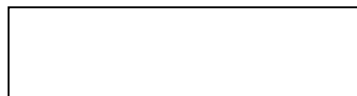
BARCODE 1.D

LAB 1.D: ……………………………..

Participant Consent

1. **"**Will you allow me to take your child's stool sample/anal swab for testing in the laboratory?"

$$\text{Yes} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots.. \; 1$$

$$\text{No} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots \; 2$$

2. "Will you allow us to keep bacteria isolates got from your child's sample for later testing?"

$$\text{Yes} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots \; 1$$

$$\text{No} \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots... \; 2$$

3. "Will you allow us to export bacteria isolates got from your child's sample to the United Kingdom for further molecular testing?"

$$\text{Yes} \dots\dots\dots\dots\dots\dots\dots\dots\dots \; 1$$

$$\text{No} \dots\dots\dots\dots\dots\dots\dots\dots\dots... \; 2$$

Date…./……/………………

Signature of the Mother/ Guardian: ………………………………………….

Signature of Witness (If Mother/Guardian is illiterate): …………………..

Lab Tech Name/ ID: ………………………………………………………….

Signature of Laboratory Technician: ………………………………….......

Appendix 3   Kiswahili translated informed consent

Ridhaa ya washiriki (Kujazwa na Mama / Mlezi wa mtoto chini ya miaka 5**)**

Mada ya Utafiti: Mlinganisho wa mifumo ya *E. coli* kukataa dawa zenye asili ya β-lactam kati ya watoto wanaokabiliwa na utapiamlo mkubwa na wale wanaolishwa vyema wanaohudhuria Hospitali ya Wilaya ya Mbagathi, Nairobi.

Wakaguzi: Samuel Mwangi Njoroge: Chuo Kikuu cha Jomo Kenyatta cha Kilimo na Teknolojia, John Kiiru: KEMRI-CMR, Gideon Kikuvi: JKUAT

Madhumuni ya somo

Lengo la utafiti huu ni kulinganisha mifumo ya *E. coli* kukataa dawa zenye asili ya β-lactam kati ya watoto wanaokabiliwa na utapiamlo mkubwa na wale wanaolishwa vyema. Watoto wanaokabiliwa na utapiamlo mkubwa wana hatari hatari kubwa ya mwili kukataa dawa zenye asili ya β-lactam na tunataka kuona kama mifumo kutoka kundi hili inawahatarisha kutofanya vizuri kwa dawa na kushindwa kwa matibabu kama matokeo. Watoto wanaolishwa vizuri watatumika kama udhibiti. Utafiti huu hautafuatilia washiriki. Mshiriki atatoa sampuli moja. Utafiti huu utachukua muda wa miezi 12 kukamilika.

Taratibu

Utafiti unahusisha kupata sampuli ya kinyesi.Kama sampuli ya kinyesi hayawezi kupatikana, anal swab itakuwa mbadala. Mtoto atalazwa chali na kutolewa usufi wa puru anapotulia. Usufi wa puru utatolewa na Mpelelezi Mkuu wa utafiti huu. Sampuli ya kinyesi/ Usufi wa puru zitasafirishwa kwenda KEMRI-CMR. *E. coli* na bakteria zingine zitakuzwa. Utafiti huu tu kuzingatia bakteria *E. coli*. Mifumo ya utendakazi wa dawa itafanyika na jeni zinazofanya bakteria kukataa dawa zitatambuliwa. *E. coli* zitahifadhiwa kwa jokofu lenye baridi ya -80°C kwa ajili ya masomo ya baadaye husika.

Faida

Gharama za maabara itakuwa bure.Hii itahusisha kuangalia viini vinavyo kuhara na *E. coli* kuripoti viini vinavyo sababisha usalama wa afya kwa wakubwa wa hospitali wanaosimahia utendakazi. Umuhimu wa kubaini ni dawa gani mzuri ya kutibu mtoto atakapo zidi kuhara na dawa zenye nguvu zinapatikana kwa urahisi hapa Nchni.

Hatari

Utafiti huu hatari kama motto hatatulia ama sampuli inapotolewa kama mtoto hajalala chali wakati usufi wa puru unapotolewa lakini utafiti unawasilisha matarajio ya faida ya afya. Hakuna hatari ya kushiriki katika kupata sampuli ya kinyesi.Matibabu hayatakawia kusubiri matokeo ya maabara.

Kujiondoa Kushiriki

Kama unajisikia kama mtoto wako hawezi kutolewa sampuli, una uhuru wa kusitisha ushiriki wake. Una hiari ya kufanya hivyo na sisi bila kutujulisha nia yako ya kukosa kushiriki katika utafiti. Mtoto hatanyimwa haki ya huduma za afya unapokosa kumsajili kwenye utafiti. Malezo kuhusu mtoto hayatatumia unapomondoa kwenye utafiti.

Usiri

Taarifa tunayoipata kutoka kwenu kwa kusudi la utafiti si ya wazi kwa mtu yeyote. Habari za mtoto itawekwa kwa siri. Numba itatumiwa badala ya majina ya watoto. Majina hayatatumika kusudi katika hatua yoyote katika utafiti huu

Mawasiliano ya Habari

Kama una maswali yoyote kuhusu somo hili, tafadhali wasiliana na Samuel Mwangi, kwenye simu namba 0724842893 na maswali kuhusu haki za kushiriki utafiti yataelekezwa kwa Katibu- KEMRI /Kamati ya Maadili, P. O. BOX 54840-00200, Nairobi; Namba ya simu: 020-272 2541, 0722 205901, 0733 400003

Uhifadhi na usafirishaji wa sampuli na masomo zaidi

E. coli kutoka utafiti huu zitahifadhiwa katika jokofu lenye baridi -80°C kwa ajili ya masomo ya baadaye muhimu kwa afya bora watoto kama vile pathogenesia ya *E. coli*, mwingiliano Jeshi-bakteria na upinzani wa dawa. Kwa hiyo ni muhimu kuwa mshiriki utafiti anaelewa kuwa sampuli inaweza kusafirishwa kwa nchi nyingine katika kuwepo wa mradi huo. Kukubali kushiriki kwenye utafiti ina maana kwamba nimekubali uwezekano wa sampuli kusafirishwa katika siku za baadaye kwenda nchi nyingine kwa ajili ya uchambuzi zaidi.

Azimio

Baada ya kusoma na kuelewa lengo la somo, ninakubali kushiriki utafiti huu.

Sahihi ....**Tarehe** ....

Ilani: Kwa kuweka sahihi yako, unakubali kwamba: Umesoma fomu hii na kukubali na umepewa fursa ya kuuliza maswali

• Wewe unajua hatari na umeelezea kuridhika kwako

• Umeweza kuelewa kuwa Jomo Kenyatta University na KEMRI haina sera au mpango wa kulipa kutokana na majeraha yoyote unaweza kupata kutokana na kushiriki kikamilifu katika itifaki hii ya utafiti

Ushiriki wako katika utafiti huu ni wa kutolewa katika mapenzi yako ya bure

Shahidi

Sahihi:…………………………Tarehe: ……………………………………

Mshiriki alipata Nakala yake

Appendix 4   Ridhaa ya kusafirisha bakteria nga'mbo

Mada: Mlinganisho wa mifumo ya *E. coli* kukataa dawa zenye asili ya β-lactam kati ya watoto wanaokabiliwa na utapiamlo mkubwa na wale wanaolishwa vyema wanaohudhuria Hospitali ya Wilaya ya Mbagathi, Nairobi.

Kuhifadhi backteria

Bakteria za *E. coli* kutoka utafiti huu zitawekwa kwa chupa ndogo zenye Tryptic soy broth na asilimia kumi na tano ya glycerol kwa baridi kiasi cha -80°C kwa tafiti zenye kuboresha afya ya watoto wanaohara.

Sababu

- Kulinganisha mifumo ya bacteria kukataa dawa zilizo tolewa Mbagathi na zile zinazotoka mahali pengine duniani.

- Kujua mpangilio wa jeni zinazosababisha kukataa dawa.

- Kujua nini kinachobeba jeni za kukataa dawa.

Kusafirishwa kwa bacteria nje ya Nchi

Bakteria zinaweza safirishwa hadi Uingereza kwa utafiti zaidi wa mipangilio wa jini ndani ya bakteria. Vituo vya utafiti ni; Chuo cha tiba, chuo kikuu cha kitaifa, Taiwan ama Shirika la Wellcome Trust, Uingereza.

BARCODE 1.D
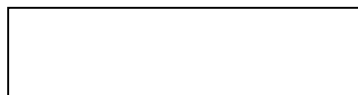
Kitambulisho cha Mahabara: …………………………..

Ridhaa ya Mshiriki

- "Je utakubali nitoe sampuli ya kinyesi ama usufi wa puru ili ipimwe kwenye mahabara?"

  - Ndio …………………………………….. 1

  - La …………………………………… 2

- "Je unakubali tuweke bakteria zilizokuzwa kutoka kwa sampuli ya mtoto wako ili ipimwe badaye?"

  - Ndio ………….................................. 1

  - La …………………………………... 2

- "Je, unakubali bakteria hizi kusafirishwa hadi Uingereza kwa Kupimwa kwa kina?"

  - Ndi ………………………………. 1

  - La ………………………………... 2

Tarehe…./……/………………

Sahihi ya mama/ Mlezi: …………………………………….

Sahihi ya Shahidi (Kama mama hawezi soma): …………………..

Jina la Lab Tech / ID: …………………………………………………….

Sahihi ya Mfanyi kazi wa mahabara: …………………………………........

Appendix 5   Drug arrangement for ESBL Phenotypic identification

Plate 1   Plate 2
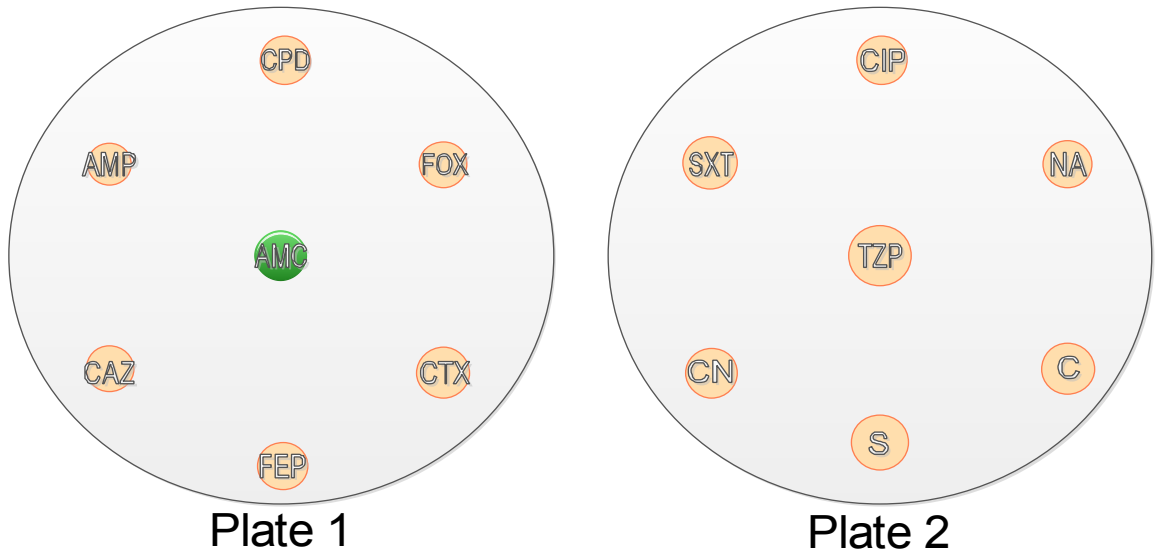
Key

CTX- cefotaxime   SXT- sulfamethoxazole/trimethoprim

AMC- amoxicilin/clavulanic acid   CIP- ciprofloxacin

CPD- cefpodoxine   TZP- tazobactam/piperacillin

AMP- ampicillin   NA- nalidixic acid

FOX –cefoxitin   C- chrolamphenicol

FEP- cefepime   S- streptomycin

CAZ- ceftazidime   CN- gentamicin

The diameter of the petri dish was 8.6cm

The diameter of an antimicrobial disc was 6mm

Appendix 6   Consensus Primer Table

TargetGene	Primer name	5'-3'sequence	Tm°C	Size (bp)	Accession no.
*bla*TEM (consensus)	TEM-F TEM-R	ATGAGTATTCAACAT TTCCG CCAATGCTTAATCAGTGAGG	55	840	EF125012-related
*bla*SHV (consensus)	SHV-F SHV-R	TTCGCCTGTGTATTATCTCCCTG TTAGCGTTGCCAGTGYTCG	50	854	AF148850-related
*bla*CTX-M (concensus)	MA1 MA2	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAYCAGCGG	60	593	Y10278-related
*bla*CMY (consensus)	CF1 CF2	ATGATGAAAAAAATCGTTATGC TTGCAGCTTTTCAAGAATGCGC	55	1200	U77414-related

Appendix 7   Scientific steering committee approval

KENYA MEDICAL RESEARCH INSTITUTE

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

ESACIPAC/SSC/100793   4th September, 2012

Samuel Njoroge

Thro'
   Director, CMR   forwarded   6/9/2012
   NAIROBI

REF: SSC No. 2382 (Revised) – Comparison of phenotypic and genotypic
      patterns of broad spectrum β-lactam resistance in faecal *E.coli*
      isolated from severely malnourished and nourished children
      attending Mbagathi District Hospital, Nairobi.

Thank you for your letter dated 3rd September, 2012 responding to the
comments raised by the KEMRI SSC.

I am pleased to inform you that your protocol now has formal scientific
approval from SSC.

The SSC however, advises that work on the proposed study can only start after
ERC approval

Sammy Njenga, PhD
SECRETARY, SSC

In Search of Better Health

Appendix 8   Ethical Review Committee approval

KENYA MEDICAL RESEARCH INSTITUTE

KEMRI/RES/7/3/1   **January 17, 2013**

TO: MR. SAMUEL NJOROGE (PRINCIPAL INVESTIGATOR)

THROUGH: DR. SAMUEL KARIUKI,
            THE DIRECTOR, CMR,
            NAIROBI

Dear Sir,

RE: SSC PROTOCOL No. 2382 – 2ND REVISION (*RE-SUBMISSION 2*): COMPARISON
       OF PHENOTYPIC AND GENOTYPIC PATTERNS OF BROAD-SPECTRUM B-
       LACTAM RESISTANCE IN FEACAL *E.COLI* ISOLATED FROM SEVERELY
       MALNOURISHED AND NOURISHED CHILDREN ATTENDING MBAGATHI
       DISTRICT HOSPITAL NAIROBI (*VERSION 1.3DATED 8TH JANUARY 2013*)

Reference is made to your letter dated January 8, 2013. The ERC Secretariat acknowledges
receipt of the revised research proposal – Version 2 dated December 2012.

This is to inform you that the Committee determines that the issue raises at the 208th meeting of
9th October 2012 and on 17th December 2012 are adequately addressed. Consequently, the study
is granted approval for implementation effective this **17th day of January 2013** for a period of
one year.

Please note that authorization to conduct this study will automatically expire on **January 16,
2014**. If you plan to continue data collection or analysis beyond this date, please submit an
application for continuation approval to the ERC Secretariat by **December 5, 2013**. The
regulations require continuing review even though the research activity may not have begun until
sometime after the ERC approval.

You are required to submit any proposed changes to this study to the SSC and ERC for review
and the changes should not be initiated until written approval from the ERC is received. Please
note that any unanticipated problems resulting from the implementation of this study should be
brought to the attention of the ERC and you should advise the ERC when the study is completed
or discontinued.

Work on this project may begin.

Sincerely,

DR. CHRISTINE WASUNNA,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

In Search of Better Health

Appendix 9   Publication

Original Article

Broad spectrum β-lactam resistance in faecal *Escherichia coli* isolated from severely malnourished and nourished children attending Mbagathi district hospital, Nairobi: A case-control study

Abstract

Context: Severely malnourished children have increased risk of being put on antibiotics due to co-morbidities. **Aim:** The study's objective was to characterize the *Escherichia coli* β-lactamase mediated resistance to the broad spectrum β-lactam antimicrobials among this population and compare them with nourished children as controls. **Settings and Design:** In this case-control, hospital-based setup, 109 *E. coli* isolates were obtained from each group, one isolate per subject. **Materials and Methods:** Stool or anal swabs were collected, enriched in buffered peptone water and cultured on MacConkey and eosin methylene blue agars. Biochemical test were used to identify *E. coli*. antibiograms to determine phenotypic resistance were determined using a panel of 14 drugs. Only the isolates showing synergy between ampicillin-calvulanic acid and one or more third generation cephalosporins were picked as extended spectrum β-lactamase (ESBL) producers. **Statistical Analysis:** Differences in ESBL rates and susceptibility percentages between cases and controls were evaluated for significance using 2-tailed Fisher's exact test. **Results:** Prevalence of ESBL phenotype was higher in severely malnourished children (39%) as compared to the controls (7%). The plasmid–encoded AmpC's (pAmpC)-like phenotype was observed in 11% isolates. **Conclusions:** Isolation of ESBL-*E. coli* among severely malnourished children is high. Surveillance of ESBL producers, both in the community and hospital settings needs to be stepped up in Kenya.

Key words:
Antimicrobial resistance, diarrhea, Escherichia coli, extended spectrum β-lactamase-Escherichia coli, severely malnourished children, Kenya

Introduction

Severely malnourished children are at a higher risk of enteric infection, making them more prone to diarrhea than healthy children.[1,2] They often have other complications such as diarrhea, pneumonia and bacteremia.[3,4] This may warrant the empiric use of antimicrobials to boost their survival, but in the case of severe acute malnutrition complicated by diarrhea only, this predisposes a child to inappropriate antimicrobial use.[5,6]

Antimicrobial resistance among *Escherichia coli* is of increasing global concern.[7] This has been associated with the emergence and spread of extended spectrum β-lactamase (ESBL)-producing *E. coli*, which are also frequently associated with resistance to quinolone and aminoglycosides.[8] Serious infections with ESBL producing *E. coli* are associated with high mortality rates as therapeutic options are limited to carbapenems.[9,10] ESBL producers are resistant to penicillins, oxyimino-cephalosporins,

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Appendix 10   Raw/Interpreted* AST results for Controls

CONTROLS

Isolate No	AMC		CPD		FOX		CTX		FEP		CAZ		AMP		SXT		CIP		TZP		NA		C		S		CN		COMMENT
109	19	S	27	S	26	S	34	S	34	S	29	S	6	R	6	R	29	S	30	S	7	R	25	S	6	R	23	S	
8B	26	S	28	S	26	S	39	S	40	S	30	S	21	S	6	R	39	S	38	S	26	S	28	S	11	R	21	S	
66	14	I	22	S	19	S	29	S	30	S	30	S	6	R	6	R	39	S	22	S	26	S	6	R	7	R	22	S	
62	20	S	28	S	29	S	33	S	30	S	33	S	6	R	6	R	36	S	28	S	22	S	26	S	11	R	22	S	
108	28	S	26	S	26	S	28	S	33	S	30	S	6	R	6	R	35	S	32	S	20	S	25	S	20	S	24	S	
50	28	S	26	S	30	S	28	S	32	S	28	S	6	R	6	R	33	S	25	S	28	S	30	S	10	R	28	S	
80	18	S	26	S	21	S	30	S	30	S	30	S	6	R	28	S	36	S	29	S	26	S	25	S	20	S	22	S	
103	29	S	29	S	30	S	31	S	28	S	28	S	14	I	6	R	35	S	40	S	25	S	30	S	16	S	24	S	
107	30	S	29	S	25	S	30	S	30	S	30	S	20	S	6	R	6	R	30	S	6	R	28	S	6	R	25	S	
52	18	S	28	S	25	S	30	S	30	S	30	S	6	R	6	R	32	S	28	S	25	S	28	S	10	R	25	S	
99	30	S	29	S	30	S	30	S	35	S	30	S	20	S	30	S	35	S	30	S	25	S	20	S	18	S	20	S	
13	28	S	22	S	30	S	30	S	30	S	30	S	20	S	6	R	40	S	30	S	28	S	22	S	20	S	25	S	
11	30	S	27	S	25	S	30	S	35	S	30	S	20	S	6	R	30	S	28	S	28	S	26	S	18	S	20	S	
67	29	S	25	S	26	S	30	S	30	S	29	S	20	S	30	S	34	S	30	S	30	S	25	S	18	S	20	S	
106	20	S	30	S	25	S	30	S	35	S	30	S	6	R	6	R	33	S	26	S	26	S	26	S	6	R	24	S	

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Remark
105	29 S	30 S	24 S	34 S	35 S	30 S	25 S	6 R	35 S	25 S	25 S	27 S	20 S	22 S	
86	23 S	21 S	27 S	29 S	30 S	30 S	6 R	6 R	30 S	28 S	23 S	25 S	18 S	6 R	ESBL
83	7 R	18 I	6 R	27 S	30 S	27 S	7 R	R	28 S	26 S	25 S	26 S	17 S	20 S	FOX RESISTANT
44	10 R	6 R	14 R	6 R	12 R	13 R	6 R	6 R	6 R	20 I	6 R	20 S	6 R	9 R	ESBL
132	8 R	18 I	6 R	28 S	30 S	29 S	6 R	6 R	30 S	29 S	25 S	25 S	18 S	21 S	FOX RESISTANT
22	7 R	18 I	6 R	25 I	32 S	26 S	6 R	29 S	32 S	26 S	S	18 S	16 S	22 S	FOX RESISTANT
143	14 I	17 I	7 R	28 S	30 S	25 S	13 R	27 S	30 S	26 S	23 S	22 S	19 S	20 S	FOX RESISTANT
90	7 R	25 S	6 R	30 S	30 S	28 S	7 R	30 S	34 S	26 S	24 S	21 S	20 S	22 S	FOX RESISTANT
37	20 S	18 I	6 R	29 S	34 S	25 S	15 I	30 S	31 S	27 S	S		15 S	22 S	FOX RESISTANT
89	8 R	20 I	6 R	29 S	32 S	27 S	6 R	6 R	20 I	27 S	15 I	24 S	10 S	22 S	FOX RESISTANT
139	21 S	26 S	25 S	35 S	30 S	28 S	19 S	28 S	31 S	26 S	23 S	25 S	17 S	22 S	
15	20 S	28 S	27 S	32 S	34 S	31 S	7 R	6 R	35 S	25 S	24 S	S		22 S	
48	18 S	27 S	25 S	32 S	32 S	30 S	6 R	6 R	R	26 S	6 R	25 S	7 R		
16	21 S	27 S	23 S	31 S	31 S	28 S	20 S	6 R	R	30 S	25 S	27 S	17 S	21 S	
10	24 S	27 S	26 S	32 S	30 S	30 S	20 S	S		27 S	23 S	26 S	15 S	22 S	
36	14 I	24 S	25 S	30 S	31 S	27 S	6 R	6 R	36 S	20 S	23 S	6 R	16 S	21 S	

120	25	S	29	S	26	S	32	S	34	S	30	S	6	R	27	S	30	S	25	S	22	S	26	S	15	S	21	S	
141	18	S	25	S	24	S	30	S	32	S	29	S	6	R	6	R	7	R	26	S	6	R	25	S	6	R	25	S	
71	23	S	25	S	25	S	30	S	30	S	26	S	21	S	6	R	33	S	28	S	25	S	24	S	16	S	21	S	
140	26	S	26	S	25	S	32	S	34	S	30	S	23	S	6	R	32	S	26	S	24	S	25	S	17	S	20	S	
35	22	S	29	S	26	S	34	S	34	S	30	S	20	S	6	R	39	S	29	S	24	S	24	S	16	S	23	S	
73	24	S	29	S	24	S	32	S	34	S	30	S	18	S	30	S	39	S	27	S	25	S	25	S	20	S	22	S	
25	22	S	26	S	24	S	34	S	34	S	30	S	20	S	6	R	32	S	28	S	25	S	22	S	13	I	21	S	
14	19	S	26	S	25	S	31	S	32	S	29	S	6	R	6	R	32	S	27	S	24	S	25	S	6	R	22	S	
38	21	S	26	S	25	S	31	S	32	S	26	S	17	S			32	S	26	S	24	S	22	S	18	S	23	S	
30	10	R	6	R	14	R	6	R	13	R	13	R	6	R	6	R	6	R	19	I	6	R	15	I	6	R	9	R	ESBL
137	21	S	26	S	26	S	34	S	34	S	32	S	19	S	6	R	39	S	30	S	29	S	28	S	18	S	20	S	
136	24	S	26	S	22	S	31	S	34	S	31	S	11	R	29	S	31	S	26	S	25	S	23	S	18	S	21	S	
23	21	S	25	S	23	S	32	S	32	S	30	S	20	S	30	S	36	S	26	S	25	S	25	S	18	S	21	S	
2	20	S	29	S	26	S	32	S	34	S	30	S	6	R			34	S	26	S	27	S	25	S	6	R	22	S	
24	20	S	28	S	26	S	35	S	31	S	28	S	6	R	6	R	34	S	30	S	26	S	10	R			22	S	
4	20	S	27	S	24	S	31	S	34	S	30	S	6	R	6	R	32	S	26	S	24	S	25	S	6	R			
18	18	S	30	S	30	S	32	S	35	S	32	S	6	R	22	S	36	S	25	S	28	S	28	S	16	S	27	S	
64	20	S	30	S	26	S	33	S	34	S	33	S	6	R	6	R	34	S	28	S	25	S	28	S	13	I	26	S	
60	20	S	28	S	27	S	32	S	35	S	31	S	20	S	6	R	35	S	27	S	25	S	25	S	17	S	24	S	

ID															ESBL
9	22 S	28 S	25 S	32 S	33 S	30 S	6 R	29 S	33 S	26 S	26 S	24 S	18 S	23 S	
53	25 S	29 S	27 S	30 S	35 S	30 S	22 S	31 S	34 S	30 S	22 S	27 S	19 S	23 S	
110	20 S	24 S	25 S	30 S	34 S	29 S	6 R	26 S	35 S	29 S	26 S	28 S	10 R	23 S	
79	21 S	30 S	27 S	35 S	35 S	31 S	23 S	31 S	36 S	30 S	27 S	30 S	21 S	23 S	
8	22 S	29 S	27 S	30 S	31 S	30 S	6 R	6 R	35 S	30 S	27 S	25 S	10 R	25 S	
56	24 S	21 S	17 I	30 S	36 S	28 S	20 S	30 S	34 S	28 S	27 S	25 S	17 S	23 S	
93	23 S	28 S	26 S	33 S	35 S	30 S	6 R	6 R	29 S	32 S	10 R	27 S	6 R	26 S	
42	22 S	26 S	24 S	34 S	34 S	20 S	18 S	6 R	39 S	26 S	27 S	25 S	17 S	24 S	
33	20 S	26 S	26 S	32 S	32 S	30 S	21 S	27 S	34 S	26 S	24 S	22 S	16 S	21 S	
88	22 S	25 S	26 S	30 S	34 S	30 S	24 S	6 R	36 S	26 S	22 S	21 S	18 S	22 S	
13B	16 I	27 S	22 S	32 S	32 S	28 S	9 R	6 R	30 S	25 S	20 S	22 S	16 S	21 S	
129	26 S	24 S	28 S	32 S	32 S	28 S	15 I	28 S	30 S	26 S	22 S	26 S	19 S	21 S	
21	20 S	24 S	22 S	30 S	30 S	29 S	16 I	6 R	40 S	26 S	26 S	25 S		26 S	
106	20 S	29 S	24 S	32 S	34 S	30 S	6 R	6 R	36 S	30 S	26 S	25 S	6 R	21 S	
49	8 R	6 R	18 S	6 R	17 I	13 R	6 R	6 R	6 R	21 S	6 R	6 R	16 S	20 S	ESBL
111	15 I	6 R	25 S	6 R	17 I	18 I	6 R	6 R	6 R	24 S	6 R	26 S	18 S	21 S	ESBL
97	6 R	23 S	26 S	30 S	30 S	30 S	6 R	6 R	35 S	30 S	24 S	27 S	16 S	25 S	ESBL
51	13 R	6 R	24 S	6 R	15 I	13 R	6 R	30 S	6 R	21 S	6 R	24 S	20 S	22 S	ESBL
77	24 S	6 R	27 S	6 R	16 I	16 R	6 R	6 R	6 R	30 S	6 R	30 S	17 S	23 S	ESBL

ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Notes
100	14 I	21 S	9 R	30 S	39 S	32 S	12 R	30 S	39 S	30 S	26 S	25 S	19 S	20 S	FOX RESISTANT
57	19 S	23 S	10 R	32 S	35 S	30 S	18 S	30 S	19 I	24 S	26 S	22 S	37 S	22 S	FOX RESISTANT
96	27 S	21 S	12 R	30 S	38 S	31 S	16 I	6 R	35 S	30 S	26 S	26 S	18 S	23 S	FOX RESISTANT
95	15 I	21 S	11 R	30 S	35 S	28 S	14 I	6 R	38 S	28 S	25 S	27 S	18 S	21 S	FOX RESISTANT
112	14 I	21 S	8 R	32 S	34 S	29 S	6 R	6 R	36 S	29 S	26 S	24 S	20 S	23 S	FOX RESISTANT
90	8 R	25 S	6 R	29 S	36 S	30 S	6 R	30 S	36 S	26 S	24 S	19 S	20 S	22 S	FOX RESISTANT
134	14 I	27 S	24 S	34 S	30 S	30 S	17 S	6 R	26 S	25 S	23 S	20 S	6 R	20 S	
126	25 S	29 S	25 S	32 S	33 S	30 S	22 S	6 R	26 S	28 S	6 R	23 S	6 R	21 S	
70	17 I	25 S	22 S	30 S	28 S	27 S	10 R	6 R	34 S	25 S	25 S	23 S	7 R	20 S	
6	20 S	25 S	24 S	30 S	32 S	28 S	19 S			26 S	22 S	23 S	17 S	20 S	
39	15 I	26 S	24 S	30 S	28 S	29 S	10 R	6 R	30 S	26 S	24 S	25 S	10 R	22 S	
142	22 S	25 S	24 S	28 S	29 S	28 S	19 S	6 R	30 S	25 S	23 S	23 S	16 S	20 S	
43	17 I	28 S	25 S	30 S	32 S	29 S	6 R	26 S	30 S	25 S	22 S	23 S	13 I	20 S	
130	16 I	27 S	24 S	32 S	33 S	28 S	6 R	6 R	32 S	27 S		25 S	18 S	22 S	
41	17 I	26 S	25 S	29 S	29 S	28 S	6 R	6 R	29 S	25 S	22 S	7 R	6 R	21 S	
39	17 I	27 S	26 S	30 S	30 S	30 S	6 R	6 R	R	25 S	26 S	25 S	11 R		

ID																												
75	22	S	25	S	22	S	29	S	28	S	27	S	18	S	26	S	32	S	24	S	23	S	22	S	22	S	22	S
40	16	I	26	S	26	S	30	S	32	S	30	S	6	R	6	R	34	S	28	S	28	S	26	S	10	R	22	S
27	14	I	24	S	22	S	30	S	28	S	28	S	6	R	6	R	6	R	24	S	6	R	26	S	6	R	22	S
78	20	S	26	S	24	S	28	S	30	S	30	S	18	S	6	R	34	S	24	S	24	S	24	S	18	S	20	S
138	19	S			26	S	29	S	32	S	29	S	6	R	30	S	30	S	28	S	24	S	28	S	20	S	20	S
128	20	S	28	S	26	S	30	S	30	S	30	S	28	S	6	R	34	S	26	S	20	S	28	S	20	S	10	R
144	20	S	30	S	26	S	34	S	30	S	30	S	6	R	6	R	34	S	24	S	22	S	24	S	18	S	20	S
76	14	I	24	S	20	S	28	S	26	S	28	S	6	R	6	R	26	S	20	I	6	R	20	S	6	R	18	S
28	20	S	22	S	26	S	30	S	28	S	28	S	20	S	28	S	30	S	26	S	28	S	28	S	20	S	20	S
125	20	S	26	S	222	S	28	S	30	S	28	S	18	S	30	S	30	S	20	I	26	S	28	S	6	R	22	S
135	18	S	28	S	20	S	30	S	28	S	28	S	6	R	6	R	6	R	20	I	6	R	6	R	18	S	20	S
45	18	S	22	S	22	S	30	S	28	S	26	S	6	R	6	R	34	S	26	S	22	S	20	S	6	R	20	S
29	18	S	26	S	22	S	28	S	28	S	26	S	6	R	6	R	30	S	26	S	10	S		R	6	R	20	S
26	20	S	28	S	26	S	28	S	28	S	28	S	6	R	25	S	24	S	24	S	24	S	24	S	6	R	18	S
104	16	I	26	S			35	S	33	S	30	S	6	R	6	R	35	S	26	S	25	S	24	S	10	R	21	S
102	24	S	30	S	26	S	34	S	36	S	30	S	11	R	26	S	32	S	27	S	27	S	6	R	14	I	23	S
59	12	R	23	S	26	S	28	S	28	S	29	S	6	R	6	R	39	S	21	S	25	S	7	R	14	I	23	S
94	22	S	20	I	24	S	28	S	32	S	28	S	17	S	30	S	32	S	27	S	26	S	25	S	19	S	20	S
55	26	S	30	S	29	S	30	S	36	S	30	S	20	S	6	R	38	S	28	S	29	S	25	S	18	S	22	S

68	22	S	25	S	26	S	30	S	36	S	30	S	18	S	28	S	39	S	26	S	25	S	25	S	13	I	20	S
69	18	S	22	S	24	S	29	S	32	S	28	S	6	R	6	R	25	S	29	S	19	S	26	S	18	S	24	S
61	22	S	25	S	26	S	32	S	34	S	30	S	16	I	6	R	31	S	30	S	24	S	28	S	18	S	23	S
54	20	S	25	S	24	S	30	S	32	S	26	S	6	R	21	S	30	S	29	S	6	R	6	R	11	R	22	S
65	21	S	29	S	21	S	34	S	32	S	32	S	18	S	26	S	39	S	30	S	27	S	28	S	18	S	20	S
E. coli ATCC 29522	19	S	25	S	22	S	30	S	31	S	28	S	17	S	30	S	28	S	29	S	26	S	25	S	16	S	26	S

*Interpratation was done using CLSI 2015 guidelines (CLSI, 2015).

Appendix 11   Raw/Interpreted* ASTresults for Cases

CASES

Study Number	AMC		CPD		FOX		CTX		FEP		CAZ		AMP		SXT		CIP		TZP		NA		C		S		CN		COMMENT
0963	14	I	6	R	21	S	10	R	16	I	10	R	6	R	6	R	6	R	24	S	6	R	6	R	13	I	17	S	ESBL
0840	18	S	26	S	28	S	19	R	36	S	32	S	6	R	6	R	42	S	30	S	32	S	30	S	20	S	28	S	ESBL
0992	9	R	6	R	21	S	9	R	18	S	16	R	6	R	6	R	6	R	25	S	6	R	28	S	9	R	11	R	ESBL
0968	18	S	6	R	28	S	13	R	22	S	20	I	6	R	6	R	6	R	24	S	6	R	6	R	10	R	15	S	ESBL
1209	13	R	6	R	25	S	6	R	14	R	13	R	6	R	6	R	6	R	24	S	6	R	20	S	11	R	7	R	ESBL
0993	17	I	6	R	29	S	11	R	20	S	20	I	6	R	6	R	6	R	24	S	6	R	30	S	13	I	11	R	ESBL
0455	14	I	6	R	30	S	10	R	20	S	18	I	6	R	6	R	6	R	25	S	6	R	30	S	15	S	11	R	ESBL
1205	13	R	6	R	24	S	7	R	15	I	15	R	6	R	28	S	6	R	22	S	6	R	19	S	14	I	8	R	ESBL
0967	20	S	6	R	28	S	11	R	20	S	18	I	6	R	6	R	6	R	24	S	6	R	28	S	20	S	14	I	ESBL
1000	16	I	6	R	26	S	7	R	16	I	14	R	6	R	30	S	6	R	24	S	6	R	18	S	14	I	8	R	ESBL
0999	13	R	6	R	25	S	7	R	15	I	15	R	6	R	30	S	6	R	22	S	6	R	15	I	14	I	7	R	ESBL
0898	11	R	6	R	15	I	6	R	13	R	15	R	6	R	24	S	6	R	22	S	6	R	22	S	6	R	7	R	ESBL

0995	15	I	6	R	25	S	10	R	18	S	23	S	6	R	6	R	6	R	30	S	6	R	26	S	14	I	10	R	ESBL
0716	15	I	6	R	27	S	12	R	21	S	18	I	6	R	6	R	7	R	24	S	6	R	6	R	9	R	13	I	ESBL
0988	11	R	6	R	25	S	7	R	15	I	14	R	6	R	10	R	39	S	30	S	26	S	30	S	15	S	25	S	ESBL
0753	14	I	6	R	28	S	6	R	14	R	10	R	6	R	6	R	6	R	22	S	6	R	27	S	10	R	7	R	ESBL
0965	13	R	6	R	28	S	11	R	26	S	17	R	6	R	6	R	22	S	23	S	16	I	6	R	17	S	8	R	ESBL
0766	10	R	6	R	15	I	6	R	9	R	8	R	6	R	6	R	6	R	22	S	6	R	6	R	8	R	8	R	ESBL
0772	13	R	6	R	26	S	8	R	9	R	15	R	6	R	6	R	31	S	22	S	24	S	24	S	19	S	9	R	ESBL
1754	12	R	6	R	26	S	10	R	18	S	16	R	6	R	6	R	6	R	25	S	6	R	29	S	16	S	14	I	ESBL
1202	13	R	6	R	25	S	6	R	14	R	12	R	6	R	6	R	6	R	21	S	6	R	14	I	8	R	6	R	ESBL
1203	15	I	6	R	26	S	6	R	14	R	14	R	6	R	6	R	6	R	23	S	6	R	19	S	11	R	7	R	ESBL
0765	10	R	6	R	26	S	6	R	13	R	11	R	6	R	6	R	30	S	20	I	28	S	6	R	10	R	11	R	ESBL
1759	12	R	6	R	26	S	7	R	17	I	17	R	6	R	6	R	6	R	24	S	6	R	26	S	13	I	11	R	ESBL
0883	13	R	6	R	25	S	7	R	14	R	17	R	6	R	6	R	6	R	27	S	6	R	29	S	21	S	15	S	ESBL
1758	10	R	6	R	26	S	6	R	11	R	10	R	6	R	6	R	6	R	23	S	6	R	15	I	9	R	7	R	ESBL
0768	6	R	6	R	23	S	6	R	11	R	13	R	6	R	6	R	6	R	24	S	6	R	27	S	7	R	10	R	ESBL
1218	15	I	6	R	24	S	6	R	14	R	15	R	6	R	6	R	6	R	23	S	6	R	28	S	12	I	10	R	ESBL
0769	17	I	6	R	25	S	11	R	17	I	18	I	6	R	6	R	6	R	25	S	6	R	6	R	11	R	15	S	ESBL
1204	16	I	6	R	23	S	6	R	11	R	14	R	6	R	6	R	20	I	24	S	18	I	6	R	6	R	13	I	ESBL
1208	17	I	6	R	25	S	6	R	7	R	11	R	6	R	6	R	6	R	22	S	6	R	15	I	7	R	7	R	ESBL

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Description
0910	11 R	6 R	25 S	6 6	22 S	19 I	6 R	6 R	23 S	26 S	7 R	26 S	12 I	25 S	ESBL
0953	17 I	6 R	25 S	6 6	20 S	18 I	6 R	6 R	6 R	26 S	6 R	6 R	10 R	16 S	ESBL
0934	15 I	6 R	25 S	6 6	20 S	16 R	6 R	6 R	30 S	22 S	27 S	6 R	13 I	13 I	ESBL
0954	14 I	6 R	24 S	6 6	15 I	16 R	6 R	6 R	6 R	24 S	6 R	25 S	19 S	13 I	ESBL
1744	24 S	6 R	29 S	6 R	26 S	24 S	6 R	6 R	6 R	34 S	6 R	32 S	21 S	10 R	ESBL
0938	16 I	6 R	25 S	6 R	19 S	15 R	6 R	6 R	24 S	25 S	6 R	6 R	7 R	12 R	ESBL
0964	16 I	6 R	26 S	6 R	20 S	18 I	6 R	6 R	6 R	23 S	6 R	6 R	10 R	15 S	ESBL
0710	13 R	6 R	10 R	6 R	6 R	6 R	6 R	6 R	6 R	23 S	6 R	6 R	15 S	13 I	ESBL and FOX resistant
0790	15 I	6 R	12 R	6 R	6 R	6 R	6 R	6 R	6 R	21 S	6 R	6 R	14 I	13 I	ESBL and FOX resistant
1214 Stool	11 R	6 R	10 R	6 R	20 S	14 R	6 R	6 R	6 R	24 S	6 R	6 R	10 R	11 R	ESBL and FOX resistant
0977	6 R	6 R	8 R	22 R	34 S	16 R	6 R	6 R	33 S	30 S	19 S	6 R	15 S	9 R	ESBL and FOX resistant
0783	17 I	6 R	12 R	6 R	6 R	6 R	6 R	6 R		23 S	6 R	6 R	14 I	14 I	ESBL and FOX resistant
0841	20 S	19 I	11 R	30 S	32 S	28 S	18 S	30 S	30 S	26 S	23 S	25 S	19 S	23 S	FOX resistant
0843	12 R	21 S	10 R	30 S	38 S	31 S	12 R	12 I	38 S	30 S	25 S	12 R	15 S	25 S	FOX

0743	8	R	20	I	8	R	28	S	32	S	30	S	6	R	6	R	36	S	32	S	26	S	28	S	9	R	25	S	FOX resistant
0858	7	R	22	S	7	R	30	S	38	S	36	S	9	R	6	R	39	S	32	S	25	S	30	S	20	S	25	S	FOX resistant
1729	12	R	20	I	9	R	26	S	33	S	30	S	6	R	14	I	33	S	29	S	25	S	24	S	6	R	25	S	FOX resistant
1733	12	R	20	I	10	R	30	S	30	S	28	S	9	R	18	S	35	S	30	S	25	S	28	S	10	R	25	S	FOX resistant
0811	13	R	22	S	7	R	22	S	37	S	32	S	6	R	6	R	32	S	30	S	26	S	22	S	6	R	24	S	FOX resistant
0828	13	R	21	S	7	R	25	S	32	S	27	S	6	R	6	R	31	S	24	S			24	S	6	R	23	S	FOX resistant
0807	12	R	28	S	7	R	28	S	36	S	34	S	20	S	30	S	36	S	31	S	28	S	26	S	21	S	25	S	FOX resistant
0877	13	R	23	S	9	R	26	S	38	S	32	S	18	S	7	R	25	S	29	S	23	S	16	I	22	S	26	S	FOX resistant
0906	10	R	22	S	8	R	26	S	36	S	30	S	6	R	6	R	32	S	28	S	25	S	28	S	21	S	24	S	FOX resistant
0801	14	I	23	S	10	R	26	S	35	S	30	S	16	I	30	S	35	S	28	S	26	S	25	S	20	S	24	S	FOX resistant
1703	12	R	24	S	8	R	26	S	35	S	28	S	13	R	33	S	33	S	24	S	26	S	26	S	20	S	25	S	FOX resistant
0932	12	R	24	S	8	R	25	S	38	S	30	S	20	S	6	R	36	S	28	S	28	S	27	S	21	S	24	S	FOX resistant

ID															Notes
0817	15 I	22 S	10 R	33 S	32 S	30 S	16 I	6 R	30 S	29 S	21 S	25 S	20 S	26 S	FOX resistant
0951	12 R	6 R	8 R	6 R	34 S	14 R	6 R	6 R	20 I	24 S	6 R	27 S	19 S	6 R	FOX resistant
0818	8 R	26 S	6 R	27 S	38 S	34 S	18 S		36 S	32 S	25 S	28 S	23 S	26 S	FOX resistant
0876	28 S	32 S	28 S	38 S	42 S	36 S	6 R	6 R	42 S	34 S	34 S	30 S	20 S	28 S	
742	18 S	23 S	30 S	17 R	34 S	30 S	6 R	6 R	34 S	25 S	29 S	28 S	6 R	23 S	
736	30 S	30 S	28 S	36 S	36 S	34 S	26 S	32 S	40 S	32 S	30 S	25 S	20 S	28 S	
0774	20 S	28 S	25 S	36 S	36 S	32 S	10 R	6 R	38 S	28 S	25 S	27 S	12 R	25 S	
0948	22 S	22 S	32 S	38 S	40 S	36 S	6 R	6 R	42 S	34 S	27 S	6 R	18 S	34 S	
0763	18 S	25 S	26 S	38 S	32 S	30 S	6 R	6 R	36 S	25 S	25 S	29 S	12 R	26 S	
0723	20 S	27 S	26 S	30 S	32 S	32 S	6 R	6 R	38 S	30 S	27 S	26 S	20 S	28 S	
0761	24 S	20 I	26 S	30 S	38 S	30 S	20 S	30 S	40 S	30 S	26 S	25 S	20 S	24 S	
987	20 S	6 R	25 S	32 S	36 S	30 S	20 S	6 R	40 S	30 S	26 S	38 S	15 S	24 S	
0914	20 S	30 S	27 S	36 S	34 S	32 S	6 R	6 R	38 S	30 S	28 S	30 S	14 I	24 S	
0826	18 S	26 S	25 S	38 S	36 S	34 S	6 R	6 R	42 S	30 S	28 S	29 S	12 R	28 S	
0971	18 S	27 S	26 S	34 S	33 S	30 S	15 I	6 R	33 S	26 S	25 S	6 R	12 R	11 R	
0736	28 S	28 S	28 S	38 S	40 S	34 S	24 S	30 S	35 S	31 S	28 S	26 S	20 S	24 S	
0825	22 S	26 S	28 S	32 S	36 S	32 S	6 R	32 S	6 R	32 S	28 S	28 S	22 S	26 S	
0752	20 S	26 S	28 S	34 S	34 S	30 S	6 R	23 S	38 S	30 S	26 S	24 S	10 R	30 S	

0955	20	S	27	S	29	S	30	S	30	S	30	S	6	R	6	R	30	S	28	S	23	S	26	S	6	R	22	S
886	20	S	30	S	30	S	36	S	35	S	36	S	6	R	6	R	36	S	30	S	27	S	28	S	14	I	24	S
1217	18	S	29	S	28	S	36	S	34	S	32	S	6	R	6	R	6	R	29	S	6	R	26	S	20	S	24	S
0988	20	S	30	S	30	S	38	S	36	S	32	S	6	R	24	S	6	R	22	S	6	R	16	I	10	R	7	R
0761	24	S	28	S	30	S	34	S	36	S	32	S	20	S	32	S	40	S	30	S	27	S	26	S	21	S	24	S
0750	18	S	28	S	28	S	36	S	34	S	32	S	6	R	6	R	34	S	20	I	26	S	30	S	13	I	24	S
0742	14	I	30	S	29	S	38	S	36	S	32	S	6	R	6	R	23	S	25	S	29	S	30	S	6	R	22	S
0900	26	S	30	S	30	S	34	S	37	S	34	S	20	S	6	R	40	S	35	S	29	S	28	S	20	S	26	S
0770	24	S	28	S	27	S	32	S	34	S	30	S	22	S	32	S	40	S	30	S	26	S	27	S	20	S	24	S
0743	14	I	28	S	29	S	34	S	30	S	32	S	6	R	6	R	6	R	27	S	6	R	29	S	20	S	27	S
1822	22	S	27	S	25	S	30	S	30	S	28	S	20	S	29	S	36	S	30	S	28	S	30	S	18	S	23	S
1201	22	S	28	S	24	S	36	S	32	S	30	S	20	S	27	S	34	S	28	S	25	S	25	S	25	S	24	S
0866	24	S	28	S	27	S	32	S	30	S	28	S	22	S	9	R	35	S	30	S	26	S	26	S	19	S	26	S
0704	10	R	25	S	25	S	32	S	30	S	27	S	6	R	6	R	36	S	25	S	25	S	24	S	13	I	25	S
0771	20	S	29	S	32	S	34	S	34	S	30	S	15	I	6	R	29	S	30	S	6	R	24	S	20	S	25	S
0874	15	I	22	S	13	R	34	S	40	S	30	S	15	I	30	S	35	S	28	S	25	S	28	S	20	S	25	S
0889	19	S	28	S	28	S	38	S	38	S	32	S	19	S	20	S	38	S	30	S	28	S	25	S	17	S	25	S
0960	22	S	28	S	27	S	30	S	30	S	31	S	6	R	6	R	40	S	29	S	26	S	30	S	21	S	25	S
0892	22	S	26	S	26	S	32	S	30	S	30	S	6	R	6	R	9	R	28	S	6	R	28	S	13	I	11	R

0887	17	I	25	S	30	S	32	S	30	S	30	S	6	R	6	R	40	S	25	S	28	S	26	S	10	R	23	S
0891	17	I	25	S	26	S	30	S	29	S	28	S	6	R	6	R	40	S	24	S	27	S	27	S	6	R	24	S
0759	22	S	26	S	25	S	32	S	30	S	32	S	6	R	6	R	26	S	27	S	7	R	27	S	14	I	25	S
0746	21	S	28	S	25	S	31	S	30	S	30	S	6	R	6	R	40	S	27	S	25	S	22	S	6	R	23	S
1752	30	S	34	S	34	S	32	S	40	S	38	S	30	S	32	S	40	S	32	S	30	S	30	S	23	S	27	S
0945	30	S	26	S	30	S	22	S	38	S	34	S	15	I	6	R	40	S	30	S	24	S	30	S	17	S	24	S
0774	30	S	28	S	30	S	22	S	36	S	35	S	13	R	6	R	34	S	29	S	23	S	26	S	16	S	23	S
0896	20	S	29	S	27	S	24	S	38	S	30	S	6	R	6	R	40	S	30	S	30	S	28	S	20	S	27	S
0819	26	S	29	S	25	S	24	S	36	S	32	S	22	S	6	R	37	S	30	S	27	S	30	S	20	S	25	S
0895	18	S	27	S	26	S	30	S	32	S	30	S	6	R	6	R	38	S	30	S	26	S	25	S	10	R	26	S
1739	20	S	27	S	24	S	34	S	34	S	30	S	6	R	6	R	40	S	26	S	28	S	25	S	10	R	26	S
0802	30	S	32	S	30	S	30	S	38	S	38	S	25	S	34	S	32	S	34	S	10	R	30	S	20	S	25	S
0821	16	I	27	S	27	S	24	S	30	S	29	S	6	R	6	R	31	S	24	S	6	R	6	R	18	S	25	S
0824	24	S	30	S	30	S	26	S	37	S	30	S	7	R	6	R	32	S	29	S	25	S	6	R	12	I	30	S
0988	30	S	30	S	29	S	25	S	40	S	34	S	22	S	6	R	40	S	29	S	25	S	25	S	20	S	25	S
0320	30	S	26	S	29	S	20	S	40	S	30	S	9	R	6	R	35	S	30	S	26	S	29	S	18	S	25	S
E. coli ATCC 25922	24	S	27	S	30	S	32	S	30	S	29	S	17	S	30	S	40	S	30	S	28	S	28	S	20	S	28	S	Control

*Interpratation was done using CLSI 2015 guidelines (CLSI, 2015)

Appendix 12   Co-carriage of other genes encoding various classes of antimicrobials

Category	MLST	Integron Associated Genes	Non-Integron Associated genes
NM,32mnths, Male		*sul1, dfrA1*	$bla_{CTX-M-15}$
NM		*dfrA16, sul1*	$bla_{KLUA-9 \text{ 'like'}}$
SM,27mnths, Female	4	*ereB, strB, strA,dfrA17, catA1, aac-(6')-lb-cr, bla$_{OXA-1}$, aph3, sul1*	*sul2, tetC, mphA,* $bla_{CTX-M-15}$
SM,10mnths, Female	38	*bla$_{OXA-1}$, catB4*	$bla_{CTX-M-15}$
SM,14mnths, Female	44	*ereB, strB, strA, dfrA17, sul1, catA, strB, bla$_{OXA-1}$, aac-(6')-lb-cr, aph3, mphA*	$bla_{CTX-M-15}$*, tetC, sul2*
SM,6mnths, Female	44	*ereB, sul1, drfA17, sul2, strA, strB, mphA, aac-(6')-lb-cr, bla$_{OXA-1}$, aph3, catA1*	$bla_{CTX-M-15}$*, tetC*
SM,14mnths, Female	131	*aac-(6')-lb-cr, bla$_{OXA-1}$, dfrA17, sul1, mphA, strA*	*tetA, sul2,* $bla_{CTX-M-15}$
SM,12mnths, Male	131	*mphA, sul1, cmlA1, aadB, aac-(6')-lb-cr, bla$_{OXA-1}$, catB3*	$bla_{CTX-M-15}$*,* bla_{TEM-1}
SM,16mnths, Male	131	*drfA17, sul1, mphA, sul2, strA, strB, aac-(6')-lb-cr, bla$_{OXA-1}$*	$bla_{CTX-M-15}$*, tetA*

SM,30mnths, Female	131	*mphA, sul1, dfrA17, strA, strB, bla*$_{OXA-1}$*, aac-(6')-lb-cr, aadB, cmlA1*	*bla*$_{TEM-1}$*, tetA, bla*$_{CTX-M-15}$*, sul2*
SM,12mnths, Male	131	*catB3, aac(6')-Ib, bla*$_{OXA-1}$*, qacDeltaE1, aadA1'*like'	*bla*$_{CTX-M-15}$
SM,2mnths, Female	131	*aadB, cmlA1, sul1, mphA, sul2, strA, strB, aac-(6')-lb-cr, bla*$_{OXA-1}$*, dfrA17*	*bla*$_{TEM-1}$*, tetA, bla*$_{CTX-M-15}$
SM,24mnths, Male	131	*sul1, qacEdelta1, catB4, aac(6')-Ib-cr*	*bla*$_{CTX-M-15}$
SM,9mnths, Male	131	*mphA, sul1, cmlA1, aadB, bla*$_{OXA-1}$*, aac-(6')-lb-cr*	*bla*$_{TEM-1}$*, bla*$_{CTX-M-15}$
SM,3mnths, Female	131	*aadB, cmlA, sul1, mphA, aac-(6')-lb-cr, bla*$_{OXA-1}$	*bla*$_{TEM-1}$*, bla*$_{CTX-M-15}$
SM,7mnths, Female	131	*AAC(3)-II, catB3, mphA and mphR, dfrA17, aadA5, sul1, QacEdelta1*	*bla*$_{CTX-M-15}$
SM,6mnths, Female	131	*dfrA14, mphA, strB, strA*	*bla*$_{TEM-158}$*, sul2, tetA, bla*$_{CTX-M-15}$
NM,22mnths, Female	131	*dfrA17, sul1, mphA, bla*$_{OXA-1}$*, aac-(6')-lb-cr*	*bla*$_{CTX-M-15}$
SM,8mnths, Female	167	*dfrA1, aac-(6')-lb-cr, bla*$_{OXA-1}$*, strA, strB, dfrA17, sul1, mphA*	*bla*$_{CTX-M-15}$*, sul2, tetB*

SM,8mnths, Female	205	*dfrA1, ereB, catA1, strA, strB, bla*$_{TEM-1}$*, sul1, dfrA17, mphA*	*tetB, sul2, bla*$_{CTX-M-15}$*, tetC, bla*$_{AmpC}$
SM,9mnths, Female	205	*dfrA1, ereB, strB, strA, catA1, dfrA17, sul1, mphA*	*bla*$_{TEM-1}$*, bla*$_{CTX-M-15}$*, tetB, sul2, tetC, bla*$_{AmpC}$
NM,46mnths, Male	405	*strB, strA, sul2, mphA, sul1, dfrA17, aac-(6')-lb-cr, bla*$_{OXA-1}$	*bla*$_{TEM-1}$*, bla*$_{CTX-M-15}$*, tetB, bla*$_{AmpC}$
SM,16mnths, Male	405	*dfrA17, sul1, strB, strA, bla*$_{OXA-1}$*, aac-(6')-lb-cr, mphA*	*bla*$_{TEM-1}$*, bla*$_{CTX-M-15}$*, tetB, sul2, bla*$_{AmpC}$
NM,17mnths, Male	405	*dfrA17, sul1, strB, strA,mphA, aac-(6')-lb-cr, bla*$_{OXA-1}$*, catB4*	*sul2, bla*$_{CTX-M-15}$*, tetB, bla*$_{TEM-1}$*, bla*$_{AmpC}$
SM,2mnths, Female	617	*qepA, dfrA12, sul1, mphA, strB, strA, sul2*	*bla*$_{CTX-M-15}$*, bla*$_{TEM-1}$*, tetB*
SM,15mnths, Female	648	*mphA, sul1, dfrA17, strB, strA, catA1, aac-(6')-lb-cr, bla*$_{OXA-1}$	*bla*$_{CTX-M-15}$*, bla*$_{TEM-1}$*, sul2, tetB, bla*$_{AmpC}$
NM,18mnths, Female	940	*drfA1, mphA, bla*$_{OXA-1}$*, catA1, strB, dfrA14*	*bla*$_{TEM-33}$*, bla*$_{CTX-M-15}$*, sul2, tetB*
SM,19mnths, Female	1642	*sul1, dfrA17, catA1, mphA, strB, strA, aac-(6')-lb-cr, bla*$_{OXA-1}$	*bla*$_{CTX-M-15}$*, tetB, bla*$_{TEM-1}$*, sul2,*
SM,14mnths, Female	1675	*strA, strB, aac-(6')-lb-cr*	*sul2, bla*$_{TEM-1}$*, bla*$_{CTX-M-15}$*, bla*$_{AmpC}$

| SM,11mnths, Female | 1722 | *catA1, dfrA17, sul1, mphA, strB, strA* | *bla*_{CTX-M-15}, *tetB, bla*_{TEM-1}, *sul2* |

SM,11mnths, Female	1722	*catA1, dfrA17, sul1, mphA, strB, strA*	*bla*$_{CTX-M-15}$, *tetB, bla*$_{TEM-1}$, *sul2*
SM,12mnths, Male	4481	*sul1, dfrA17, mphA, aac-(6')-lb-cr,* blaOXA-1	*bla*$_{CTX-M-15}$

Key

NM   - Non-malnourished
SM   - Severely Malnourished
ereB   - erythromycin resistance
strB and *strA*   - streptomycin resistance
dfrA17, drfA1, dfrA16 - trimethoprim resistance
sul1   -sulphonamide resistance
catA1, catB4, catB3   - chloramphenicol resistance
bla$_{OXA-1}$- oxacillin resistance
aac-(6')-lb-cr - aminoglycoside/ciprofloxacin resistance
aph3   - confers resistance to Kanamycin, Neomycin, Paromomycin, Ribostamycin, Butirosin, GentamycinB
mphA   - Macrolide resistance
QacEdelta1   -antiseptics with quaternary ammonium compounds resistance
AAC(3)-II   - high-level aminoglycoside resistance
aadB   - resistance to streptomycin and spectinomycin
cmlA   - chloramphenicol resistance
aadA1'like'   - resistance to streptomycin and spectinomycin
bla$_{TEM-1}$ - ampicillin resistance
bla$_{CTX-M-15}$ -3rd generation cephalosporin resistance
tetA, *tetB*,*tetC* - tetracycline resistance
bla$_{AmpC}$ - cephamycin resistance
qepA   - quinolone resistance