ANTIMICROBIAL RESISTANCE PROFILES AND GENETIC CHARACTERIZATION OF ESCHERICHIA COLI ISOLATED FROM WATER IN ATHI RIVER, MACHAKOS

PERIS WANJIRU WAMBUGU

A MASTER OF SCIENCE

(Medical Microbiology)

JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

2016

Antimicrobial Resistance Profiles and Genetic Characterization of *Escherichia coli* Isolated From Water in Athi River, Machakos

Peris Wanjiru Wambugu

A thesis submitted in partial fulfillment of the degree of Master of Science in Medical Microbiology, in the Jomo Kenyatta University of Agriculture and Technology

2016

DECLARATION

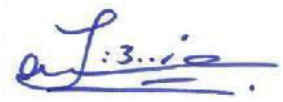
This thesis is my original work and has not been presented for the award of a degree in any academic institution.

21/09/16

Signature: …….. Date: ……

Peris Wanjiru Wambugu

This thesis has been submitted for examination with our approval as University Supervisors.



20th sept 2016

Signature: ……

Date…….

Dr. John Kiiru (PhD)

KEMRI, Kenya

21/09/16

Signature: ……. Date…….

Prof. Viviene Matiru

JKUAT, Kenya

DEDICATION

This thesis is dedicated to my entire family for their endless support. Their prayers and moral support have been the driving momentum that has enabled me to achieve the targeted goal in my studies.

ACKNOWLEDGEMENTS

I would like to begin by thanking and acknowledging almighty God for having given me the strength, knowledge, understanding and resilience to undertake this project. To my supervisors Dr. John Kiiru and Prof. Viviene Matiru who shared a lot of their expertise and research insights with me. Their advice, insightful criticisms and patient encouragement aided in completion of this thesis.

I would also like to thank to Prof Samuel Kariuki, the Director at the Centre of Microbiology Research for allowing me to do my experiments in the CMR facilities as well as all the CMR staff who helped in all ways they could.

I gratefully acknowledge my sponsor, The National Commission for Science, Technology and Innovation for the research grant they offered to make the research work possible.

Most importantly, I wish to thank my mother for all her prayers and rest of my family for their moral support.

Finally, to all those who tirelessly supported me morally, socially and any other sought of input, may God bless you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

ARGs	Antimicrobial resistance genes
aggR	Aggregative gene
Bfp	Bundle forming pili
bla	Beta- lactamase gene
CFUs	Colony forming units
CLSI	Clinical Laboratory Standards Institute
CMR	Centre for Microbiology Research
CMTs	Complex mutant TEMs
CMY	Cephamycinase Enzymes
CTX-M	CefoTaXimases ‘ Munich’
DAEC	Diffusely adherent *E. coli*
DNA	Deoxyribonucleic acid
Eae	Intimin adherence protein
EAEC	Enteroaggregative *E. coli*
E. coli	*Escherichia coil*
EHEC	Enterohemorrhagic *E. coli*
EDTA	Ethylene diamine-tetra acetatic acid
EIEC	Enteroinvasive
ESBLs	Extended spectrum B-lactamases
EPEC	Enteropathogenic

ETEC	Enterotoxigenic *E. coli*
Inv	Invasive gene
IpaH	Invasion plasmid antigen H
IRT	Inhibitor resistant TEM (Temoneira Enzymes)
LT	Heat labile toxin
KEMRI	Kenya Medical Research Institute
MICs	Minimal Inhibitory Concentrations
MDR	Multi Drug Resistance
NSBLs	Narrow spectrum β-lactamase phenotypes
OXA	Oxacillinase Enzymes
PAmp C	Plasmid encoded Amp C
PCR	Polymerase Chain Reaction
SHV	Sulfhydrl Variable Enzymes
ST	Heat stable toxin
Taq	*Thermus aquaticus*
TEM	Temoneira Enzymes
VGs	Virulence genes
WHO	World Health Organization
WWTP	Waste water treatment plant

ABSTRACT

Antimicrobial use in agriculture, livestock and in human health has increased over the years leading to increase in antimicrobial resistance among strains that can also find their way into the aquatic environments. Rivers can act as reservoirs of highly resistant strains and facilitate the dissemination of multidrug resistant (MDR) strains to animals and humans using the water. The objective of the study was to determine antimicrobial resistance profiles and genetic characterization of *Escherichia coli* isolated from water in Athi River, Machakos. The study design was cross-sectional. A total of 318 *E. coli* isolates (53 from each of the size site) were analysed for antibiotic susceptibility and characterized using polymerase chain reaction (PCR). Descriptive analysis using mean, frequency and proportion were used to analyse the data. Chi square test, odds ratio and confidence intervals (set at 95%) were used to assess drug susceptibility between the sites. Student's t-test was also computed to compare the means of coliform count during dry and wet season. Sewage effluent site most highly contaminated (mean of $9.5x10^3$ CFUs/ml) and virgin land with the least CFUs (mean $9.5x10^2$CFUs/ml). *E. coli* isolates were most resistant to ampicillin (63.8%) and most susceptible to gentamicin (99.4%). The prevalence of Multi drug resistant (MDR) strains (resistance to ≥3 classes of antimicrobials) was 65.4% in all sites. The prevalence of extended spectrum β-lactamases (ESBLs) was 2.2% out of 318 and their distribution was not related to the sites. Based on PCR results, 22% had bla_{TEM}, 33% had $bla_{\text{CTX-M}}$ and 28% had bla_{CMY}. The prevalence of typical Enteropathogenic *E. coli* (EPEC) strains (carrying both *eae* and *bfp* genes) was 5% while the prevalence of atypical EPEC (carrying only *eae*) was 1.8%. The prevalence of Enteroaggregative *E. coli* (EAEC) carrying the *aggR* gene was 11%. The prevalence of Enterotoxigenic *E. coli* (ETEC) encoding only *lt* toxin was16 (5%) and while those carrying only *st* toxin was 6.9%. The prevalence of Enteroinvasive *E. coli* strains (EIEC) encoding as *IpaH* was 5% while that of strains, adherent invasive *E. coli* (AIEC), carrying adherent invasive gene *inv* was 8.7%. PCR analysis also showed that 36% isolates were positive for class 1integrons. It can be concluded that water from Athi River is not safe for human consumption and region near the sewage treatment plant requires most attention for decontamination. Better management of

sewage treatment plant should be encouraged as well as reduction of anthropogenic activities near water systems.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Rise in resistance to multiple antimicrobials among *E. coli* is a major concern both in developed and developing countries. Evolutionary pressure arising from widespread use of antimicrobials has contributed to the emergence of multidrug-resistant (MDR) strains and the spread of resistance between and among bacterial species. The major cause of the emergence of MDR strains is the misuse and overuse of antimicrobials. Antimicrobial resistance has also been influenced by activities along a water body such as misuse of antimicrobials in humans and veterinary industries and as a result of drainage of sewage containing heavy metal. This presents a strong selection pressure for resistant strains. In addition, contamination of water sources as a result of pollution with fecal bacteria has potential to spread highly resistant and pathogenic strains (Kummerer, 2009).

Water bodies and aquatic systems have great potential as sources of infectious bacteria to people who use the water for recreational activities, fishing, drinking, bathing and irrigation of crops, especially those eaten raw (Liu *et al*., 2006). *E. coli* is an important indicator organism for fecal pollution in environmental waters and has also been useful in monitoring antimicrobial resistance patterns in gram-negative bacteria and therefore, pollution of water bodies with MDR strains, especially those suspected to be of human origin requires evaluation (Hawkey *et al*., 2009; WHO, 2006).

Some *E. coli* strains have acquired virulence genes that allow them to cause various infections such as diarrhea and hemolytic-uremic syndrome (HUS), (Xi *et al*., 2009). If such pathotypes find their way into the water systems, their potential for spread could be multiplied. Clinical isolates can find their way into water systems through fecal matter from humans and animals. This is particularly the case if the sewerage systems are not properly designed or where water treatment is poor. The MDR strains can be a challenge due to public use of untreated water.

In Africa, multidrug resistance in fecal coliforms in water has been reported in Nigeria, Ethiopia with limited studies in Kenya (Ali *et al*., 2012; Felicitas *et al*.,

2010). When it comes to resistance of isolates to antimicrobials, β-lactams have been highly affected. Most importantly is the emergence of Extended-spectrum β-lactamases (ESBLs) which affects the health sector. ESBLs are a rapidly evolving group of β-lactamases which share the ability to hydrolyze third-generation cephalosporins and aztreonam yet are inhibited by clavulanic acid. Typically are derived from genes $bla_{\text{TEM-1}}$, $bla_{\text{TEM-2}}$ or $bla_{\text{SHV-1}}$ by mutations that alter the amino acid configuration near the active site of these β-lactamases. This affects the spectrum of β-lactam antibiotics susceptible to hydrolysis by these enzymes. The presence of ESBLs, especially those that are plasmid-encoded is of tremendous clinical significance. Plasmids responsible for ESBL production frequently carry genes encoding resistance to other antimicrobial classes (for example, aminoglycosides and fluoroquinolones). Therefore, options available for the treatment of ESBL-producing organisms are extremely limited (Paterson & Bonomo 2005).

The presence of antimicrobial resistance genes on mobile genetic elements leads to their dissemination and possible development of multi-resistance phenotype. The dissemination of resistance is associated with genetic mobile elements, such as plasmids, that may also carry virulence determinants. A combination of resistance genes and virulence factors enable a host to replicate and disseminate these genes to other hosts with ease. As a result of using antimicrobials, bacteria can evolve resistance that can be passed to commensal and other pathogens sharing the same ecosystem, *e.g*. the human gut. It is assumed that virulent MDR strains are more difficult to control than other strains and this impacts on patient's chemotherapeutic success. Presence of such bacteria in healthcare, animal and environmental setting is a major public health concern because such bacteria are highly virulent and untreatable using antimicrobials (Holt *et al*., 2015).

Among resistance determinants, integrons have received significant attention. Integrons are genetic elements that are borne on plasmids and/or chromosomes and containing site-specific recombination sequences that mediate, capture and mobilize resistance genes known as cassettes. At present, three classes of integrons are known to have a role in the dissemination of antibiotic resistance genes. These classes are distinguished by their respective genes (*int* I*)*, encoding class-specific integrases.

Classes, 1, 2 and 3, are strongly associated with resistance to multiple antibiotics, with the class 1 integrons being the most prevalent. The high prevalence of class 1 integron is partially due to their association with *Tn*21 transposon which is more promiscuous and associates with multiple conjugative plasmids (Dakic *et al*., 2007). The ability of *E. coli* to carry plasmid-borne integron 1 suggests that the encoded antimicrobial resistance genes can easily be transferred among bacteria and even between pathogenic commensal strains and environment *E. coli* trains. It is of interest to note that if such highly resistant strains enter aquatic systems, their chances of spread are highly increased.

Rivers and aquatic systems are important environments for exchange of resistance determinants among enteric and environmental isolates due to activities along a water body such as drainage of sewage containing heavy metal that result in natural selection of resistant strains, humans' extensive use of antibiotics in agriculture and health. This promotes displacement of susceptible strains with resistant ones which find their way to the aquatic environments as well as use of detergents which can select for MDR strains in sections of rivers where domestic activities such as washing clothes and household items with detergents take place (Coutinho *et al.,* 2013).

Athi River in Machakos County is a heavily polluted water system mainly as a result of contamination from sewerage originating from Westlands and Kasarani areas in Nairobi (Musyoki *et al.*, 2013). However, little is known about the carriage of *E. coli*, antimicrobial susceptibility patterns and the genetic basis of resistance from this river. The aim of this cross-sectional study was therefore to determine the carriage of *E. coli* as an indicator of pollution and antimicrobial susceptibility profiles among strains isolated from different sections of this river. It also determined the molecular basis of resistance to selected antimicrobials and carriage of virulence genes and integron 1 gene among the isolates.

1.2 Statement of the problem

Antimicrobial use in agriculture, livestock and in human health has increased over the years leading to an increase in antimicrobial resistance that can also find its way to the aquatic environments. Rivers can also act as reservoirs of highly resistant strains and can facilitate the dissemination of MDR strains including ESBL-

producers. This process, involving notable co-selection events, could cause an ecological imbalance leading to the dominance of resistant bacteria and their eventual disturbance in multiple global ecosystems. Human activities along water bodies affect microbial contamination levels as a result of pollution of the water ways with fecal matter, agricultural and industrial activities. Antimicrobial resistance has also been influenced by activities along a water body such as drainage of sewage containing heavy metal that result in natural selection of resistant strains, extensive use of antibiotics by humans in agriculture and health that promote displacement of susceptible strains with resistant ones finding their way to the aquatic environments. Water systems are one of the major sources of diarrhoeic pathogens and drainage of untreated waste water from sewage plants into surface water can lead to spread of resistant isolates that can result in serious waterborne diseases with limited antimicrobials available for treatment. In Kenya, antimicrobial resistance has been determined in very limited studies and most of these studies mainly focus on clinical isolates. Limited studies have been done to show the extent of microbial contamination in Athi River (Musyoki *et al*., 2013). Furthermore, only scanty information is available on carriage of *E. coli* in this water and the resistance mechanisms among water isolates have not been elucidated. Until now, no study has determined how human activity impacts on microbial contamination of this river whose untreated water is used for commercial and domestic purpose and therefore can be a source of infectious strains to the public. In the absence of this data, it is difficult to recommend any suitable interventions concerning contamination and to recommend treatment regimens in case these strains are implicated in human infections.

1.3 Justification

A high prevalence MDR strains has been reported in many parts of the world including Kenya. This trend presents a great challenge to public health because some pollutants such as detergents can select for MDR strains. In Kenya there is limited data regarding antimicrobial resistance phenotypic encountered among *E. coli* from aquatic systems and the genetic basis of such resistances are yet to be elucidated. There is therefore a need to determine contamination levels of various aquatic systems and proportions of MDR strains. It is also important to determine the genetic

basis of resistance and the major groups of elements that encode such resistances. The aim of this study was to assess whether Athi River carries a significant burden of MDR *E. coli* and what pathotypes are represented in this ecosystem. The data presented in this work will be useful in addressing public health concerns about the potential risk to health of the communities living near the contaminated water as well as help in formulation of intervention policies and recommendations applicable locally.

1.4 Research questions

1. What is the occurrence of *E. coli* as an indicator of pollution at different sections of the Athi River?

2. What are the resistance profiles of *E. coli* isolates recovered from different sections of Athi River?

3. What is the prevalence of multi drug resistance and proportion of extended spectrum β- lactamases producers?

4. What is the prevalence of various *E. coli* resistance genes, pathotypes and common resistance phenotypes among such pathotypes and isolates carrying class 1 integrons?

1.6 Objectives

1.6.1 General Objective

To determine antimicrobial resistance profiles, resistance genes, virulence genes and integron 1 from *E. coli* isolated from water in Athi River, Machakos.

1.6.2 Specific Objectives

1. To investigate the occurrence of *E. coli* as an indicator of pollution at different sections of the Athi River.

2. To assess resistance profiles of *E. coli* isolates recovered from different sections of Athi River.

3. To determine the prevalence of multidrug resistance and proportion of isolates exhibiting resistance to extended spectrum β-lactamases.

4. To determine the prevalence of various *E. coli* resistance genes, pathotypes, common resistance phenotypes among such pathotypes and if these isolates carry class 1 integrons.

1.7 Scope of the study

E. coli occurrence were determined and the resistance phenotypes of the isolates at different sections of the river. MDR and ESBL proportions and carriage of virulence genes and class 1 integrons among these isolates were also determined. The six sampling sites were determined by the type of activities occurring near or on the river which were: domestic use (water used for domestic purposes such as drinking and washing), settlements (houses built very close to the river bed, less than 200 meters), virgin land (with no apparent evidence of recent or past human activity including farming and settlement), Sewage site near sewage influent/effluent from sewage treatment plant, and sections near road as well and those near industrial installations. The sites were sampled during the wet and the dry seasons over a six month period.

1.8 Limitations of the study

Due to lack of sophisticated equipment to reach the middle of the river, sampling was restricted to the banks collecting run offs and shallow water that was easy to access.

1.9 Expected benefits of the Research

The data generated from the study will be used to advise NEMA on the true state of pollution of Athi River as well as alert Ministry of health (MoPHs). The data from this work also provided a platform for assessing the level of MDR contamination in the environment and prevalence of *E. coli* pathotypes. This knowledge will be useful in determining treatment therapy options for individuals coming from this study area.

CHAPTER TWO

LITERATURE REVIEW

2.1 Pollution and contamination of river waters

Approximately a quarter of the diseases facing mankind today occur due to prolonged exposure to water pollution mainly from the discharge of industrial effluent into water bodies causing environmental contamination and degradation in many cities, especially those in developing countries (Smith & Coast 2002). Water quality can be measured by use of indicators of pollution such as fecal coliform bacteria naturally present in the human digestive tract, but rare or absent in unpolluted water. *E. coli* is one of fecal flora in many species including humans and is an excellent indicator for water contamination. Rainfall and run off have been implicated in water borne disease outbreaks in both developed and developing countries due to increase in microbial pollution (Shehane *et al.*, 2005).

2.2 Antimicrobial resistance in *E. coli*

It should be noted that the pollution of water can select antibiotic resistant bacteria. This process, involving co-selection events, could cause an ecological imbalance leading to the dominance of resistant bacteria strains and their global dissemination. The development of antibiotic resistances very likely occurs naturally in the environment, but factors like pollution, especially of water bodies, could increase the volume of the genes and hasten strains evolution. Improved water quality could limit this phenomenon. Despite this, the status of the water quality especially untreated water that is used for domestic purposes in many developing countries is poorly investigated (Lupo *et al*., 2012).

Human activities along water bodies affect microbial contamination levels as a result of pollution of the water ways with faecal matter, agricultural and industrial activities. Untreated sewage or manure coming from human or animals treated with antibiotics can contaminate aquatic and soil environments and may lead to emergence of antimicrobial-resistant strains (Kummerer 2009; Lupo *et al.,* 2012; Suzuki *et al.,* 2012). Some antibiotics may not be fully eliminated or deactivated during the sewage treatment process and these can provide sufficient evolutionary

pressure to select for MDR strains. Therefore, aquatic environments such as rivers can act as reservoirs of highly resistant strains (Kummerer 2004; Lupo *et al*., 2012).

Antimicrobial resistance has also been influenced by activities along a water body such as drainage of sewage containing heavy metals that result in natural selection of resistant strains, extensive use of antibiotics in agriculture and human health that erode commensal bacteria leaving resistant ones. Studies carried out in China, Brazil and US areas showed differences in site contaminations along rivers and varying antimicrobial susceptibilities among strains. Statistical differences were found between antimicrobial susceptibilities of isolates obtained from different sites further suggesting that activities along the river contribute to different results. These studies show anthropogenic activities, waste discharge and industrial activities propagate antibiotic resistant bacteria in aquatic environment (Coutinho *et al*., 2013; Hamelin *et al*., 2007; Tao *et al*., 2010).

Resistant *E. coli* in aquatic environment has been reported from different water sources such as hospital effluents, sewage, sludge, De Boeck, ocean and rivers. This is a public health concern because *E. coli* is an indicator for environmental contamination with microbes from the gut ecosystem (Hamelin *et al*., 2007). Factors that contribute to antimicrobial resistance in aquatic environment are the over-usage of antibiotics in clinics which is been believed to be the principal element involved in the rise of new resistances. More recent evidence suggests that habitats such as rivers and streams are ideal environments for the antibiotic resistance dissemination. Here, the propagation of bacteria harbouring antibiotic resistance genes can occur spatially along the river. Furthermore, the dispersion of these bacteria in the environment favours the interaction with the autochthonous microbiota, creating new scenarios for the evolution of antibiotic resistances (Lupo *et al*., 2012). Presence of heavy metals and cations due to industrial pollution are known to co-select for MDR strains (Baker-Austin *et al*., 2006).

In strong contrast to clinics, there are no data available on the epidemiology of antibiotic resistances in the environment. This in turn makes it extremely difficult to predict the risk of spread and emergence of new resistant strains in non-human ecosystems. For this reason, a better knowledge on the environmental reservoir of

resistances is fundamental to predict the emergences of new resistances of clinical concern (Amaya *et al*., 2012).

2.3 Bacterial mechanisms of antibiotic resistance

Pathogenic and potentially pathogenic bacteria from human and animals are constantly released from waste water into the aquatic environment. Many of these organisms harbour antimicrobial resistant genes, especially those inserted into genetic platforms such as self-transmissible plasmids and transposons and also in integrons (Chen *et al*., 2011). Investigating strains that harbor resistance gene, virulence genes and integrons is essential in understanding the epidemiology of resistance in the environment. β-lactam drugs mechanism of action is inhibition of the last stage of bacterial cell wall synthesis and are the largest family of antimicrobial agents and the most widely used in current clinical practice (Kong *et al*., 2010). More so, β-lactam antimicrobials are widely used in Kenya where treatment with alternative or more effective agents may be beyond the reach of majority of patients due to cost and therefore their resistance levels in aquatic environment are of key importance *(*Xi *et al.,* 2009).

2.3.1 ß-lactam resistance

β-lactam antibiotics consist of a broad class of antibiotics containing a β-lactam ring in their molecular structures.This includes penicillin and its derivatives, cephalosporins, monobactams, and carbapenems. β-lactams constitute the main therapeutic choices to treat serious infections including those caused by *E. coli* (Zhang & Chang 2015).

2.3.2 Molecular basis of resistance to β-lactams

There are several hundred types of β-lactamase enzymes, coded for by different β-lactamase (*bla*) genes. Their individual differences lie in the amino-acid structure of the protein molecules. These enzymes are categorized into families, based on genetically similar characteristics. Several of these genes are specific which confirm the ESBL phenotype. ESBLs are a rapidly evolving group of β-lactamases which share the ability to hydrolyze third generation celaphosporins and aztreonam yet are inhibited by clavulanic acid and the most common genes they exhibit are $bla_{\text{CTX-M}}$,

bla$_{TEM}$, and *bla*$_{SHV}$ families. Each ESBL bacterium may possess genes for one or more of these enzymes (Rashid *et al*., 2015). Phylogenetically similar *bla* genes with structural likeness are grouped together within these gene families and are often targeted for detection and identification using PCR. For instance, *bla* genes coding for the *bla*$_{TEM-1}$ enzyme are found within group 1 of the *bla*$_{TEM}$ family. Some ESBL types, such as *bla*$_{TEM}$ and *bla*$_{OXA}$, have also been shown to possess resistance genes against β-lactamase inhibitors (*e.g. IRT* genes), making such species more of a threat. Furthermore, since *bla* genes are usually found encoded on mobile vectors such as plasmids (*bla*$_{CTX-M}$, *bla*$_{SHV}$) and transposons (*bla*$_{TEM}$), the transfer of resistance between bacteria is easily facilitated. This implies the easy spread of disease in the event of outbreaks (Tissera & Lee 2013). β-lactamases are diverse with their own substrate spectrums which are Narrow Spectrum β-lactamases (NSBLs), Extended spectrum ß-lactamases (ESBLs), Complex Mutant TEMs (CMTs), and ampCs that are discussed below.

2.3.3 Narrow Spectrum β-lactamase Phenotypes (NSBLs)

Based on resistance to β-lactam antibiotics, bacteria strains may be conveniently categorized into various resistant phenotypes. Strains exhibiting Narrow Spectrum β-lactamase phenotypes (NSBLs) normally produce *bla*$_{TEM-1}$ and/or *bla*$_{SHV-1}$ enzymes that effectively degrade basic antibiotics such as penicillins but are susceptible to other classes of β-lactams. However, mutations on the promoter region of the gene encoding *bla*$_{TEM-1}$ may result to over-production of these otherwise narrow-spectrum enzymes. This overproduction may in turn confer resistance to other classes of β-lactams besides penicillins. Point mutations on these enzymes may also generate inhibitor resistant enzymes such as the Inhibitor Resistant TEMs (IRTs) that degrade penicillins not cephalosporins but are not sensitive to by β-lactamase inhibitors such clavulanic acid or sulbactam (Bennet,2008 ; Kiiru *et al*., 2012).

2.3.4 Complex Mutant TEMs (CMTs)

Complex Mutant TEMs (CMTs) are also derived from TEM-1 or TEM-2 and degrade most β-lactams but are susceptible to advanced classes β-lactamase inhibitors such ad tazobactam but not to clavulanic acid or sulbactam. The CMTs are also susceptible to cephamycins and carbapenems (Jacoby *et al*., 2006).

2.3.5 AmpC β-lactamases

Plasmid–encoded AmpC (pAmpC) such as bla_{CMY}s mediate resistance to most classes of β-lactams except to fourth generation cephalosporins and carbapenems (Kiiru *et al*., 2012). AmpC β-lactamases are clinically important cephalosporinases encoded on the chromosomes of many of the Enterobacteriaceae and a few other organisms, where they mediate resistance to cefoxitin, cephalosporins most penicillins, and most β-lactamase inhibitor-β-lactam combinations. In many bacteria, AmpC enzymes are inducible and can be expressed at high levels by mutation. Over-expression confers resistance to broad-spectrum cephalosporins including cefotaxime, ceftazidime, and ceftriaxone and is a problem especially in infections due to *Enterobacter aerogenes* and *Enterobacter cloacae*, where an isolate initially susceptible to these agents may become resistant upon therapy. Transmissible plasmids have acquired genes for AmpC enzymes, which consequently appear in bacteria *E. coli* that lack or poorly express chromosomal bla_{AmpC} genes. Resistance due to plasmid-mediated AmpC enzymes is less common than extended-spectrum β-lactamase production in most parts of the world but may be both harder to detect and broader in spectrum (Jacoby, 2009).

2.3.6 Extended spectrum ß-lactamase enzymes (ESBLs)

Extended spectrum ß-lactamase enzymes (ESBLs) are modified ß-lactamases which are mainly plasmid-borne and have evolved from point mutations on TEM 1/2, SHV-1. Other ESBLs include bla_{CTX-M} that are plasmid-borne and are not derived from TEM or SHV enzymes (Clermont *et al*., 2009). The ESBL enzymes are widespread all over the world, but their prevalence among clinical isolates may vary between geographical regions (Jacoby *et al.,* 2006; Sirot *et al*., 1991). ESBLs exhibit a wide hydrolytic ability to different generations of cephalosporins but remain susceptible to β-lactamase inhibitors.

In recent years, ESBL-producing *Enterobacteriaceae* isolates have shifted from the hospital to the community and the environment. ESBL-producing bacteria have been recovered from different sources in the community, including food and companion animals and one recent study from India reported that a substantial number of tap water samples were contaminated with carbapenemase bla_{NDM-1} producing organisms (De Boeck & Sacramento 2012). Dissemination of ESBLs producing genes can

occur through horizontal gene transfer from resistant pathogens in waste water to environmental non pathogenic bacteria. Environmental non-pathogenic bacteria would then serve as a reservoir of resistance genes. The risk of contamination is greatest when wastewater is discharged directly into the environment (Armine, 2013).

According to Storberg who summarized Africa ESBL reports published 2008-2012, he found $bla_{\text{CTX-M-15}}$ was the most prevalent gene in a high proportion of the samples, disregarding the country and were usually combined with other types of $bla_{\text{CTX-M,}}$ bla_{TEM}, and bla_{SHV} genes. In the community setting, ESBL-producing *Enterobacteriaceae* had a lower prevalence than in the hospital; however genes involved in the community were the same as in the hospital setting (Storberg, 2014).

2.4 Dissemination of *bla* genes in aquatic systems

Water systems may harbour isolates resistant to β-lactams (Blaak *et al*., 2014). Genes that have been identified as mediators of β-lactam resistance among isolates recovered from aquatic systems include $bla_{\text{CTX-M}}$, bla_{TEM} and bla_{CMY}. A study in Portugal reported that the occurrence and diversity of $bla_{\text{CTX-M}}$ genes were clearly different between polluted and unpolluted ecosystems; these findings favored the hypothesis that natural environments are reservoirs of resistant bacteria and resistance genes, where anthropogenic-driven selective pressures may be contributing to the persistence and dissemination of genes usually relevant in clinical environments (Tacão *et al*., 2012).

In aquatic environment, positive AmpC beta-lactamase gene from *E. coli* was isolated from hospital wastewater in city of Mainz, Germany (Schwartz, 2003). In Kenya, studies have been restricted to clinical isolates and there are no published studies showing presence of bla_{AmpC} gene in water.

Some of the commensals as well as pathogenic *E. coli* that are excreted into the environment may have the capacity to produce ESBL. ESBL-producing *E. coli* have been detected in surface water in United Kingdom River (Dhanji, 2011). Human exposure to these bacteria may occur, for instance during recreation in contaminated surface water, or indirectly, when contaminated surface water is used for irrigation of (raw consumed) crops, therewith contributing to community-associated

dissemination of ESBL-producing *E. coli*. Additionally, contaminated surface water might contribute to exposure of animals that drink from it (Blaak *et al*., 2014).

In a study in China, twenty aquatic isolates contained a single *bla* gene including $bla_{CTX\text{-}M}$ (17 strains), bla_{TEM} (2 strains) and bla_{SHV} (1 strain). Forty six isolates had more than one type of β-lactamase gene. It was concluded that contamination with ESBL producing Enterobacteriaceae in rural water environment was closely related to local animal farms and anthropogenic activities (Zhang & Chang 2015). In Kenya, $bla_{TEM\text{-}1}$, bla_{SHV}*-1*, $bla_{CTX\text{-}M}$, bla_{CMT} and $bla_{OXA\text{-}1}$ have been previously recovered from clinical isolates collected over 18 year period (Kiiru *et al*., 2012). Unfortunately, there are no published reports of β-lactam resistance in water yet.

2.5 Options for treating infections caused by β-lactamase-producers

Unfortunately ESBL producing organisms often possess resistance determinants to other important antibiotic groups, such as fluoroquinolones, leaving an extremely limited range of effective agents (Kariuki *et al*., 2005).

The 7α-methoxy cephalosporins, such as cefoxitin, cefmetazole, cefotetan and latamoxef, are often effective against Enterobacteriaceae producing bla_{TEM}- and bla_{SHV}-derived ESBLs. However, *Klebsiella pneumoniae* strains are prone to cephamycin resistance as a result of porin loss. Cefipime is active against most ESBLs, but is reported to show a marked inoculum effect (Pitout *et al*., 1998). The choice of β-lactam antibiotics for the treatment of infections caused by ESBL-positive isolates thus appears limited to cephamycins and carbapenems; or for the producers of AmpC-type β-lactamases or cephamycinases, to carbapenems only. Carbapenems are the only β-lactam antibiotics uniformly active against isolates producing ESBLs and plasmid-mediated AmpC-type β-lactamases. Carbapenems are, however, susceptible to carbapenemases.

Bla$_{TEM}$ and *bla*$_{SHV}$ derived ESBLs are generally sensitive to β-lactamase inhibitor combinations such as amoxicillin-clavulanic acid and these have been recommended as treatment. However, sulbactam which is also a β-lactamase inhibitor is less effective than clavulanate for the inhibition of *bla*$_{SHV}$ enzymes, a marked inoculum effect has been noted and combinations are not always successful in the treatment of experimental infections. The viability of tazobactam against SHV-derived ESBL

producers is controversial. Their use is thus advocated with caution, taking cognisance of the inoculum effect and the family of ESBLs produced (Livermore, 1995).

ESBLs are typically encoded by plasmids carrying multi-resistant genes conferring resistance to aminoglycosides, chloramphenicol, sulfonamides, trimethoprim and other antimicrobials, severely limiting even alternative therapies. Extensive susceptibility testing before the institution of antibiotic therapy is thus vital (Essack, 2000). Fluoroquinolones are often used to treat infections caused by ESBL-producers

2.5.1 Fluoroquinolones

Broad use of fluroquinolones has been followed by emergence of resistance, which has been due mainly to chromosomal mutations in genes encoding the sub-units of the antimicrobial target, enzymes, DNA gyrase and topoisomerase IV, and in the genes that affect the expression of diffusion channels in the outer membrane and multidrug-resistant efflux systems. The reported resistance rate of *E. coli* to ciprofloxacin is still very low at less than 3%. As we approach the halfway point of second decade of fluoroquinone use, resistance has already emerged in many bacterial species (Arslan, 2005; Hooper, 2001).

Fluoroquinolone resistance has been reported in *E. coli* belonging to O25b:H4-ST131 clone from river Thames in London as well as environmental samples in Mexico (Amábile-Cuevas *et al*. 2010; Dhanji *et al*., 2011). In Sweden, quinolone resistance genes were detected in 42% of well water, 100% and 18% in Swedish river sediments respectively (Rutgersson *et al*., 2014).

In Kenya, quinolone resistance has only been reported in clinical isolates where resistance resulted from combination of the presence of *qnr*A, *qnr*B and ciprofloxacin acetylating enzyme designated *aac*(6')-*lb-cr* and mutations in the two amino acid substitutions (Kariuki *et al*., 2005). There are no published reports of fluoroquinolone resistance in water.

Antimicrobial resistance in water can be due to integrons, β-lactamases, fluoroquinolone resistance genes and other genes that can be found in plasmids. The ability of plasmids to harbour resistance genes as well as virulence genes which can

then be acquired by commensal susceptible bacterial isolates creates need to ascertain whether *E. coli* isolates from water can have virulence genes. Bacterial isolates that are pathogenic and MDR are of unique clinical significance. Bacterial isolates have shown they are capable of carrying both resistance and virulence genes and it is therefore important to investigate whether aquatic bacteria harbour virulence genes which would render them pathogenic and able to cause disease.

2.6 Occurrence of *E. coli* pathotypes in aquatic environments

Intestinal *E. coli* consists of a diverse group of bacteria. Pathogenic *E. coli* strains are categorized into more than six pathotypes based on phenotypic characteristics and virulence properties associated with diarrhoea and collectively are referred to as diarrheagenic *E. coli* (Jafari *et al*., 2012; Kaper *et al*., 2004; Nataro & Kaper, 1998).

2.6.1 Enteroaggregative *E. coli* (EAEC)

It is found only in humans. So named because they have fimbriae which aggregate tissue culture cells, EAEC bind to the intestinal mucosa to cause watery diarrhea without fever and are non-invasive. They produce a hemolysin and a Shiga-like Toxin (*ST*) similar to that of ETEC. EAEC has been shown to have significant resistance to trimethoprim-sulfamethoxazole and ampicillin (Abe *et al*., 2001). These pathogens have been isolated from aquatic environments in Detroit and South Africa (Hamelin *et al*., 2007; Ndlovu *et al*., 2015). In Kenya, studies have focused on clinical isolates from patients and children (Sang *et al.,* 2012; Sang *et al*., 2012) but no publication to the same in Kenya.

2.6.2 Enteropathogenic *E. coli* (EPEC)

EPEC causes diarrhea in humans, rabbits, dogs, cats and horses. Like ETEC, EPEC also causes diarrhea, but the molecular mechanisms of colonization and etiology are different. EPEC lack fimbriae, Shiga-like Toxin (*ST*) and Labile Toxin (*LT*), but they utilize an adhesin known as intimin to bind host intestinal cells. This pathotype has an array of virulence factors that are similar to those found in *Shigella*, and may possess a shiga toxin. Adherence to the intestinal mucosa causes a rearrangement of actin in the host cell, causing significant deformation (Mellies, 2001). EPEC has

been isolated in aquatic environments including South Africa but in Kenya studies have been limited to clinical isolates (Ndlovu *et al*., 2015; Sang *et al*., 2012).

2.6.3 Enteroinvasive *E. coli* (EIEC)

EIEC is found only in humans. EIEC infection causes a syndrome that is identical to Shigellosis, with profuse diarrhea and high fever. Although little work has been done to characterize EIEC virulence factors by molecular typing, it is assumed that they are virtually identical to those of *Shigella* species (Kaper *et al*., 2004). These pathogens have been isolated from water in other countries across the world including South Africa but no published reports are found in Kenya (Ndlovu *et al*., 2015).

2.6.4 Enterohemorrhagic *E. coli* (EHEC)

EHEC is found in humans, cattle, and goats. Enterohaemorrhagic *E. coli* have emerged as a serious gastrointestinal pathogen in many countries. Although the mode of transmission is mainly through the consumption of contaminated meat, outbreaks associated with water-borne enterohaemorrhagic *E. coli* have also been described (Musyoki *et al*., 2013; Páll *et al*., 2013). The clinically significant member of this pathotype is strain O157:H7, which causes bloody diarrhea but no fever. EHEC can cause hemolytic-uremic syndrome and sudden kidney failure. It uses bacterial fimbriae for attachment (*E. coli* common pilus, ECP) and is moderately-invasive and possesses a phage-encoded Shiga toxin (phage encoded virulence factor stx_1 and stx_2 that can elicit an intense inflammatory response (Jafari *et al*., 2012). These pathogens have been isolated across the world including Iran and a study in South Africa which found different serotypes in waste water but published reports using water samples lack in Kenya. However, this study did not analyse this group of *E. coli* pathotype (Jafari *et al*., 2012; Doughari *et al*., 2011).

2.6.5 Enterotoxigenic *E. coli* (ETEC)

ETEC is a causative agent of diarrhea (without fever) in humans, pigs, sheep, goats, cattle, dogs, and horses. ETEC uses fimbrial adhesins (projections from the bacterial cell surface) to bind enterocyte cells in the small intestine. ETEC can produce two proteinaceous enterotoxins. The larger of the two proteins, *LT* enterotoxin, is similar

to cholera toxin in structure and function. The smaller protein, *ST* enterotoxin causes Cyclic guanosine monophosphate (cGMP) accumulation in the target cells and a subsequent secretion of fluid and electrolytes into the intestinal lumen ETEC has been isolated from water in other parts of the world including South Africa but published reports still lack in Kenya (Jafari *et al*., 2012; Ndlovu *et al*., 2015).

2.6.6 Diffusely adherent *E. coli* (DAEC)

Diffusely adherent *E. coli* can cause acute diarrhoea in children and are characterized by the diffuse adherence pattern on cultured epithelial cells HeLa or HEp-2. Approximately 75% of DAEC harbor adhesins from the *Afa/Dr* family, responsible for this adherence phenotype. The adhesins of *Afa/Dr* family have been implicated in DAEC pathogenesis. They include adhesins found in uropathogenic strains, like the *Dr* adhesin, in addition to *AfaE-I, AfaE-II, AfaE-III, AfaE-V* and *F1845*, which occur in diarrheagenic DAEC strains (Mansan-Almeida *et al*. 2013).These pathogens have also been isolated in aquatic environments in Peru and South Africa with an exception to Kenya where there is no published data (Guion *et al*., 2008; Ndlovu *et al*., 2015).

2.7 Integrons

Integrons are mobile DNA elements with the ability to capture gene cassettes, notably those encoding antibiotic resistance, by site-specific recombination. Integrons have an intergase gene (*int*), a nearby recombination site (*attI*), and a promoter. The nine classes of resistance integrons are distinguished based on their respective integrase (*int*) genes (Gillings *et al*., 2015).

In *E. coli* isolates harbouring integrons, the level of antimicrobial resistance depends on the origin and habitat and differs among isolates cultured from clinical, sewage and river water samples. Moreover the discharge of treated waste water may contribute to elevated frequency of integron-positive *E. coli* isolates because after waste water is treated through waste water treatment plant, the resistant bacteria can enter the reservoir that receives the waste water treatment plant effluent in the river and may broaden their resistance range (Koczura *et al*., 2012).

Three classes of integrons are mainly responsible for multidrug resistance phenotype with class 1being the most ubiquitous among Gram-negative bacteria and is considered to play a major role in the emergence and wide dissemination of resistance cassettes (Nemergut *et al*., 2008).

2.7.1 Class 1 integron

Class 1 integrons consist of a variable region bordered by 5' and 3' conserved regions. The 5' region is made up of the *int* gene encoding *attI (*the recombination site*),* and a promoter that drives transcription of genes within the variable region. The 3' region consists of a truncated ethidium bromide resistance locus (*qacE*D1), a sulfonamide resistance gene (*sulI*). Class I integrons are frequently associated with the Tn*21* transposon family (Gillings *et al*., 2015).

Integron class 1-mediated resistance has been reported in environmental Gram-negative bacteria (Koczura *et al*., 2012). More so, a study has shown a link between a high prevalence of class 1 integrons and anthropogenic pollution. These studies suggest that carriage of these integrons confer unique advantages to the host bacteria in the presence of selective pressure emanating from pollution. Class 1 integrons also confer resistance to disinfectants, and heavy metals (Gillings *et al.,* 2015).

2.7.2 Integron gene cassette

Cassettes which contain antimicrobial resistance genes are defined as discrete units by comparison of the sequences flanking a limited number of genes which are either found alone or in different positions in naturally occurring cassette arrays (Hall & Collis 1995). Diverse antibiotic-resistance gene cassettes, *aadA1, aadA2, aadA4, aadA5, dfrA1, dfrA5, dfrA7, dfrA12, dfrA16, dfrA17, dfrA28, dfrA30, dfr-IIe, blaIMP-9, aacA4, Ac-6'-Ib, oxa1, oxa10* and *arr2* were detected in 64 isolates facultative oligotrophic bacteria in India(Chakraborty *et al*., 2013). A recent study of *Aeromonas* from water streams and beaches of California found that all integrons detected in these isolates belonged to class 1 and the most common gene cassettes contained *dfrA*12, *dfrA*15, *dfrA*17 (conferring resistance to trimethoprim) and *aad*A2 and *aad*B (conferring resistance to aminoglycosides) (Rosser & Young 1999). Additionally, in another study done in river Tay, United Kingdom, it was revealed

that integron structures were prevalent in bacteria in natural habitats. Nineteen integrons contained additional or other gene cassettes in their variable region, including those encoding resistance to trimethoprim (*dfr*A1, *dfr*B3, *dfr*4 *dfr*7, *and dfr12*), chloramphenicol (*cat*B3, *cat*B5), aminoglycosides (*aad*A2, *aac*A4, *and aacC1*), β-lactamases (*oxa*2) and erythromycin (*ere*A). It was concluded the frequent identification of 'empty' integrons supported the view that antibiotic selective pressure plays a significant role in promoting the incorporation and maintenance of cassettes in the variable region of integrons with a possibility that cassettes are excised from integron in the absence of sustained antibiotic pressures resistance (Armine, 2013).

The predominance of selected aminoglycoside and trimethoprim resistance gene cassettes however, indicated that some gene cassettes were more stably incorporated within integron structures than others (Chakraborty *et al.*, 2013; Rosser & Young 1999). In Kenya, most research has focused on integron cassettes in clinical isolates with limited studies published on integron cassettes present in aquatic environments.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site and specimen source

The study was carried out along the banks of River Athi in Athi River town, Machakos County. River Athi is the second longest river (390 km) in Kenya after River Tana (1000kms) and drains a basin area of 70,000 km². The river arises at 1° 42' S on Aberdare's or Mt Kenya as River Athi and enters the Indian Ocean as River Galana (also known as River Sabaki). The River flows across the Kapiti and Athi plains, through the Athi River town, takes a northeast direction and is met by the Nairobi River. Near Thika, it forms the Fourteen Falls and turns south-south-east under the wooded slopes of the Yatta ridge, which ends in its basin on the east (Figure 3.1).

The Athi River Township, catchment area of this river, experiences recurrent sewage outbursts and poor sanitation due to increasing number of people living in slum areas. The town also has poor water sanitation as a result of direct use of untreated river water for washing, drinking as well as irrigation.

Population from this area to water pollutants and disease include the fact that the river passes through human settlements with poor sanitation infrastructure such as slums. The town also has medical facilities and hospitals that may discharge medical wastes into the river. However, the possibility that this river is contaminated with such wastes has not been determined so far. This river also runs near industrial establishments therefore presenting a possibility of contamination with industrial wastes that may contain heavy metals and cations which are known to co-select for MDR strains (Baker-Austin *et al*., 2006). Figure 3.1 shows the study site and sources of water that was collected from the River when passing near human settlements, waste effluent and arid areas. These are shown in detail in Figures 3.2, 3.3, 3.4, 3.5, 3.6 and 3.7.

Figure 3-1: Google earth picture showing the flow of River Athi along sites

Domestic human settlements, virgin land , sewage effluent, near road and farming and industrial zone in Athi River town.

Figure 3-2: Domestic use

Due to easy access of the river at this point, people use the water for washing clothes, swimming, fetching for home use and other domestic purposes

Figure 3-3: Human settlements

People have built very close to river with domestic water effluents directed towards the river

Figure 3-4: Virgin land

No apparent human activity in site

Figure 3-5: Sewage effluent

Sewage treatment plant in close proximity to the river

Figure 3-6: Near road and farming

Area used for farming and easily accessible

Figure 3-7: Industrial zone

The River is near a busy industrial part of Athi River Town

3.2 Sampling and study design

3.2.1 Study design

The study was a cross-sectional in which environmental samples were obtained at random from the banks of River Athi in Machakos County during wet and dry periods.

3.2.2 Sampling sites

The sampling points were selected based on prevailing human activities such as washing, drinking points for livestock, points where residents fetch water for domestic use, and points contaminated with industrial effluent from industries nearby. Sampling was also done along sections of the river passing through virgin lands that have no obvious evidence of recent interference by human activity, agriculture or settlement as shown in Table 3.1 and Figure 3.1.

Table 3-1: Activities on different sites where water was sampled

Site	Activities on site
Domestic use	Water for drinking and for other domestic use such as washing clothes
Human settlements	Informal and formal human settlements
Virgin land	No apparent human, industrial or agricultural activities
Sewerage effluent	Sewage treatment plant in close vicinity
Near road and farming	Close to tarmac road. Water also used for flower farming
Industrial zone	Near the industrial zone (cement factory in close vicinity), water used for bathing, watering flowers, livestock watering in shallow edges of the river.

3.3 Sample size determination

From previous studies carried out in Nairobi river and other water sources, the prevalence of *E. coli* was 29.4% (Njugu, 2011) which was used for estimation of the number of isolates required to provide a statistically significant sample size using Fishers exact test (Jung, 2014) as shown below:

$n = (Z_{1-\alpha})^2 X (PXQ) / d^2$

Where:

N= the sample size needed

$Z_{1-\alpha} = Z_{0.95}$=1.96 (95% Confidence Limit)

P= 0.294 (29.4% Prevalence of *E. coli* from various water sources (Njugu 2011)

Q= (1-P); d= Absolute precision required= 0.05%

$n= (1.96)^2*(0.294*0.706) =0.7974/ (0.05)^2=318$

The minimum sample size that would yield statistically significant number of *E. coli* isolates to generate meaningful data was 318.

The minimum *E. coli* isolate size of 318 was used to determine the number of water samples that would be significant in the study. The number 318 was conveniently divided by the six sampling sites therefore in each site; 53 samples were collected in total per site. From which, 26 were from rainy season and 27 from dry season per site.

One site = 53 water samples in total

Rainy season 26 water samples

Dry season 27 water samples

Therefore 53 water samples per site × 6 sites = 318 water samples were collected.

Rainy season (26x6) =156, Dry season (27x6)= 162 water samples

3.3.1 Sample collection

Water samples were collected from each site at varying dates from September 2014 to January 2015. Sampling of each site was done only once. From each site, a total of 53 samples (500ml each) were taken during dry and wet seasons and also varied by time of day. Water samples were collected using sterile plastic bottles. These bottles were lowered into the water body near the river banks where water was surface run off that was settled and not flowing with the river current to a maximum depth of 30cm before withdrawal. The samples were transported to Kenya Medical Research Institute Centre for Microbiology laboratory in insulated cool boxes (4-8° C) and processed within 24 hrs.

3.4 Laboratory procedures

3.4.1 Determination of colony forming units

To determine presence of coliforms in water samples, multiple tube fermentation technique was used. The dilution factor was determined. Afterwards, Each water sample was inoculated directly on three plates of colony count agar plates using a 10µl sterile wire loop and then incubated for 24 hours under 37° C. CFUs/ml were then determined and the means calculated.

3.4.2 Isolation of *E. coli*

For isolation of *E. coli*, 25 ml of sample water was inoculated into 225 mL of buffered peptone water (BPW) (Oxoid, UK) and incubated at 37°C for 24 hrs. A loop-full of broth (10µl) was then streaked on MacConkey's agar (Oxoid, UK), (preparation shown in appendix 3) and incubated at 37°C for 24 hrs. Suspect colonies colonies (medium sized pink non-mucoid colonies) were then picked from the plates by use of sterile wire loop (3 colonies per plate) were identified as *E. coli* using standard morphological and biochemical tests for Enterobacteriaceae.

3.4.3 Biochemical identification of bacteria

A total of 954 *E. coli* isolates that were presumptively identified as *E. coli* based on their growth characteristic on MacConkey agar and were further identified by biochemical typing as shown in Figure 4.5. Colony morphology and sugar fermentation reactions were used for presumptive identification of the isolates on the culturing plates. All Biochemical typing media used were obtained from Oxoid, UK. The biochemical characteristics were determined on Triple Sugar Iron agar, Sulphur Indole motility agar, Simmons citrate agar, Methyl Red Voges-Proskauer (MRVP) broth and urea agar (interpretation of the tests shown in appendix 4). *E. coli* isolates identified were stocked in 1ml capacity vials containing 15% glycerol and tryptic soy broth, stored at -80°C for further analysis.

3.4.4 Antimicrobial susceptibility testing

E. coli isolates analysed for antimicrobial susceptibility testing were selected from the 954 isolates stored. From this stock, one colony was picked from the three

colonies per water sample in random to a total of 318 *E. coli* isolates that were a representative of each water sample collected from the six sampling sites.

Antibiotic susceptibility was done using the disk diffusion technique on Mueller-Hinton agar (Oxoid, UK),(Appendix 2). Quality control was performed by including *E. coli* ATCC 25922 in each batch of testing. The panel of antimicrobial used comprised of amoxicillin/clavulanic acid (AMC, 20/10µg), ampicillin (AMP,10µg), aztreonam (ATM, 30µg), cefotaxime (CTX,30µg) cefepime (FEP,30µg), cefoxitin (FOX, 30µg), ceftazidime (CAZ,30µg), cefpodoxime (CPD,10µg), tetracycline (Te,30µg), gentamicin (Cn, 10µg), trimethoprim (W,5µg), chloramphenicol (C, 30µg), sulfamethoxazole (SMX, 50ug), ciprofloxacin (Cip,5 µg), nalidixic acid (NA, 30µg) and streptomycin (S,10µg).

Double disc synergy (m-DDS) test was used for the determination of the ESBLs phenotype (Karisik *et al*. 2006).The synergy was determined by placing a disk of augumentin (AMC; 20µg of amoxicillin plus 10 µg of clavulanic acid) and antibiotic discs representing the four generations of β-lactam antibiotics at inter-disc distance (center to center of 24 mm) which included the following;

(moderate- spectrum: - ampicillin (AMP,10 µg) monobactam: - aztreonam (ATM, 30 µg) broad spectrum 3rd generation cephalosporins : - cefotaxime (CTX, 30 µg) ceftazidime (CAZ, 30 µg) cefpodoxime (CPD 10 µg) broad spectrum 4th generation cephalosporin: - cefepime (FEP,30 µg) and cephamycins: - cefoxitin (FOX, 30 µg)

The double-disk synergy test was considered positive when a clear extension of the edge of cephalosporin disc zone was towards the amoxicillin/ clavulanic acid.

3.4.6 Isolation of DNA by boiling method

E. coli isolates that were done antimicrobial susceptibility testing were used for molecular analysis. From a total of 318 *E. coli* isolates, each isolate was suspended in 300 µl of DNA extraction buffer (99ml deionised water, 1ml PH8.0Tris and 200µl-0.5M EDTA) and boiled for 15 minutes at 95°C using a heating block. After lysis, centrifugation was done at 14,000 rmp for 5 minutes at 4°C. The DNA containing

3.4.7 PCR amplification for virulence, resistance genes

PCR amplification was carried out in 25µL reaction volumes containing 1x *Taq* standard buffer 12.5µl [20 mM Tris/HCl (pH 8·9@ 25°C), 22 mM KCl, 1.8 mM $MgCl_2$, 22Mm NH_4Cl,0.2 mM DNTPs, 5% glycerol, 0.06% IGEPAL^R CA-630, 0.05% Tween^R 20, 25 units/ml One *Taq* DNA Polymerase], template DNA 1µl , 10 µM forward primer 0.5 µl ,10 µM reverse primer 0.5µl and nuclease-free water to 25µl. The thermo-cycler PCR conditions were primer specific. Table 3.2 shows the PCR annealing temperatures (°C) and primers used

Table 3-2: PCR primers and annealing temperature used in PCR amplification

Oligonucleotide name	Oligonucleotide 5'-3'	Target gene	PCR annealing TM	Product Size (bp)	Gene Accession Number/ Reference
	Resistance genes				
blaTEM	ATGAGTATTCAACAT TTC CG-F CCAATGCTTAATCAG TGA-R	*bla*$_{TEM}$	55	840	EF125012
blaCMY	ATGATGAAAAAATCGTTATGC-F TTGCAGCTTTTCAAGAATGCG-R	*bla*$_{CMY}$	55	1200	U77414
blaCTX-M	ATGTGCAGYACCAGTAARGTK-F ATGGCRAARTARGTSACCAGA-R	*bla*$_{CTX-M}$	60	593	Y10278
eae	CTGAACGGCGATTACGCGAA-F CGAGAGACGATACGATCCAG-R	eae	54	917	(Aranda *et al*., 2007)
bfp	AATGGTGCTTGCGCTTGCTGC-F GCCGCTTTATCCAACCTGGTA-R	bfp	54	326	(Aranda *et al.,* 2007)
	Virulence genes				
Aggr	GTATACACAAAGAAGGAAGC-F ACAGAATCGTCAGCATCAGC-R	Aggrksa1 Aggrksa2	54	254	(Aranda *et al*., 2007)
lt	GCACACGGAGCTCCTCAGTC-F TCCTTCATCCTTTCAATGGCTTT-R	lt	54	218	(Vidal et *al*., 2005)
st	GCTAAACCAGTAGASTCTTCAAAA-F CCCGGTACARGCAGGATTACAACA-R	st	54	147	(Aranda et al., 2007)
Ipa H	CTCGGCACGTTTTAATAGTCTGG-F GTGGAGAGCTGAAGTTTCTCTGC-R	*ipaH*	54	933	(Vidal *et al*., 2005)
inv	ATATCTCTATTTCCAATCGCGT-F GATGGCGAGAAATTATATCCCG-R	inv	54	382	(Vidal et al., 2005)
Int 1	TCGGTCAAGGTT-F AACTTTCAGCACATG-R	int1	50	923	U12338

PCR annealing temperatues that were used and the specific primers for resistance genes, virulence genes and integron 1 genes. Example of primer specific themo cycler condition is CMY consensus which was Initial denaturation was at 94°C for 5 minutes, Denaturation at 94°C for 1 minute, annealing temperatures stated on the table (55°C) for 1 minute,extension at 72°C for I munite and final extension at 94°C for 35 more times and storage at 72°C for 10 minutes . After which PCR products were to be incubated at 10°Cfoerver.

3.4.8 Gel electrophoresis.

3.4.8.1 Gel preparation

Electrophoresis was carried out in 1.5% agarose (wt/vol) in Tris-borate EDTA (TBE) (sigma) shown in appendix 6. A small volume of 0.5µl ethidium bromide solution was added to the agarose. 10ul of the PCR product was mixed with 3 µl of loading buffer and added to the wells for electrophoresis. Three micro-litre of molecular weight marker (Hyper Ladder I; Bioline USA Inc., Canton, MA) was loaded in the first lane of the gel to aid in the estimation of the PCR product sizes. Electrophoresis was performed at 100 V for 1hr. The gel was observed under UV light and image captured using a digital camera.

3.5 Data management

Laboratory procedures and results were recorded in a note book. The data was collected in a spreadsheet and stored in the hard disk with flash disk backups. The notebook and flash disks were kept in a securely locked cabinet when not in use and a password used for data in the computer hard disk drive.

3.6 Data analysis

Data presentation was done by use of tables, bar graphs and pie chart. Statistical Package for Social Sciences (SPSS) software version 20.0 was used for analysis of the data. Descriptive analysis using mean, frequency and proportion were used. Chi square test and odds ratio with corresponding 95% confidence intervals were used to assess drug susceptibility between the sites. Virgin land was selected as reference because it had no apparent human or industrial activities. Student's *t* test was also computed to compare the means of coliform count during dry and wet season. P values < 0.05 were considered significant.

3.7 Ethical considerations

Laboratory procedures were performed in accordance to the standard operating procedures. No human subjects were involved in the study. However, ethical requirements were sought from Kenya Medical Research Institute (KEMRI) Scientific Steering Committee (SSC) and Ethical Review Committee (ERC).

CHAPTER FOUR

RESULTS

4.1 Samples

A total of three hundred and eighteen samples were collected from six sampling points along Athi River. From each site, 53 samples were collected at random during wet and dry seasons. Figure 4.1 shows site used for domestic purposes after rainfall.

Figure 4-1: Area used for Domestic purposes after rainfall

4.2 Presumptive *E. coli* counts during dry and wet season across sites

The total CFU counts were more near to the sewage treatment plant and domestic use in a rainy season when compared to dry period ($P \leq 0.01$). However, in the industrial zone the total counts were more in dry season than rainy period ($P < 0.001$) as shown in Figure 4.2

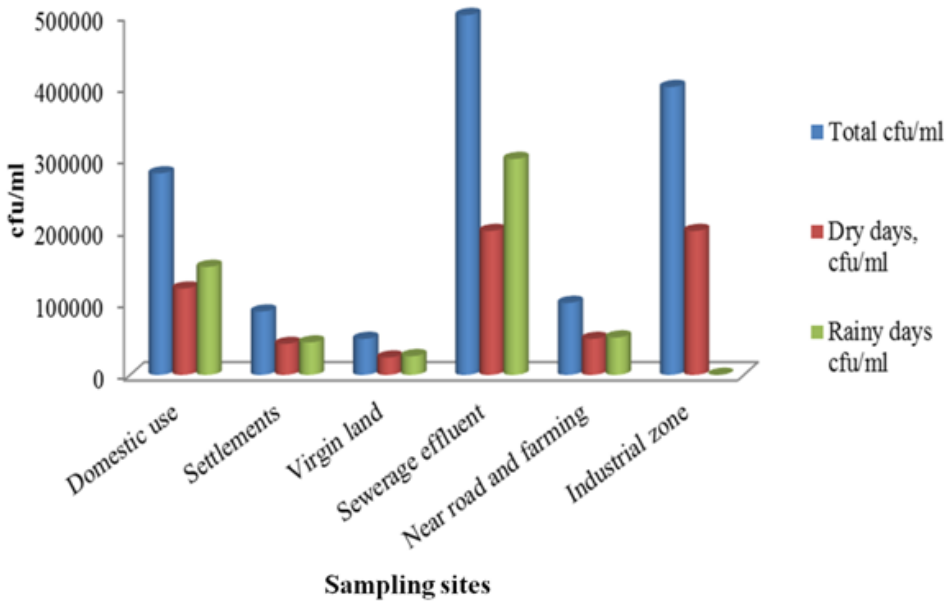


Figure 4-2: Total *E. coli* (CFUs/ml) across the six sites during wet and dry season

Total numbers of *E. coli* were obtained from colony count agar plates triplicate counts where three plates were inoculated and CFUs counted and average of the three plates determined

4.2.1 Mean of presumptive *E. coli* counts

The mean of *E. coli* isolates from the total counts during rainy season were significantly higher ($P \leq 0.01$) than the dry period. Samples collected from near sewage treatment plant and domestic use had significantly higher ($P \leq 0.01$) *E. coli* mean counts after rains than dry period. In industrial zone additionally, the mean counts were also significantly more ($P < 0.001$) in the dry season compared to rainy season. Sewage effluent area had the highest mean *E. coli* counts of 9.5×10^3 (SD $\pm 2.3 \times 10^3$). However, areaof the river which had no activities, had the lowest mean as shown by Figure 4.3

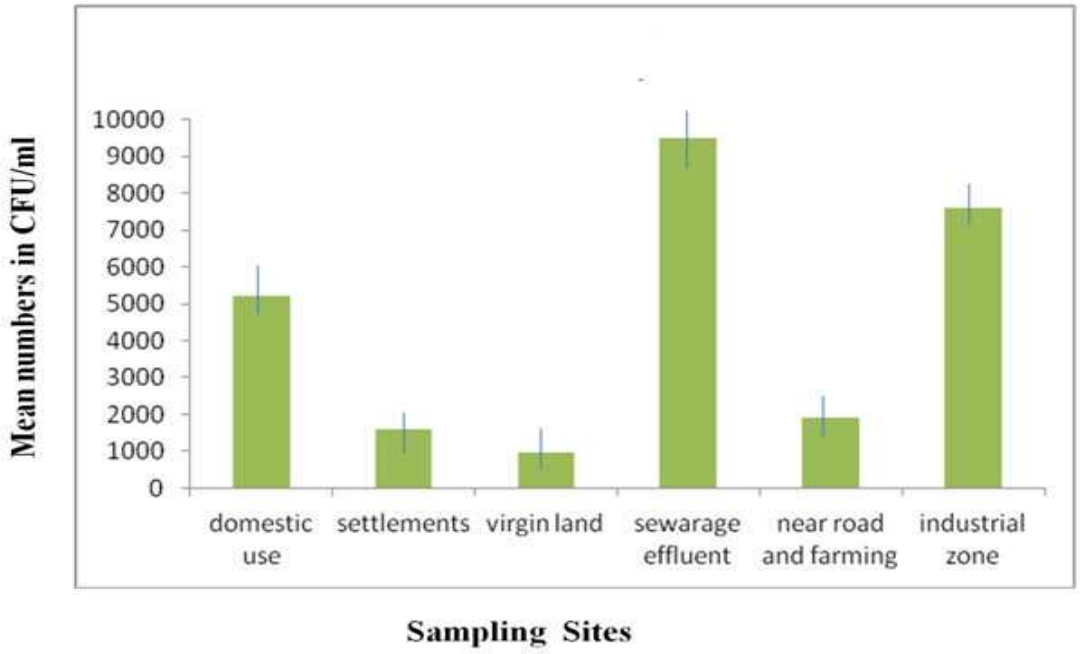


Figure 4-3: Mean *E. coli* (CFUs/ml) across sites in Athi River

The mean was obtained from colony count agar plates triplicate counts where three plates were inoculated, CFUs were counted and means determined for both dry and wet season. SD bars were obtained from the mean.

4.3 Isolation and identification of *E. coli*

The total cfus of *E. coli* were isolated on MacConkey agar after enrichment of water in buffered peptone water at 37°C overnight. Figure 4.4 shows the colony morphology of *E. coli* obtained.

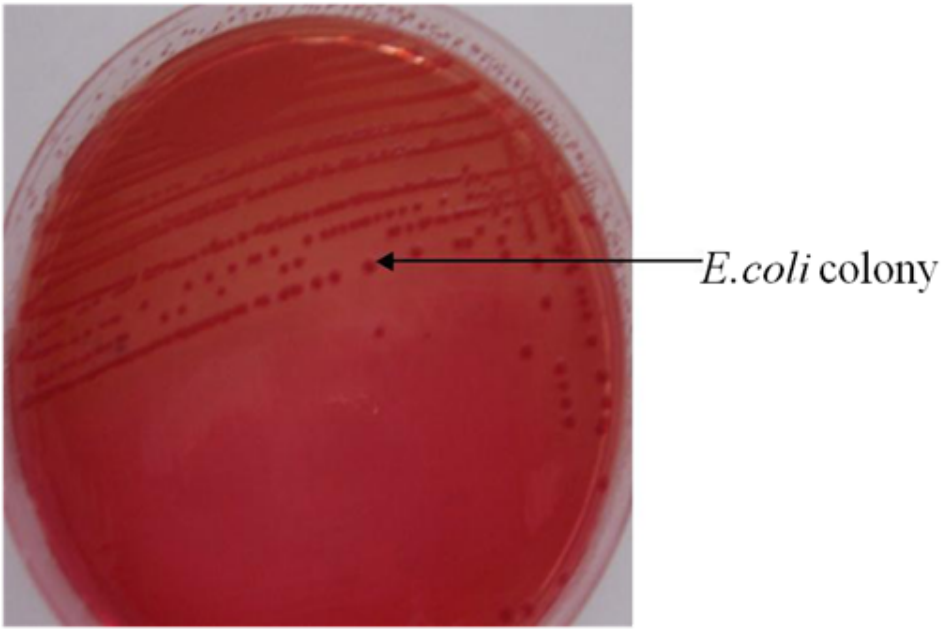


Figure 4-4: Morphology of *E. coli* colony

A plate showing *E. coli* morphology from water sample 25 collected from site having human settlement characteristic

Three discrete colonies with characteristic pink (lactose fermenters – characteristic of *E. coli*) were picked and confirmed using biochemical tests as shown in Figure 4.5.

Figure 4-5: Biochemical typing characteristics of *E. coli*

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

4.4 Antimicrobial susceptibility among *E. coli* isolates

The diameter of the inhibition zones (zone without growth) was measured and interpreted to resistant (R) or sensitive (S). A standard chart was used for interpretation (Appendix 4). The inhibition zones appeared as shown in Figure 4.6 below. Synergy is shown in second plate which is phenotypic characteristic of ESBL production by *E. coli* isolate no. 526

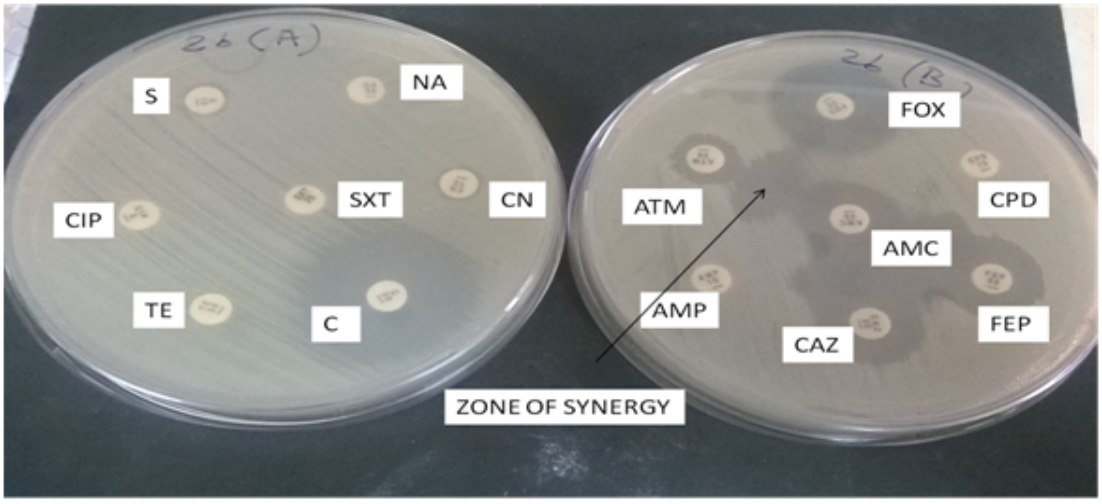


Figure 4-6: Disk diffusion technique for antibiotic susceptibility testing

The plate(1) on the left, *E. coli* isolate isolated from water near industrial zone was resistant to most antimicrobials with exception to chloramphenicol while on right plate(2), zone of synergy was noted between amoxicillin/clavulanic acid (AMC) in the middle and cefoxtin (FOX) and aztreonam (ATM).

In *E. coli* isolate no.7 below in Figure 4.7, the isolate was highly resistant to cefoxitin drug on the right plate while susceptible to other β-lactam antimicrobials. This was also observed in significantly high numbers of isolates tested.

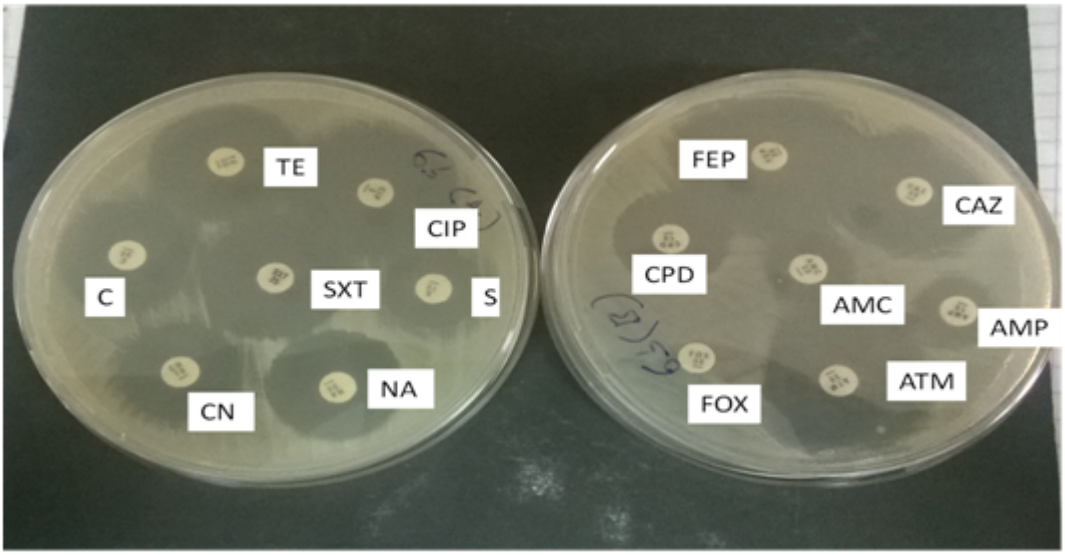


Figure 4-7: *E. coli* resistant to cefoxitin

The plates showed susceptibility of the isolate to the antimicrobials tested and interpreted using standard chart. *E. coli* isolate tested was isolated from water near virgin land

Distribution of antimicrobial resistance of *E. coli* from Athi River is presented in Figure 4.8. A total of 318 isolates were tested for resistance. High resistances were observed against ampicillin (63.8%), cefoxitin (46.9%), amoxicillin/clavulanic acid (46.2%) and sulfamethazole (44%). Least resistances were observed against gentamicin (<1%), cefepime (1.9%), ceftazidime (2.5%) and chloramphenicol (3.8%).

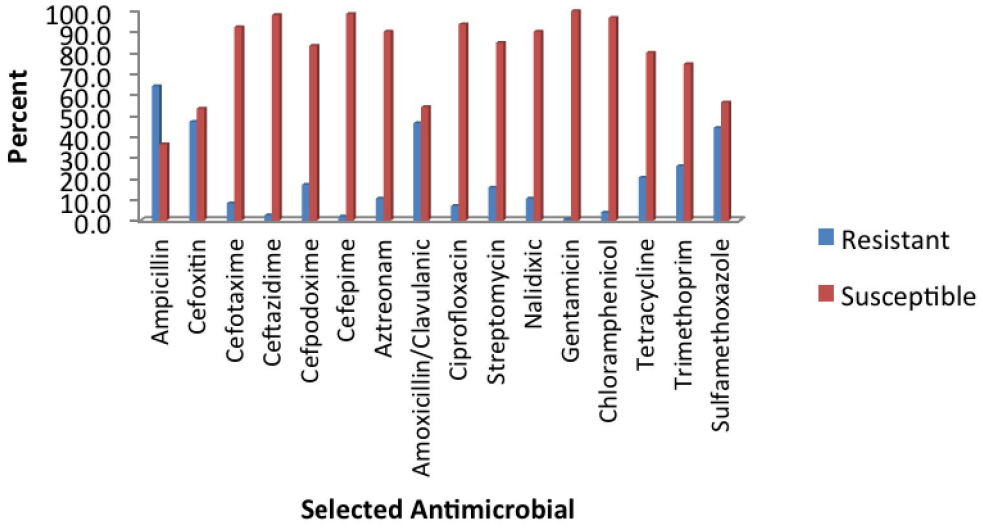


Figure 4-8: Percentage of resistance and susceptibility in *E. coli* isolates to various antimicrobials

In general most isolates were susceptible to the antimicrobials used.

4.4.1 Resistance of *E. coli* isolates across the sampling points (sites)

Antimicrobial resistance patterns among isolates recovered from each of the six sampling sites are shown in Table 4.1. Highest resistance to ampicillin (79.2%) was observed among isolates recovered from sections of the river close to tarmac road where water was used for flower farming and industrial zone. However, *E. coli* isolates isolated from water used for domestic purposes had the lowest observed resistance to ampicillin (47.2%). Resistance to cefoxitin was highest among isolates from the industrial zone (73.6%), and lowest among those from the virgin land (30.2%). Resistance to amoxicillin/clavulanic acid was highest among isolates from the sections near the industrial zone (81.1%) and lowest among isolates from the section of the river near formal and informal settlements 32.1%). Resistance to sulfamethazole was highest among isolates from the sections through the virgin land (52.8%) and lowest in the informal settlement site (34%). Highest resistance to ciprofloxacin (20.8%) was observed among isolates obtained from water utilized for domestic purposes and lowest (0%) in flower farming site where water was utilized for farming and was closest to the tarmac road. Interestingly, highest resistance to

cefepime (a fourth generation cephalosporin) was recorded among isolates from the virgin land (9.4%) and lowest in all the other sites including the industrial zone and the section contaminated with sewerage effluent. Highest resistance to aztreonam was recorded among isolates obtained from water utilized for domestic purposes (13.2%) while the lowest resistances were observed among isolates recovered from the river section closest to the tarmac road where water was also utilized for flower farming (5.7%).

Table 4-1: Distribution of resistance across sites

Sampling sites	Total n	*E.coli* isolates resistant to various antimicrobials (%)															
Sites	**(n)**	**Amp**	**Fox**	**Ctx**	**Caz**	**Cpd**	**Fep**	**Atm**	**Amc**	**Cip**	**S**	**Na**	**Cn**	**C**	**Te**	**Smx**	**W**
Domestic use	53	25	25	10	3	10	1	7	20	11	4	6	1	4	17	26	18
		-47.2	-47.2	-18.9	-5.7	-18.9	-1.9	-13.2	-37.7	-20.8	-7.5	-11.3	-1.9	-7.5	-32.1	-49.1	-34
Settlements	53	30	21	4	0	9	0	5	17	1	5	5	0	1	8	18	8
		-56.6	-39.6	-7.5	0	-17	0	-9.4	-32.1	-1.9	-9.4	-9.4	0	-1.9	-15.1	-34	-15.1
Virgin land	53	35	16	3	4	9	5	9	18	4	11	4	1	4	10	28	13
		-66	-30.2	-5.7	-7.5	-17	-9.4	-17	-34	-7.5	-20.8	-7.5	-1.9	-7.5	-18.9	-52.8	-24.5
Sewerage		29	17	7	0	8	0	5	22	4	8	5	0	2	18	22	17
effluent	53	-54.7	-32.1	-13.2	0	-15.1	0	-9.4	-41.5	-7.5	-15.1	-9.4	0	-3.8	-34	-41.5	-32.1
Near road and		42	31	1	0	7	0	3	27	0	6	4	0	1	5	19	15
farming	53	-79.2	-58.5	-1.9	0	-13.2	0	-5.7	-50.9	0	-11.3	-7.5	0	-1.9	-9.4	-35.8	-28.3
Industrial zone	53	42	39	1	1	11	0	4	43	2	16	9	0	0	6	27	11
		-79.2	-73.6	-1.9	-1.9	-20.8	0	-7.5	-81.1	-3.8	-30.2	-17	0	0	-11.3	-50.9	-20.8

Amp:Ampicillin, Fox:Cefoxitin, Ctx:Cefotaxime, Caz:Ceftazidime, Cpd:Cefpodoxim, Fep:Cefepime, Atm:Aztreonam, Amc:Amoxicillin/clavulanic acid, Cip:Ciprofloxacin, S:Streptomycin, Na:Nalidixic, Cn:Gentamicin, C: Chloramphenicol, Te: Tetracycline, Smx:Sulfamethoxazole,W:Trimethoprim. The percentages were based on 318 *E. coli* isolates isolated from water samples across the six sites.

4.2.2 Distribution of resistance strains

Antimicrobials can be grouped into several groups or classes depending on the type of antimicrobial. For this study, the antimicrobials used belonged to the following classes: Aminogycosides - streptomycin and gentamicin, B-lactams- aztreonam, ampicillin, cefoxitin, cefotaxime, ceftazidime, cefpodoxime, cefepime, amoxicillin/clavulanic acid, Fluoroquinolones - nalidixic acid and ciprofloxacin, Chloramphenicol, Tetracyclines and Nucleic acid inhibitors - trimethoprim and sulfamethazole.

E. coli isolates that were resistant to more than three groups/classes of antimicrobials were termed MDR. Isolates near virgin land and sewage effluents had a high number of isolates resistant to four to five antimicrobials (37.1%) and in general, majority of isolates from any given site were resistant to 4-5 classes of antimicrobials. Water for domestic use had the highest counts of strains resistant to > 5 classes. Antimicrobial resistant phenotypes that were resistant to different classes of antimicrobials are presented in Table 4.2.

Table 4-2: Percentage of *E. coli* showing resistance to classes of antimicrobials

		Antimicrobial Resistance Phenotype, n (%)					
	Total n	**Fully susceptible,**	**MDR (1)**	**MDR (2)**	**MDR (3)**	**MDR (4-5)**	**MDR (>5)**
All sites	318	17(5.3)	41(12.9)	52(16.4)	65(20.4)	**118(37.1)**	25(7.9)
Domestic use	53	1(1.9)	6(11.3)	15(28.3)	5(9.4)	18(34.0)	8(15.1)
Settlements	53	7(13.2)	10(18.9)	10(18.9)	9(17.0)	16(30.2)	1(1.9)
Virgin land	53	0	10(18.9)	10(18.9)	9(17.0)	**20(37.7)**	4(7.5)
Sewerage effluent	53	4(7.5)	6(11.3)	10(18.9)	10(18.9)	**20(37.7)**	3(5.7)
Near road and farming	53	4(7.5)	7(13.2)	5(9.4)	19(35.8)	15(28.3)	3(5.7)
Industrial zone	53	1(1.9)	2(3.8)	2(3.8)	13(24.5)	29(54.7)	6(11.3)

N.B: MDR 1: Resistance to one antimicrobial, MDR 2: Resistance to two antimicrobials, MDR 3: Resistance to three antimicrobials, MDR 3: Resistance to three antimicrobials, MDR 4-5: Resistance to four and five antimicrobials, MDR >5: Resistance to five or more antimicrobials

4.2.3 Association between antimicrobial resistance and sampling sites

Virgin land was selected as the reference site to compare differences in antimicrobial susceptibilities to other sites because it had no apparent human or industrial

activities. Antimicrobials selected to be compared were those whose resistances were above 15%.

4.2.3.1 Association of drug resistance between virgin land site and domestic site

Table 4.3 shows the bivariate analysis of relationship in antimicrobial resistance between virgin land and domestic use. However, there was no significant association (P<0.05) observed between the virgin land and domestic use sites.

Table 4-3: Virgin land and domestic use

Susceptibility of drugs	Resistant, n (%)	Susceptible, n (%)	OR (95%CI)	P value
		Ampicillin		
Domestic use	25 (47.2%)	28 (52.8%)	0.46 (0.21-1.01)	0.052
Virgin land	35 (66.0%)	18 (34.0%)		
		Cefoxitin		
Domestic use	25 (47.2%)	28 (52.8%)	2.07 (0.93-4.58)	0.075
Virgin land	16 (30.2%)	37 (69.8%)		
		Cefpodoxime		
Domestic use	10 (18.9%)	43 (81.1%)	1.14 (0.42-3.07)	0.8
Virgin land	9 (17.0%)	44 (83.0%)		
		Amoxicillin/clavulanic acid		
Domestic use	20 (37.7%)	33 (62.3%)	1.18 (0.53-2.61)	0.686
Virgin land	18 (34.0%)	35 (66.0%)		
		Streptomycin		
Domestic use	4 (7.5%)	49 (92.5%)	0.31 (0.09-1.05)	0.06
Virgin land	11 (20.8%)	42 (79.2%)		
		Tetracycline		
Domestic use	17 (32.1%)	36 (67.9%)	2.03 (0.83-4.98)	0.122
Virgin land	10 (18.9%)	43 (81.1%)		
		Sulfamethoxazole		
Domestic use	26 (49.1%)	27 (50.9%)	0.86 (0.40-1.84)	0.698
Virgin land	28 (52.8%)	25 (47.2%)		
		Trimethoprim		
Domestic use	18 (34.0%)	35 (66.0%)	1.58 (0.68-3.69)	0.287
Virgin land	13 (24.5%)	40 (75.5%)		

OR=Odds ratio; CI= Confidence interval

4.2.3.2 Association of drug resistance between virgin land and settlements

Table 4.4 shows the bivariate analysis of relationship in drug resistance between virgin land and settlements. However, there was no significant association (P<0.05) observed between the virgin land and human settlements.

Table 4-4: Virgin land and settlements

Susceptibility of drugs	Resistant, n (%)	Susceptible, n (%)	OR (95%CI)	P value
Ampicillin				
Settlements	30 (56.6%)	23 (43.4%)		
Virgin land	35 (66.0%)	18 (34.0%)	0.67 (0.31-1.47)	0.32
Cefoxitin				
Settlements	21 (39.6%)	32 (60.4%)		
Virgin land	16 (30.2%)	37 (69.8%)	1.52 (0.68-3.39)	0.309
Cefpodoxime				
Settlements	9 (17.0%)	44 (83.0%)		
Virgin land	9 (17.0%)	44 (83.0%)	1.00 (0.36-2.96)	1
Amoxicillin/clavulanic acid				
Settlements	17 (32.1%)	36 (67.9%)		
Virgin land	18 (34.0%)	35 (66.0%)	0.928 (0.41-2.06)	0.836
Streptomycin				
Settlements	5 (9.4%)	48 (90.6%)		
Virgin land	11 (20.8%)	42 (79.2%)	0.40 (0.13-1.24)	0.111
Tetracycline				
Settlements	8 (15.1%)	45 (84.9%)		
Virgin land	10 (18.9%)	43 (81.1%)	0.76 (0.28-2.12)	0.606
Sulfamethoxazole				
Settlements	18 (34.0%)	35 (66.0%)		
Virgin land	28 (52.8%)	25 (47.2%)	0.46 (0.21-1.01)	0.052
Trimethoprim				
Settlements	8 (15.1%)	45 (84.9%)		
Virgin land	13 (24.5%)	40 (75.5%)	0.55 (0.21-1.46)	0.227

OR=Odds ratio; CI= Confidence interval

4.2.3.3 Association of drug resistance between virgin land and sewerage effluent

Table 4.5 shows the bivariate analysis of relationship in drug resistance between virgin land and sewerage effluent. However, there was no significant association ($P<0.05$) observed between the virgin land and sewerage effluent sites.

Table 4-5: Virgin land and sewerage effluent

Susceptibility of drugs	Resistant, n (%)	Susceptible, n (%)	OR (95%CI)	P value
Ampicillin				
Sewerage effluent	29 (54.7%)	24 (45.3%)	0.62 (0.28-1.36)	0.235
Virgin land	35 (66.0%)	18 (34.0%)		
Cefoxitin				
Sewerage effluent	17 (32.1%)	36 (67.9%)	1.09 (0.48-2.49)	0.834
Virgin land	16 (30.2%)	37 (69.8%)		
Cefpodoxime				
Sewerage effluent	8 (15.1%)	45 (84.9%)	0.87 (0.31-2.46)	0.791
Virgin land	9 (17.0%)	44 (83.0%)		
Amoxicillin/clavulanic acid				
Sewerage effluent	22 (41.5%)	31 (58.5%)	1.38 (0.63-3.04)	0.423
Virgin land	18 (34.0%)	35 (66.0%)		
Streptomycin				
Sewerage effluent	8 (15.1%)	45 (84.9%)	0.68 (0.25-1.85)	0.449
Virgin land	11 (20.8%)	42 (79.2%)		
Tetracycline				
Sewerage effluent	18 (34.0%)	35 (66.0%)	2.21 (0.91-5.40)	0.081
Virgin land	10 (18.9%)	43 (81.1%)		
Sulfamethoxazole				
Sewerage effluent	22 (41.5%)	31 (58.5%)	0.63 (0.29-1.37)	0.244
Virgin land	28 (52.8%)	25 (47.2%)		
Trimethoprim				
Sewerage effluent	17 (32.1%)	36 (67.9%)	1.45 (0.62-3.40)	0.39
Virgin land	13 (24.5%)	40 (75.5%)		

OR=Odds ratio; CI= Confidence interval

4.2.3.4 Association of drug resistance between virgin land and near road and farming

Table 4.6 shows the bivariate analysis of relationship in drug resistance between virgin land and near road and farming. There was a significant difference observed on cefoxitin between the virgin land and near road and farming with odds ratio of 3.26 (CI 1.46-7.26), P value of 0.004.

Table 4-6: Virgin land and near road and farming

Susceptibility of drugs	Resistant, n (%)	Susceptible, n (%)	OR (95%CI)	P value
Ampicillin				
Near road and farming	42 (79.2%)	11 (20.8%)	1.96 (0.82-4.71)	0.13
Virgin land	35 (66.0%)	18 (34.0%)		
Cefoxitin				
Near road and farming	31 (58.5%)	22 (41.5%)	**3.26 (1.46-7.26)**	**0.004**
Virgin land	16 (30.2%)	37 (69.8%)		
Cefpodoxime				
Near road and farming	7 (13.2%)	46 (86.8%)	0.74 (0.26-2.17)	0.588
Virgin land	9 (17.0%)	44 (83.0%)		
Amoxicillin/clavulanic acid				
Near road and farming	27 (50.9%)	26 (49.1%)	2.02 (0.92-4.42)	0.079
Virgin land	18 (34.0%)	35 (66.0%)		
Streptomycin				
Near road and farming	6 (11.3%)	47 (88.7%)	0.49 (0.17-1.43)	0.449
Virgin land	11 (20.8%)	42 (79.2%)		
Tetracycline				
Near road and farming	5 (9.4%)	48 (90.6%)	0.45 (0.14-1.41)	0.171
Virgin land	10 (18.9%)	43 (81.1%)		
Sulfamethoxazole				
Near road and farming	19 (35.8%)	34 (64.2%)	0.50 (0.23-1.09)	0.08
Virgin land	28 (52.8%)	25 (47.2%)		
Trimethoprim				
Near road and farming	15 (28.3%)	38 (71.7%)	1.22 (0.51-2.89)	0.66
Virgin land	13 (24.5%)	40 (75.5%)		

OR=Odds ratio; CI= Confidence interval

4.2.3.5 Association of drug resistance between virgin land and industrial zone

Table 4.7 shows the bivariate analysis of relationship in drug resistance between virgin land and industrial zone. There was a significant difference on cefoxitin (odds ratio of 6.44 (95%CI 2.76-15.02) and P value of 0.000 and amoxicillin/ clavulanic acid (odds ratio of 8.36 (95% CI 3.43-3.43) and P value of 0.000.

Table 4-7: Virgin land and industrial zone

Susceptibility of drugs	Resistant, n (%)	Susceptible, n (%)	OR (95%CI)	P value
Ampicillin				
Industrial zone	42 (79.2%)	11 (20.8%)		
Virgin land	35 (66.0%)	18 (34.0%)	1.96 (0.82-4.71)	0.13
Cefoxitin				
Industrial zone	39 (73.6%)	14 (26.4%)		
Virgin land	16 (30.2%)	37 (69.8%)	**6.44 (2.76-15.02)**	**0**
Cefpodoxime				
Industrial zone	11 (20.8%)	42 (79.2%)		
Virgin land	9 (17.0%)	44 (83.0%)	1.28 (0.48-3.40)	0.62
Amoxicillin/clavulanic acid				
Industrial zone	43 (81.1%)	10 (18.9%)		
Virgin land	18 (34.0%)	35 (66.0%)	**8.36 (3.43-3.43)**	**0**
Streptomycin				
Industrial zone	6 (11.3%)	47 (88.7%)		
Virgin land	11 (20.8%)	42 (79.2%)	0.49 (0.17-1.43)	0.449
Tetracycline				
Industrial zone	6 (11.3%)	47 (88.7%)		
Virgin land	10 (18.9%)	43 (81.1%)	0.55 (0.18-1.64)	0.282
Sulfamethoxazole				
Industrial zone	27 (50.9%)	26 (49.1%)		
Virgin land	28 (52.8%)	25 (47.2%)	0.93 (0.43-1.99)	0.846
Trimethoprim				
Industrial zone	11 (20.8%)	42 (79.2%)		
Virgin land	13 (24.5%)	40 (75.5%)	0.81 (0.32-2.01)	0.643

OR=Odds ratio; CI= Confidence interval

4.2.3.6 Association of multiple antibiotic resistance between virgin land and the other different sites

Table 4.8 shows the bivariate analysis of relationship of multi drug resistance between virgin land and the other sites. There was a significant difference in industrial zone compared to virgin land with an odds ratio of 5.82 (95% CI1.99-17.06), and P value of 0.001.

Table 4-8: MDR isolates from virgin land and other sites

Sites	MDR (>3 drugs), n (%)	Non MDR (<3 drugs), n (%)	OR (95%CI)	P value
Domestic use	31 (58.5%)	22 (41.5%)		
Virgin land	33 (62.3%)	20 (37.7%)	0.85 (0.39-1.86)	0.691
Settlements	26 (49.1%)	27 (50.9%)		
Virgin land	33 (62.3%)	20 (37.7%)	0.58 (0.27-1.27)	0.172
Sewerage effluent	33 (62.3%)	20 (37.7%)		
Virgin land	33 (62.3%)	20 (37.7%)	1.00 (0.46-2.19)	1
Near road and farming	39 (73.6%)	14 (26.4%)		
Virgin land	33 (62.3%)	20 (37.7%)	1.69 (0.74-3.85)	0.214
Industrial zone	48 (90.6%)	5 (9.4%)		
Virgin land	33 (62.3%)	20 (37.7%)	**5.82 (1.99-17.06)**	**0.001**

OR=Odds ratio; CI= Confidence interval

4.2.4 Distribution of resistance phenotypes across sites

The resistance isolates from the antimicrobials tested were placed into groups starting with the most susceptible antimicrobial to the most resistant and distribution across the sites. Table 4.9 below summarizes the groupings.

Table 4-9: Prevalence and distribution of resistance phenotypes across sites

Group of antimicrobials	Total, n	Domestic use	Settlements	Virgin land	Sewage effluent	Near road and farming	Industrial zone
Cn, Fep, Caz, C, Cip, Na, Atm, S, Cpd, Te, W, Smx, Amc, Fox, Amp	2	1	0	1	0	0	0
Fep, Caz, C, Cip, Ctx, Na, Atm, S, Cpd, Te, W, Smx, Amc, Fox, Amp	5	1	0	4	0	0	0
Caz, Cip, Ctx, Na, W, Amc, Fox, Amp	4	2	0	1	0	0	1
C, Cip, Na, Atm, S, Cpd, Te, W, Smx, Amc, Amp	9	2	1	3	2	1	0
Cip, Ctx, Na, Atm, S, Cpd, Te, W, Smx, Amc, Fox, Amp	17	9	0	2	4	0	2
Ctx, Na, Atm, S, Cpd, Te, W, Smx, Amc, Fox, Amp	19	6	4	2	6	1	0
Na, Atm, Cpd, Te, W, Smx, Amc, Fox, Amp	22	3	3	1	2	4	9
Atm, S, Cpd, Te, W, Smx, Amc, Fox, Amp	20	2	3	5	4	2	4
S, Cpd, Te, W, Smx, Amc, Fox, Amp	32	2	2	6	3	6	13
Cpd, Te, W, Smx, Amc, Fox, Amp	31	4	5	5	3	6	2
Te, W, Smx, Amc, Fox, Amp	25	4	3	5	9	2	2
W, Smx, Amc, Fox, Amp	24	7	5	2	5	3	2
Smx, Amc, Fox, Amp	38	5	7	7	6	3	10
Amc, Fox, Amp	27	1	5	0	1	13	7
Fox, Amp	12	1	4	2	3	2	0
Amp	14	2	0	6	0	5	1

N.B: Amp: Ampicillin, Fox:Cefoxitin, Ctx: Cefotaxime, Caz: Ceftazidime, Cpd: Cefpodoxim, Fep: Cefepime, Atm: Aztreonam, Amc: Amoxicillin/clavulanic acid, Cip: Ciprofloxacin, S: Streptomycin, Na: Nalidixic, Cn: Gentamicin, C: Chloramphenicol, Te: Tetracycline, Smx: Sulfamethoxazole, W: Trimethoprim

4.2.4.1 Six resistance profiles selected

From a total 301 *E. coli* isolates grouped to resistance profiles in table 4.9 above, six resistance profiles were randomly selected to present profiles that were most commonly found in Athi River. Table 4.10 below shows the six resistance profiles and the number of isolates from each site.

Table 4-10: Six Resistance profiles of selected isolates

Resistance profile	Total number	Domestic use	settlements	Virgin land	Sewage effluent	Near road and farming	Industrial zone
C, Cip, Na, Atm, S, Cpd, Te, W, Smx, Amc, Amp	9	2	1	3	2	1	0
Cip, Ctx, Na, Atm, S, Cpd, Te, W, Smx, Amc, Fox, Amp	17	9	0	2	4	0	2
Na, Atm, Cpd, Te, W, Smx, Amc, Fox, Amp	22	3	3	1	2	4	9
S, Cpd, Te, W, Smx, Amc, Fox, Amp	32	2	2	6	3	6	13
Smx, Amc, Fox, Amp	38	5	7	7	6	3	10
Amc, Fox, Amp	27	1	5	0	1	13	7

N.B: Amp:Ampicillin, Fox:Cefoxitin, Ctx:Cefotaxime, Caz:Ceftazidime, Cpd:Cefpodoxim, Fep:Cefepime, Atm:Aztreonam, Amc:Amoxicillin/clavulanic acid, Cip:Ciprofloxacin, S:Streptomycin, Na:Nalidixic, Cn:Gentamicin, C: Chloramphenicol, Te: Tetracycline, Smx: Sulfamethoxazole, W:Trimethoprim

4.5 ESBLs proportion

There were a total of 7out of 318 (2.2%) *E. coli* isolates that were ESBL- producers. Table 4.11 shows their resistance profile and site where they are isolated from.

Table 4-11: Resistance profiles of ESBL isolates across sites

Resistance profile	Total, n	Domestic use	Settlements	Virgin land	Sewage effluent	Near road and farming	Industrial zone
Cip, S, Na, Cn, Smx, Te, Amt, Amp, Fep, Caz, Cpd	2	0	1	0	0	0	1
S, Na, Cn, Smx, C, Te, Amp, Atm, Fep, Caz, Cpd, Amc	1	0	1	0	0	0	0
Cip, S, Na, C, Te, Amp, Amc	1	0	0	1	0	0	0
S, Na, Smx, Atm, Fep, Caz	1	0	0	0	1	0	0
Cip, S, Na, Cn, Smx, C, Te, Atm, Amp, Caz, Fep, Cpd, Amc	2	0	0	0	1	0	1

N.B: Amp: Ampicillin, Fox: Cefoxitin, Ctx:Cefotaxime, Caz:Ceftazidime, Cpd:Cefpodoxim, Fep:Cefepime, Atm:Aztreonam, Amc:Amoxicillin/clavulanic acid, Cip:Ciprofloxacin, S:Streptomycin, Na:Nalidixic, Cn:Gentamicin, C: Chloramphenicol, Te: Tetracycline, Smx: Sulfamethoxazole, W:Trimethoprim

Double-disk synergy

Figure 4.9 below shows an isolate that is an ESBL producer. ESBLs hydrolyse third generation cephalosporins and azreonam but are inhibited by clavulanic acid as seen in the figure below.

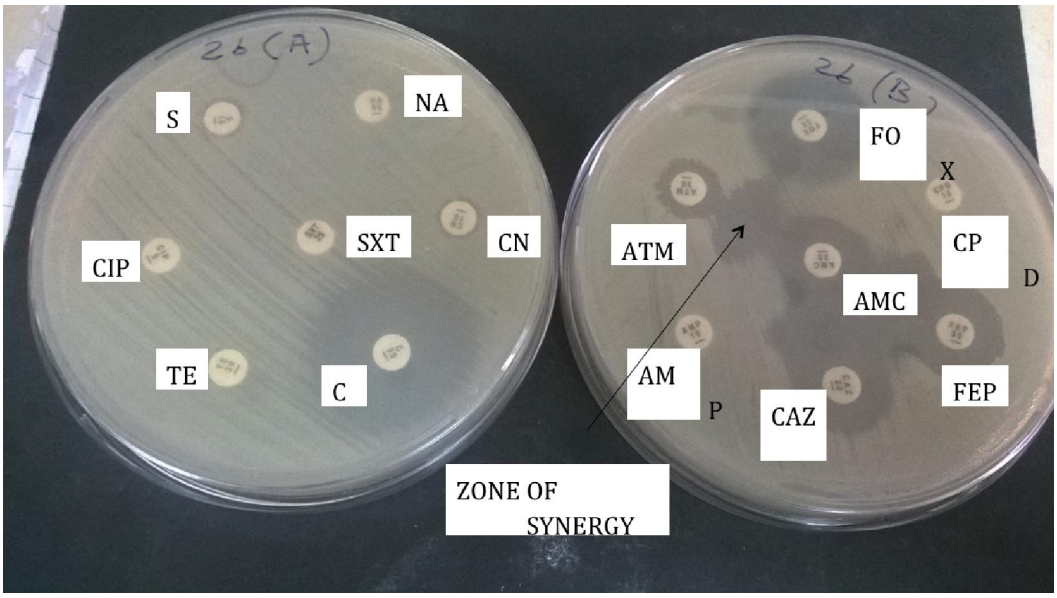


Figure 4-9: Disk diffusion showing a phenotypic ESBL producer

The plate(1) on the left, *E. coli* isolate isolated from water near industrial zone was resistant to most antimicrobials with exception to chloramphenicol while on right plate(2), zone of synergy was noted between amoxicillin/clavulanic acid (AMC) in the middle and cefoxtin (FOX) and aztreonam (ATM).

4.6 Resistance genes

4.6.1 Distribution of resistance genes *bla*$_{TEM}$, *bla* $_{CTX-M}$, *bla* $_{CMY}$

Out of 318 *E. coli* isolates, those that had resistance genes *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{CMY}$ were 265(83%) and the rest had no resistance genes. B*la*$_{CTX-M}$ had the highest prevalence of 106 (33%) (CI= 27.8-38.2) while *bla*$_{TEM}$ had lowest prevalence of 70 (22%) (CI =17.45-26.55) as shown in Figure 4.10.

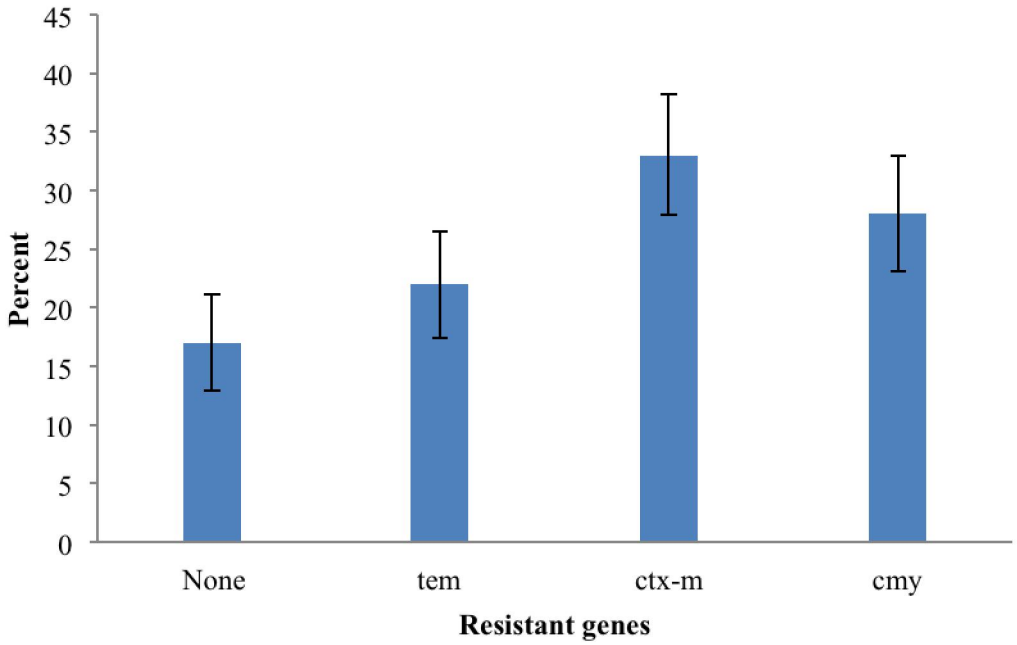


Figure 4-10: Distribution of resistance genes

PCR analysis of bla_{TEM}, bla_{CTX-M} and bla_{CMY} genes.318 isolates were analyzed. These genes are responsible for resistance against ampicillin, cefotaxime and cefoxitin respectively. The graph also shows error bars of percentage values at 95% CI.

4.6.2 Distribution of resistance genes across sites

Resistance genes bla_{TEM}, bla_{CTX-M} and bla_{CMY} were highest in sewage effluent and near road and farming while bla_{CMY} gene was relatively higher near sewage effluent 32(12%) and near road and farming 27(10.2%). bla_{TEM} had lower prevalence compared to the other two across sites as shown in Figure 4.11.

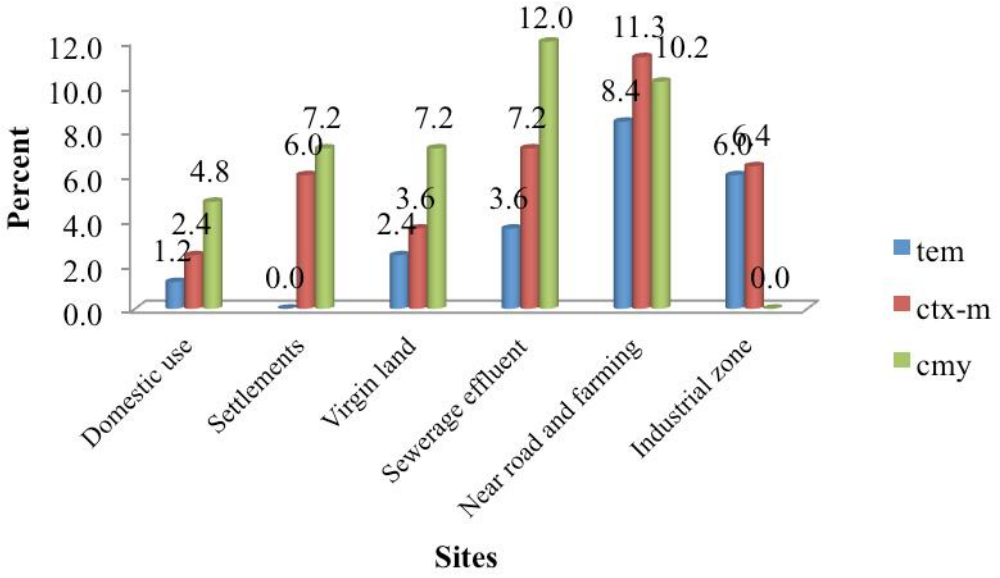


Figure 4-11: Distribution of resistance genes across sites

PCR analysis of *bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{CMY}$ genes. 318 isolates were analyzed. The values on bars are the exact percentages obtained. These genes are responsible for resistance against ampicillin, cefotaxime and cefoxitin respectively

4.6.3 Distribution of resistance genes during wet and dry period

bla$_{CMY}$ gene during dry period were 29(10.9%) (CI=7.2-14.7) and in wet season were 62 (23.4%) (18.3-28.5) while *bla*$_{TEM}$ were 19 (7.2) (CI=4.1-10.3) in dry season and 51 (19.2%) (CI=14.5-23.9). This is shown in Figure 4.12 below. Rainy season had more *bla* genes than dry season (P<0.004).

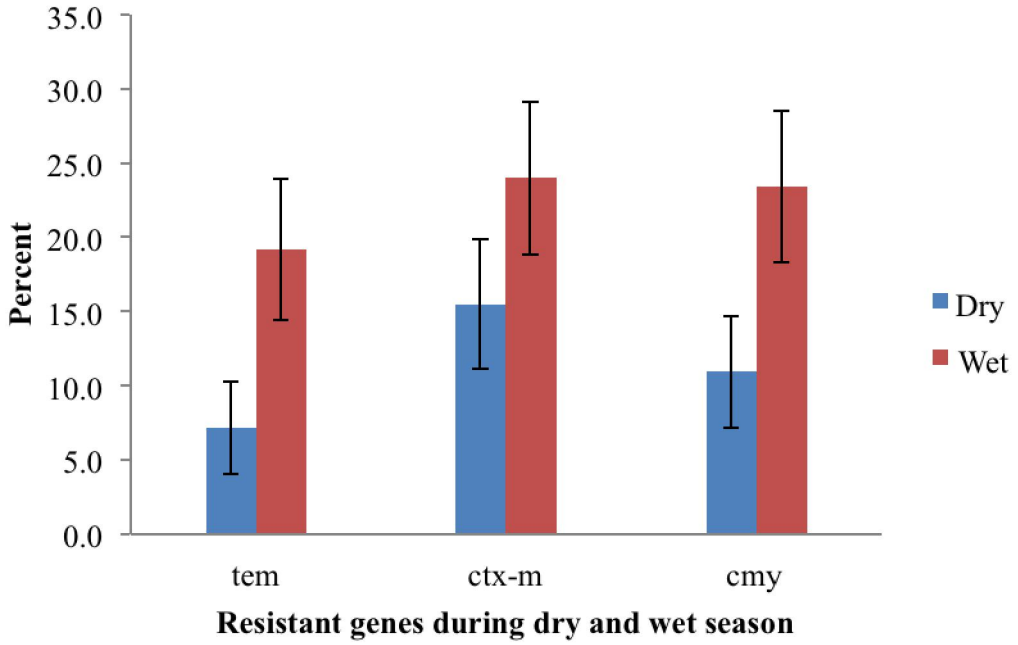


Figure 4-12: Resistance genes during wet and dry season

PCR analysis of bla_{TEM}, bla_{CTX-M} and bla_{CMY} genes.318 isolates were tested. These genes are responsible for resistance against ampicillin, cefotaxime and cefoxitin respectively. The graph shows error bars of percentage values at 95% CI.

4.6.4 Resistance profile of *bla*$_{TEM}$ positive isolates

Out of the 318 isolates 70 (22%) were found to be positive for bla_{TEM} gene. Table 4.12 below shows the resistance profiles for some of the isolates.

Table 4-12: Resistance profile of *bla*$_{TEM}$ isolates

Resistance profile	Total number	Domestic use	settlements	Virgin land	Sewage effluent	Near road and farming	Industrial zone
C, Cip, Na, Atm, S, Cpd, Te, W, Smx, Amc, Amp	1	0	0	0	0	0	0
Cip, Ctx, Na, Atm, S, Cpd, Te, W, Smx, Amc, Fox, Amp	5	1	0	1	1	0	2
Na, Atm, Cpd, Te, W, Smx, Amc, Fox, Amp	7	2	0	1	0	1	3
S, Cpd, Te, W, Smx, Amc, Fox, Amp	4	0	0	0	2	2	0
Smx, Amc, Fox, Amp	7	1	0	1	1	0	2
Amc, Fox, Amp	4	0	0	0	0	1	0

N.B: Amp: Ampicillin, Fox: Cefoxitin, Ctx: Cefotaxime, Caz: Ceftazidime, Cpd: Cefpodoxim, Fep: Cefepime, Atm: Aztreonam, Amc: Amoxicillin/clavulanic acid, Cip: Ciprofloxacin, S: Streptomycin, Na: Nalidixic, Cn: Gentamicin, C: Chloramphenicol, Te: Tetracycline, Smx: Sulfamethoxazole, W: Trimethoprim

4.6.5 Resistance profile of *bla$_{CTX-M}$* positive isolates

Isolates that were found positive for *bla $_{CTX-M}$* genes were 106 out of 318 isolates (33%). Table 4.13 below shows the distribution of some of the isolates on the phenotypes that were most common.

Table 4-13: Resistance profile of *bla$_{CTX-M}$* positive isolates

Resistance profile	Total number	Domestic use	settlements	Virgin land	Sewage effluent	Near road and farming	Industrial zone
C, Cip, Na, Atm, S, Cpd, Te, W, Smx, Amc, Amp	6	1	0	2	2	1	0
Cip, Ctx, Na, Atm, S, Cpd, Te, W, Smx, Amc, Fox, Amp	10	8	0	1	1	0	0
Na, Atm, Cpd, Te, W, Smx, Amc, Fox, Amp	12	0	2	0	1	3	6
S, Cpd, Te, W, Smx, Amc, Fox, Amp	18	1	1	6	1	0	9
Smx, Amc, Fox, Amp	19	4	2	3	3	3	4
Amc, Fox, Amp	16	0	2	0	1	8	5

N.B: Amp: Ampicillin, Fox: Cefoxitin, Ctx: Cefotaxime, Caz: Ceftazidime, Cpd: Cefpodoxim, Fep: Cefepime, Atm: Aztreonam, Amc: Amoxicillin/clavulanic acid, Cip:Ciprofloxacin, S: Streptomycin, Na: Nalidixic, Cn: Gentamicin, C: Chloramphenicol, Te: Tetracycline, Smx: Sulfamethoxazole, W: Trimethoprim

4.6.6 Resistance profile of *bla$_{CMY}$* positive isolates

The number of isolates that were positive for *bla$_{CMY}$* gene were 89 out of 318 isolates (28%). Table 4.14 below summarizes the common phenotype which had the gene and their distribution across the sites.

Table 4-14: Resistance profile of *bla$_{CMY}$* positive isolates

Resistance profile	Total number	Domestic use	Settlements	Virgin land	Sewage effluent	Near road and farming	Industrial zone
C, Cip, Na, Atm, S, Cpd, Te, W, Smx, Amc, Amp	2	1	0	1	0	0	0
Cip, Ctx, Na, Atm, S, Cpd, Te, W, Smx, Amc, Fox, Amp	2	0	0	0	2	0	0
Na, Atm, Cpd, Te, W, Smx, Amc, Fox, Amp	3	1	1	0	1	0	0
S, Cpd, Te, W, Smx, Amc, Fox, Amp	10	1	1	0	0	4	4
Smx, Amc, Fox, Amp	12	0	3	3	2	0	4
Amc, Fox, Amp	7	1	0	0	0	4	2

N.B: Amp: Ampicillin, Fox: Cefoxitin, Ctx: Cefotaxime, Caz: Ceftazidime, Cpd: Cefpodoxim, Fep: Cefepime, Atm: Aztreonam, Amc: Amoxicillin/clavulanic acid, Cip: Ciprofloxacin, S: Streptomycin, Na: Nalidixic, Cn: Gentamicin, C: Chloramphenicol, Te: Tetracycline, Smx: Sulfamethoxazole, W: Trimethoprim

4.7 Gel images for resistance genes

4.7.1 *bla*$_{\text{CTX-M}}$

Gel electrophoresis of *bla*$_{CTX-M}$ PCR products are shown in Figure 4.13.

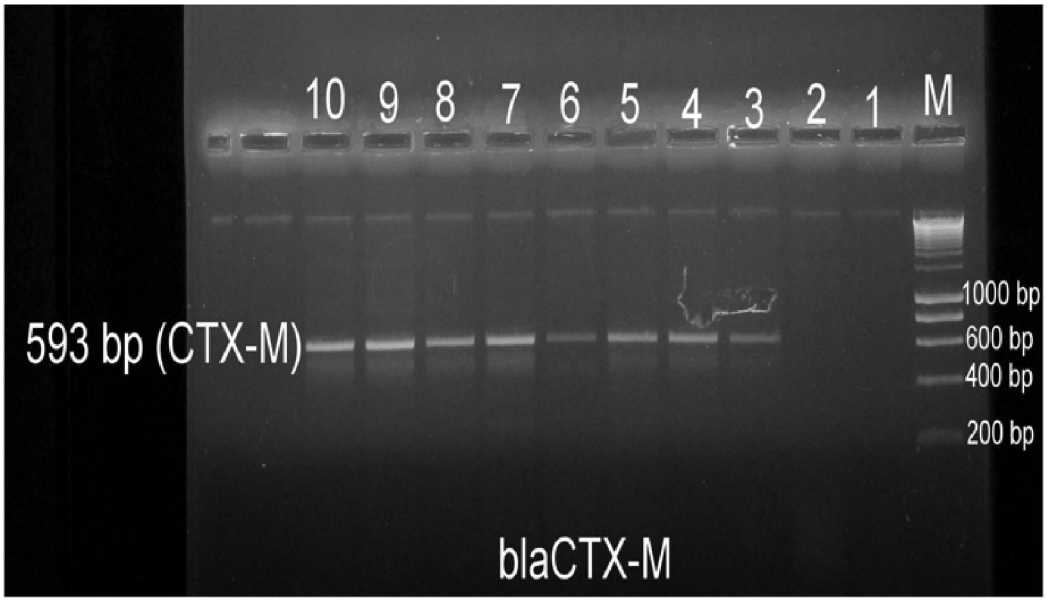


Figure 4-13: Gel image showing *bla*$_{CTX-M}$ gene

(M) -ladder, well 1 empty, 2-negative control, 3-6 positive isolates from sewage effluent and positive isolates 7-9 from site near road and farming, 10-positive control

4.7.2 *bla*$_{\text{TEM}}$

Figure 4.14 shows a sample of gel electrophoresis of *bla*$_{\text{TEM}}$ PCR

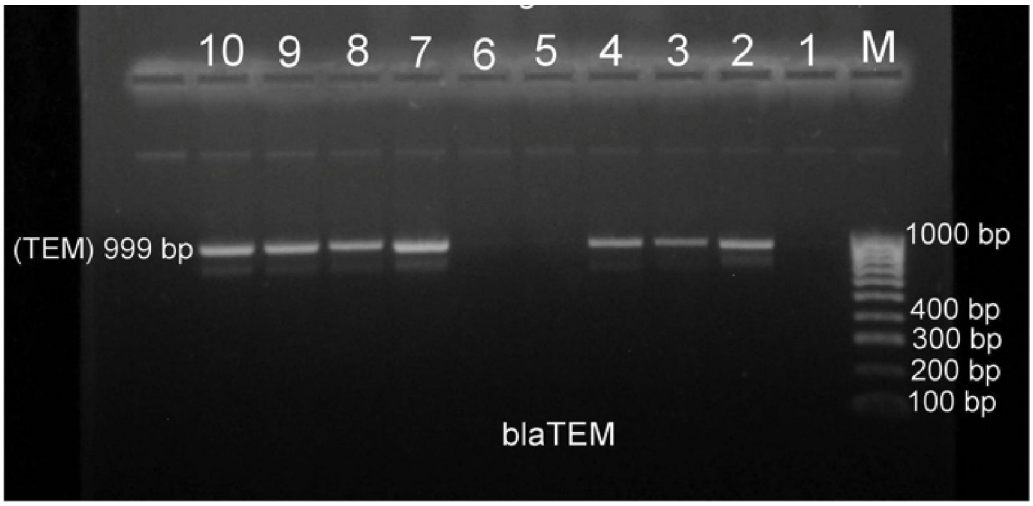


Figure 4-14: Gel image showing *bla$_{TEM}$* gene

(M)-ladder 1-negative control, 2-positive control 3 and 4 positive isolates from human settlements, isolate 7 from sewage effluent and isolates 8 and 9 from near road and farming, wells 5 and 6 were empty

4.7.3 *bla*$_{CMY}$

Figure 4.15 shows a sample of gel electrophoresis of *bla*$_{CMY}$ *PCR*.

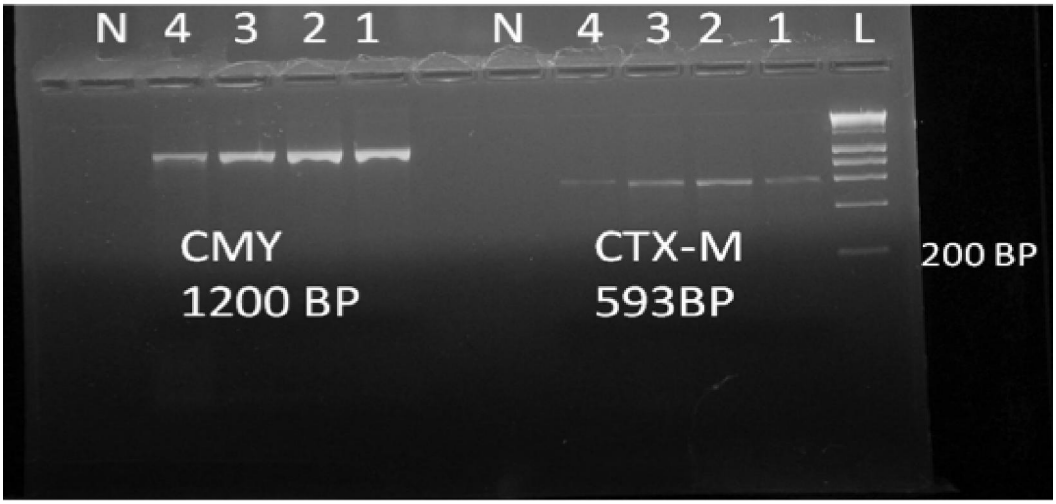


Figure 4-15: Gel image showing *bla$_{CMY}$* and *bla$_{CTX-M}$* genes:

L-ladder, N-negative control for *bla$_{CMY}$* gene on the left, 1 to 3 are positive isolates from seawage effluent. 4-positive control while on right *bla$_{CTX-M}$* gene 1 to 3 isolates positive from sewage effluent and 4-positive control

4.8 Carriage of *E. coli* pathotypes

4.8.1 Prevalence of virulence genes

Isolates that harbored virulence genes were 140 (44%) while those that did not have any virulence genes were 178 (56%) as shown in Figure 4.16.

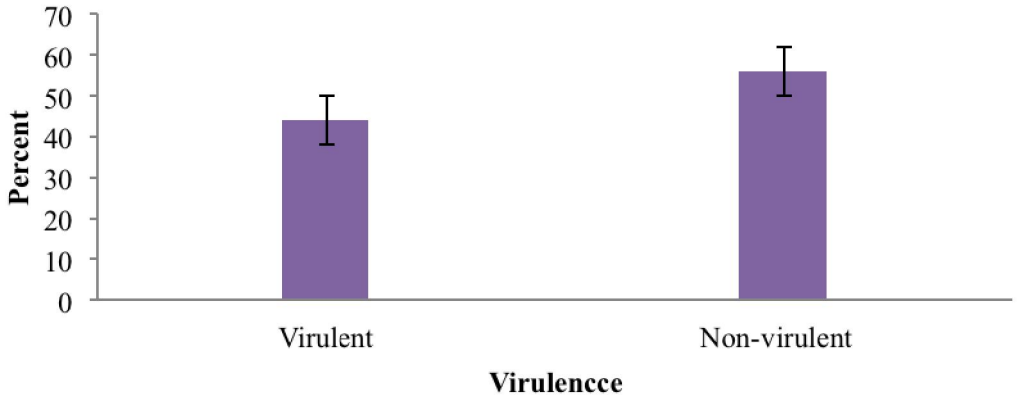


Figure 4-16: Prevalence of virulence genes

PCR analysis using primers for virulence genes. A total of 318 isolates were analyzed. The bar graph shows error bars from percentage using 95% CI

4.8.2 Distribution of selected virulence genes

EAEC pathotype had the highest prevalence of 35(25.0%) (CI17.8-32.2) while atypical EPEC had the lowest prevalence of 6(4.3%) (CI=0.9-7.6) as shown in Figure 4.17.

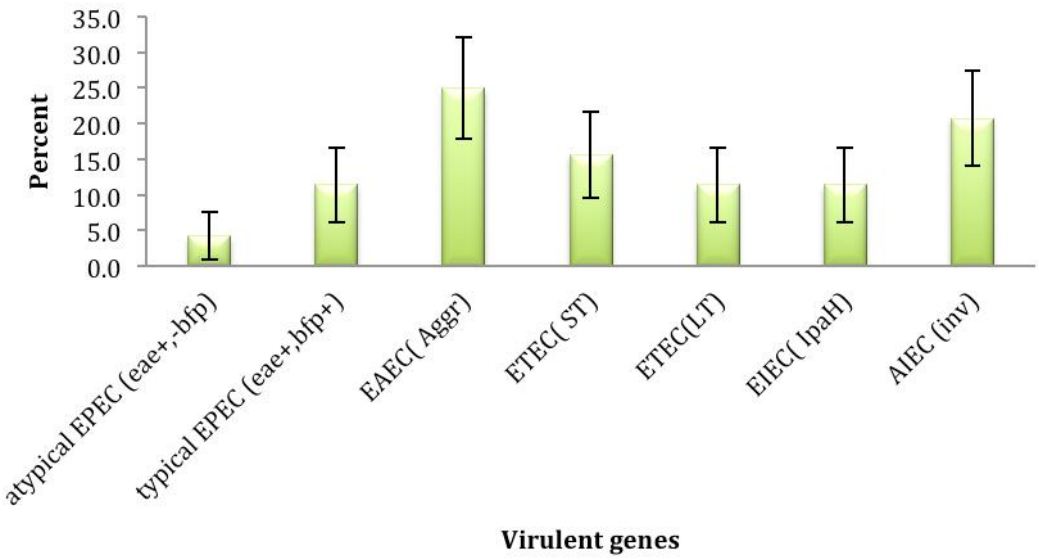


Figure 4-17: Distribution of virulence genes

PCR analysis using primers for virulence genes. A total of 318 isolates were analyzed. The bar graph shows error bars from percentage using 95% CI.

4.8.3 Distribution of virulence genes across sites

EAEC pathotype was highest near road and farming site and were not isolated in site used for domestic purposes. AIEC pathotype with *inv* gene were most in virgin land and not present in industrial zone. This is shown in Figure 4.18 below

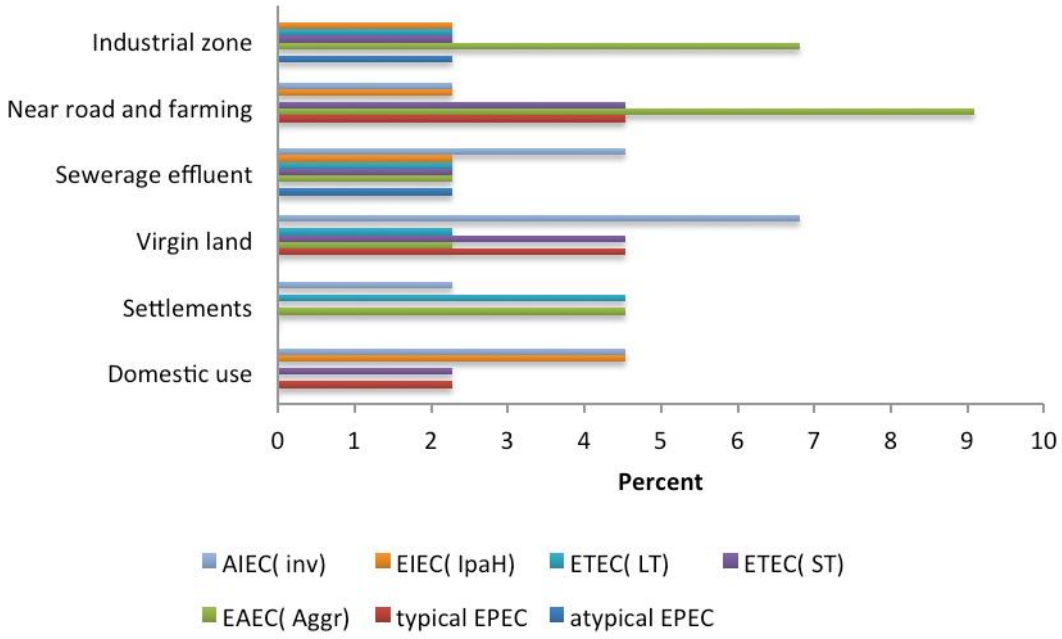


Figure 4-18: Distribution of virulence genes across sites

Distribution of *inv, ipaH, lt,st,aggr,bfp* and *eae* genes across sites sampled in the study. *Aggr* gene for EAEC pathotype has highest in site near road and farming

4.8.4 Distribution of virulence genes during dry and wet period

Virulence genes were recovered more during the rainy season than dry ($P < 0.001$) with an exception of aggregative gene which were more during dry period ($P \leq 0.005$) Figure 4.19 and Table 4.15 summarize these differences.

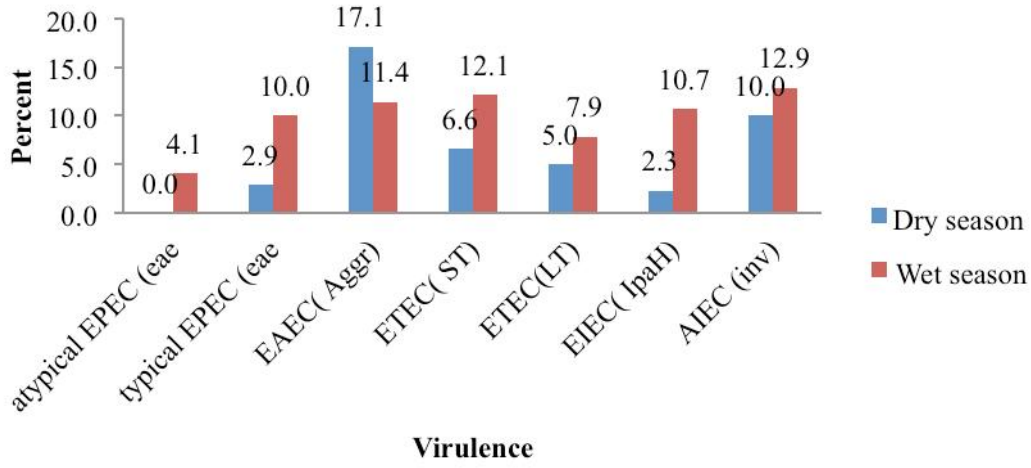


Figure 4-19: Distribution of virulence genes across sites

Virulence genes *inv, ipaH, lt,st,aggr,bfp* and *eae* were isolated more during the rainy season than dry with *inv* gene for AIEC highest during rainy season

Table 4-15: Distribution of virulence genes across sites during wet and dry period

Virulence	Domestic use n(%)		Settlements n(%)		Virgin land n(%)		Sewerage effluent n(%)		Near road and farming		Industrial zone n(%)	
	dry	wet	dry	wet	dry	wet	dry	wet	dry	wet	dry	wet
atypical EPEC eae+bfp-	0	0	0	0	0	0	0	3(2)	0	0	0	3(2)
typical EPEC eae+bfp+	3(2)	0	0	0	0	6(4)	0	0	0	6(4)	0	0
EAEC (aggr)	0	0	6(4)	0	3(2)	0	3(2)	0	0	14(10)	10(7)	0
ETEC (st)	3(2)	0	0	0	3(2)	0	0	3(2)	0	6(4)	0	3(2)
ETEC (lt)	0	0	6(4)	0	0	3(2)	0	3(2)	0	0	0	3(2)
EIEC (IpaH)	0	6(4)	0	0	0	0	0	3(2)	0	3(2)	3(2)	0
AIEC (inv)	7(5)	0	0	3(2)	0	10(7)	6(4)	0	0	3(2)	0	0

Tabulated results of the distribution of the virulence genes across each of the sampled sites in the study

4.8.5 Resistance phenotypes of virulence genes detected in *E. coli*

4.8.5.1 EAEC pathotype: Aggregative gene (*aggR*)

Isolates that were positive for *aggR* were assessed for their resistance phenotype and is summarized in Table 4.16.

Table 4-16: Resistance phenotype of *aggR* positive isolates

Resistance profile	Total number	Domestic use	Settlements	Virgin land	Sewage effluent	Near road and farming	Industrial zone
C, Cip, Na, Atm, S, Cpd, Te, W, Smx, Amc, Amp	1	0	0	1	0	0	0
Cip, Ctx, Na, Atm, S, Cpd, Te, W, Smx, Amc, Fox, Amp	0	0	0	0	0	0	0
Na, Atm, Cpd, Te, W, Smx, Amc, Fox, Amp	2	0	1	0	0	0	0
S, Cpd, Te, W, Smx, Amc, Fox, Amp	1	0	0	0	0	1	0
Smx, Amc, Fox, Amp	6	0	1	1	1	2	1
Amc, Fox, Amp	4	0	0	1	0	1	2

N.B: Amp: Ampicillin, Fox: Cefoxitin, Ctx: Cefotaxime, Caz: Ceftazidime, Cpd: Cefpodoxim, Fep: Cefepime, Atm: Aztreonam, Amc: Amoxicillin/clavulanic acid, Cip: Ciprofloxacin, S: Streptomycin, Na: Nalidixic, Cn: Gentamicin, C: Chloramphenicol, Te: Tetracycline, Smx: Sulfamethoxazole, W:Trimethoprim

4.8.5.2 Typical EPEC pathotype : eae+ and bfp+ genes

Those isolates positive for eae+ and bfp+ had the following resistance phenotype shown by Table 4.17.

Table 4-17: Resistance phenotypes of *eae*+ and *bfp*+ positive isolates

Resistance profile	Total number	Domestic use	settlements	Virgin land	Sewage effluent	Near road and farming	Industrial zone
C, Cip, Na, Atm, S, Cpd, Te, W, Smx, Amc, Amp	0	0	0	0	0	0	0
Cip, Ctx, Na, Atm, S, Cpd, Te, W, Smx, Amc, Fox, Amp	2	0	0	1	0	1	0
Na, Atm, Cpd, Te, W, Smx, Amc, Fox, Amp	1	1	0	0	0	0	0
S, Cpd, Te, W, Smx, Amc, Fox, Amp	3	0	0	2	0	1	0
Smx, Amc, Fox, Amp	1	0	0	0	0	1	0
Amc, Fox, Amp	4	0	0	0	4	0	0

N.B: Amp: Ampicillin, Fox: Cefoxitin, Ctx: Cefotaxime, Caz: Ceftazidime, Cpd: Cefpodoxim, Fep: Cefepime, Atm: Aztreonam, Amc: Amoxicillin/clavulanic acid, Cip: Ciprofloxacin, S: Streptomycin, Na: Nalidixic, Cn: Gentamicin, C: Chloramphenicol, Te: Tetracycline, Smx: Sulfamethoxazole, W: Trimethoprim

4.8.5.3 ETEC pathotype:st and lt genes

Isolates that were *st* and *lt* positive had resistance phenotype as shown in Table 4.18.

Table 4-18: Resistance phenotype for *st* and *lt* positive isolates

Resistance profile	Total number	Domestic use	Human settlement	Virgin land	Sewage effluent	Near road and farming	Industrial zone
C, Cip, Na, Atm, S, Cpd, Te, W, Smx, Amc, Amp	2	0	0	0	1	0	1
Cip, Ctx, Na, Atm, S, Cpd, Te, W, Smx, Amc, Fox, Amp	2	0	0	0	1	1	0
Na, Atm, Cpd, Te, W, Smx, Amc, Fox, Amp	5	0	0	0	3	2	0
S, Cpd, Te, W, Smx, Amc, Fox, Amp	1	0	0	0	1	0	0
Smx, Amc, Fox, Amp	2	0	0	0	2	0	0
Amc, Fox, Amp	8	0	0	0	1	7	0

N.B: Amp: Ampicillin, Fox: Cefoxitin, Ctx: Cefotaxime, Caz: Ceftazidime, Cpd: Cefpodoxim, Fep: Cefepime, Atm: Aztreonam, Amc: Amoxicillin/clavulanic acid, Cip: Ciprofloxacin, S: Streptomycin, Na: Nalidixic, Cn: Gentamicin, C: Chloramphenicol, Te: Tetracycline, Smx: Sulfamethoxazole, W: Trimethoprim

4.8.5.4 EIEC pathotype; IpaH gene

Table 4.19 shows the resistance phenotypes for *ipaH* positive isolates.

Table 4-19: Resistance phenotypes for isolates positive for *IpaH* gene

Resistance profile	Total number	Domestic use	settlements	Virgin land	Sewage effluent	Near road and farming	Industrial zone
C, Cip, Na, Atm, S, Cpd, Te, W, Smx, Amc, Amp	2	1	0	0	1	0	0
Cip, Ctx, Na, Atm, S, Cpd, Te, W, Smx, Amc, Fox, Amp	4	2	0	0	2	0	0
Na, Atm, Cpd, Te, W, Smx, Amc, Fox, Amp	0	0	0	0	0	0	0
S, Cpd, Te, W, Smx, Amc, Fox, Amp	0	0	0	0	0	0	0
Smx, Amc, Fox, Amp	2	1	0	0	1	0	0
Amc, Fox, Amp	5	3	0	0	1	1	1

N.B: Amp: Ampicillin, Fox: Cefoxitin, Ctx: Cefotaxime, Caz: Ceftazidime, Cpd: Cefpodoxim, Fep: Cefepime, Atm: Aztreonam, Amc: Amoxicillin/clavulanic acid, Cip: Ciprofloxacin, S: Streptomycin, Na: Nalidixic, Cn: Gentamicin, C: Chloramphenicol, Te: Tetracycline, Smx: Sulfamethoxazole, W: Trimethoprim

4.8.5.5 AIEC pathotype: inv gene

Isolates that were positive for *inv* gene had resistance phenotypes shown in Table 4.20 below.

Table 4-20: Resistance phenotypes for *inv* positive isolates

Resistance profile	Total number	Domestic settlements use		Virgin land	Sewage effluent	Near road and farming	Industrial zone
C, Cip, Na, Atm, S, Cpd, Te, W, Smx, Amc, Amp	2	0	0	0	2	0	0
Cip, Ctx, Na, Atm, S, Cpd, Te, W, Smx, Amc, Fox, Amp	1	0	0	1	0	0	0
Na, Atm, Cpd, Te, W, Smx, Amc, Fox, Amp	1	0	0	0	1	0	0
S, Cpd, Te, W, Smx, Amc, Fox, Amp	1	0	1	0	0	0	0
Smx, Amc, Fox, Amp	1	0	0	0	0	1	0
Amc, Fox, Amp	3	0	1	1	0	1	0

N.B: Amp: Ampicillin, Fox: Cefoxitin, Ctx: Cefotaxime, Caz:Ceftazidime, Cpd: Cefpodoxim, Fep: Cefepime, Atm:Aztreonam, Amc: Amoxicillin/clavulanic acid, Cip: Ciprofloxacin, S: Streptomycin, Na: Nalidixic, Cn: Gentamicin, C: Chloramphenicol, Te: Tetracycline, Smx: Sulfamethoxazole, W: Trimethoprim

4.9 Gel images of virulence genes

4.9.1Aggregative gene (*aggR*)

From gel electrophoresis, isolates positive for *aggR* gene were depicted as Figure 4.20 below.

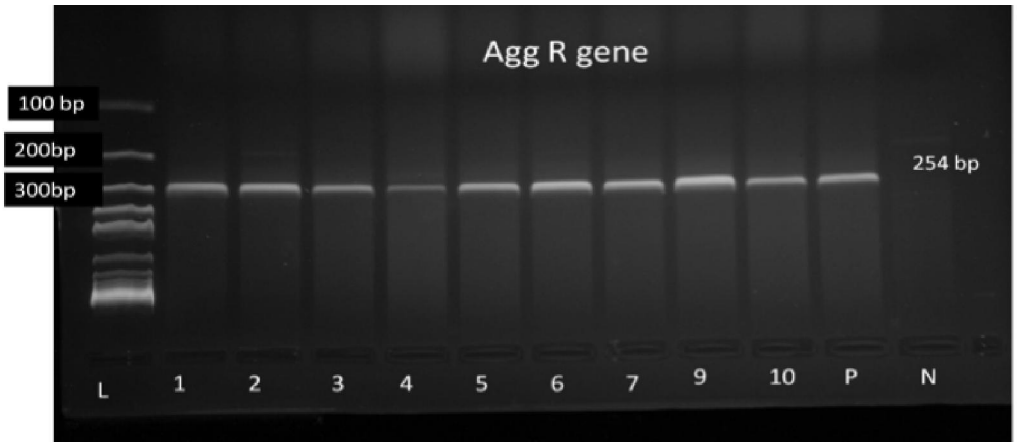


Figure 4-20: Gel image showing aggregative gene (*aggR*) *E. coli* isolates

EAEC pathotype carrying Aggregrative gene (aggr) bp 254 from *E. coli* isolated from close proximity to sewage treatment plant (L) ladder (P) positive control (N) negative control (1-10) *E. coli* isolates

4.9.2 Inv,lt,bfp and st genes

Image of *inv, bfp, lt* and *st* genes is represented in Figure 4.21.

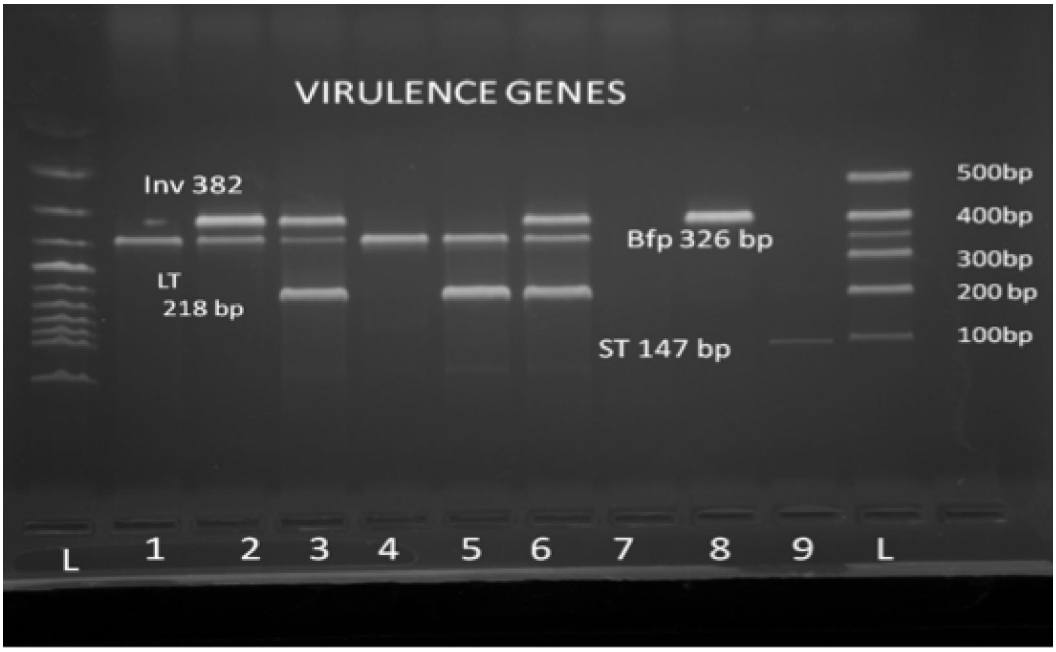


Figure 4-21: Gel image representing *inv, lt, st* and *bfp* virulence genes

Summary gel showing multiplex PCR for various pathotypes: AIEC pathotype *inv* positive at bp 382 (isolate 2, 3 and 6; 8-positive control,9-negative control) and ETEC pathotype with *lt* gene at 218 bp (positive control isolate 3, negative control 4; isolates 5 and 6 from human settlements and *ST* gene at 147 bp, typical EPEC that had *bfp* gene at 326 bp (1-5) positive isolates from sewage effluent 6-positive control, 7-negative control (L) ladder

4.9.2 Eae and ipaH genes

Isolates that were positive for *eae* and *ipaH* genes were captured on gel image and shown in Figure 4.22.

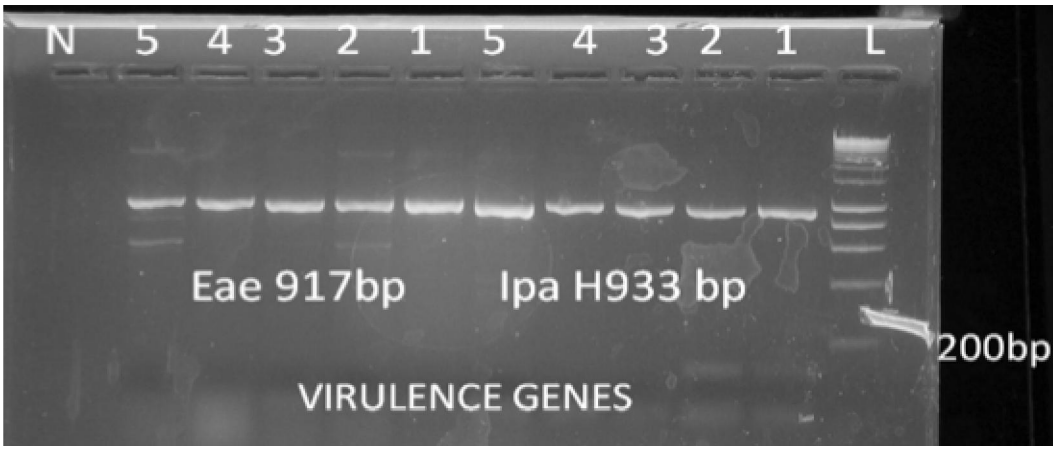


Figure 4-22: Gel image showing *eae* and *Ipa H* genes

E. coli isolates isolated from site near sewage treatment plant (N) Negative control (L) ladder, From the right, 1-4 isolates positive For *ipaH* gene(EIEC) 5- positive control; 1-4 isolates positive for *eae* gene (EPEC) 5- positive control.

4.10 Carriage of Integron 1

4.10.1 Prevalence of integron I gene

From 318 isolates analyzed, 114 (36%) had integron 1 while the rest did not have integron 1. The distribution of integron 1 gene across sites is as shown in Figure 4.23 below.

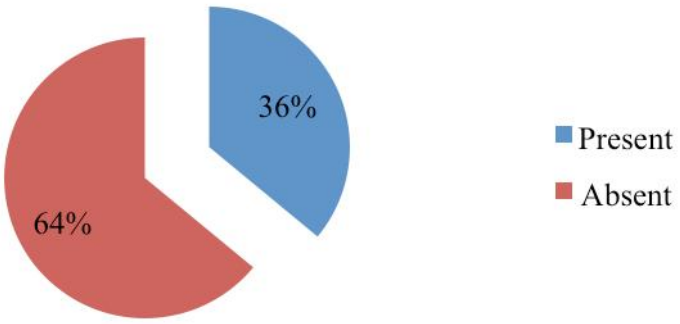


Figure 4-23: Prevalence of integron 1

4.10.2 Distribution of integron 1 across sites

Integron 1 gene was highest in near sewage effluent 11(30.6%) (CI=15.5-45.6%) and lowest in site where water is used for domestic purposes 3(8.3%) (CI= -0.7-17.3) as shown in Figure 4.24.

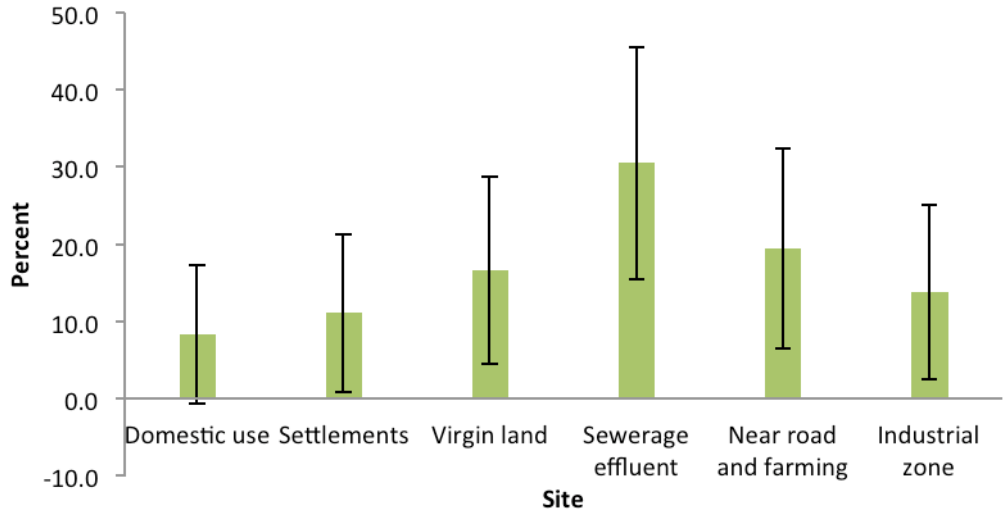


Figure 4-24: Distribution of integron 1 across sites

PCR analysis using primers for *int1* gene. A total of 318 isolates were analyzed. The bar graph shows error bars from percentage using 95% CI.

4.10.3 Distribution of integron 1 during wet and dry season

Integron 1 was isolated more during the rainy season than dry season (P<0.001). During rainy period, integron 1 was highest in sewage effluent 19.4%. During the dry period, integron 1 was highest in industrial zone (13.9%) and lowest in water used for domestic purposes and human settlement at 2.8% as shown in Figure 4.25.

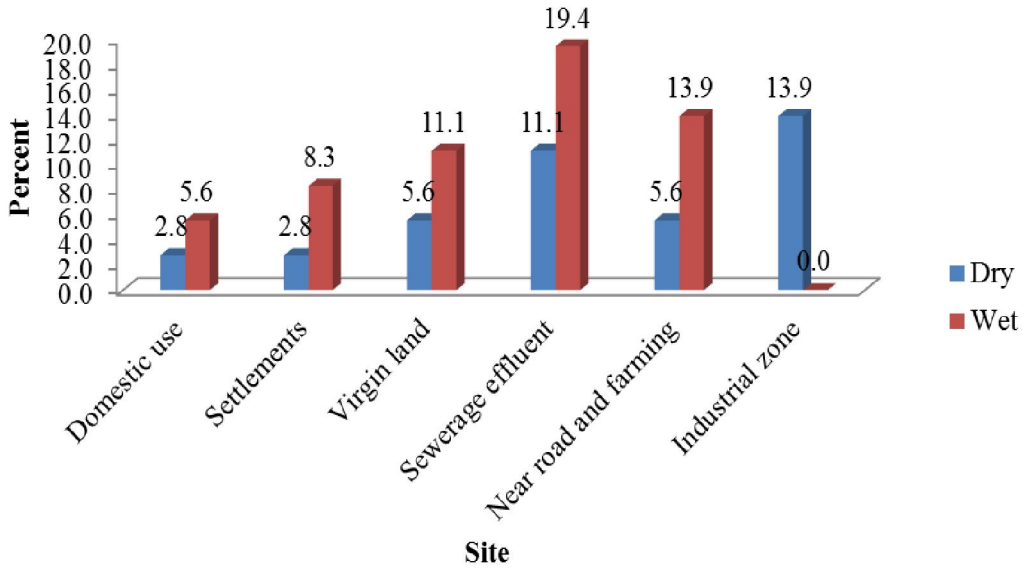


Figure 4-25: Distribution of integron 1 during wet and dry season

PCR analysis using primers for *int1* gene. A total of 318 samples were analyzed. The bar graph shows distribution of int1 gene during wet and dry period

4.10.4 Prevalence of isolates with both integron 1 gene and *bla* genes

The number of isolates which had both a *bla* gene and integrase 1 gene from the 318 *E. coli* isolates analyzed were bla_{TEM} and *int 1* 32 (17%), bla_{CTX-M} and *int1* 72 (32%) and bla_{CMY} and *int 1* 45 (22%) as shown in Figure 4.26

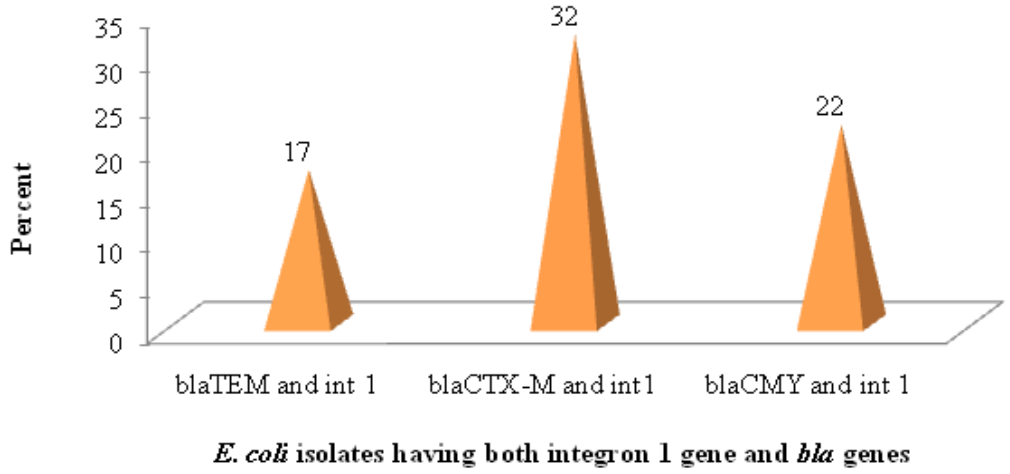


Figure 4-26: *E. coli* isolates having both integron 1 gene and *bla* genes

The figure shows isolates that had both resistance genes and integron 1 gene. Those isolates having *bla* $_{CTX-M}$ gene and integron 1 were more than those isolates with integron 1 and *bla* $_{TEM}$ and *bla* $_{CMY}$

4.10.5 Resistance profiles of integron 1 positive isolates

From the 318 isolates that were tested, 114 isolates were positive for *int1* gene which presents 36% of the isolates positive with integron class 1. Table 4.21 summarizes the resistance profiles of the positive isolates.

Table 4-21: Resistance profiles for *int 1* positive isolates

Resistance profile	Total number	Domestic use	settlements	Virgin land	Sewage effluent	Near road and farming	Industrial zone
C, Cip, Na, Atm, S, Cpd, Te, W, Smx, Amc, Amp	7	1	1	2	2	1	0
Cip, Ctx, Na, Atm, S, Cpd, Te, W, Smx, Amc, Fox, Amp	10	7	0	0	1	0	2
Na, Atm, Cpd, Te, W, Smx, Amc, Fox, Amp	11	0	1	0	2	4	4
S, Cpd, Te, W, Smx, Amc, Fox, Amp	20	0	0	1	2	5	12
Smx, Amc, Fox, Amp	27	0	0	1	6	3	7
Amc, Fox, Amp	21	0	1	0	0	13	7

N:B Amp: Ampicillin, Fox: Cefoxitin, Ctx: Cefotaxime, Caz: Ceftazidime, Cpd: Cefpodoxim, Fep: Cefepime, Atm: Aztreonam, Amc: Amoxicillin/clavulanic acid, Cip:Ciprofloxacin, S: Streptomycin, Na: Nalidixic, Cn: Gentamicin, C: Chloramphenicol, Te: Tetracycline, Smx: Sulfamethoxazole, W: Trimethoprim

4.10.6 Gel images of integron 1

From gel electrophoresis, Figure 4.27 below shows positive PCR products of *int1* gene amplification.

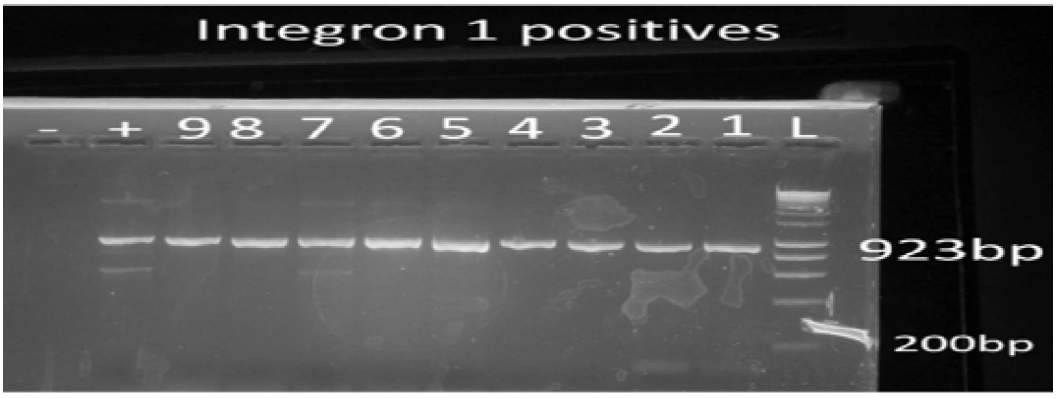


Figure 4-27: Positive PCR products of *int1* gene amplification

E. coli isolates positive for *int 1* from near settlements and industrial zone sampled. (-) Negative control, (+) positive control, (L) ladder (1-9) isolates positive for *int 1* at 923 bp

CHAPTER FIVE

DISCUSSION

This study showed higher total number of CFUs in areas with sewage effluent ($5.0x10^5$) industrial ($4.0x10^5$) and agricultural ($1.0x10^5$) activities, than those near virgin land ($5.0x10^4$) during wet season than dry ($P \leq 0.01$). These observations are similar to those obtained in previous studies and suggest a possible increase in the amount of contaminated surface run offs into the river system during the rainy period (Musyoki *et al*., 2013; Varghese & Roymon 2013).

Interestingly, the area near human settlements had lower number of CFUs as shown in Figure 4.2. This phenomenon cannot be explained. More so, areas located near a sewerage treatment plant recorded significantly high CFUs compared to other sites ($P \leq 0.01$). Better management of the sewage treatment should be encouraged because the study indicates its failure. Similar results have been obtained in a study conducted in Germany where microbial contamination of water passing through megacities was assessed (Abraham, 2011). Upstream from this area, the colony counts declined with those of human settlement showing least contamination. As expected, these results showed that water near the sewerage plant was more polluted. This observation indicates possible inefficiencies of sewage treatment plant and its management.

The current study also reported a high number of resistant bacteria especially to ampicillin, cefoxitin, amoxicillin/clavulanic acid and sulfamethazole. These results are close to those published in related studies conducted in Mexico, India, Kenya and Nigeria (Castillo *et al*., 2013; Kariuki *et al*., 2005; Varghese & Roymon 2013; Felicitas *et al*., 2010). This is a public health concern because increase in resistant bacteria may complicate treatment of infections arising from such strains. From the study, resistance to cefoxitin was 46.9% as shown in Figure 4.8 which was lower when compared to a study carried out in Philippines that sampled water hand pumps and reported resistance to cefoxitin of 59% (Fanuncio & Nuñeza, 2014).

High resistance to cefoxitin with concomitant low resistance to cephalosporins may indicate that majority of these isolates carry inducible chromosomal encoded AmpCs genes as suggested in other studies carried out in India (Mohamudha *et al*., 2010; Sreeshma *et al*.,2015). AmpC β-lactamases can confer resistance to aminopenicillins,

cephalosporins, oxyimino-cephalosporins (e.g. ceftriaxone, cefotaxime, and ceftazidime), cephamycins (e.g cefoxitin and cefotetan), and monobactams. Cloxacillin and 3-aminophenylboronic acid inhibit AmpC β-lactamases, while AmpC β-lactamase activity is not affected by the ESBL inhibitor clavulanic acid (Peter-Getzlaff *et al*., 2011). However, this study did not investigate whether the high resistance to cefoxitin was due to Amp C β-lactamases.

Generally, ciprofloxacin resistance was 6.9% as shown in Figure 4.8. When sites were compared to the virgin land, Ciprofloxacin resistance was highest in section of river used for domestic purposes. Resistance to third generation cephalosporins was observed such as cefotaxime (8.2%), ceftazidime (2.5%,) and to cefepime (1.9%). These antibiotics are used in treating complicated systemic bacterial infections and therefore, their potency is of immense clinical value (Sekowska *et al*., 2010). Similar results have been reported in related studies in Turkey, United Kingdom and in China (Arikan & Aygan 2009; Leonard *et al*., 2015; Zhang & Chang 2015). More so, β-lactams make up to 40% of the prescribed antimicrobials in an outpatient setting with cephalosporins making up 14% of prescriptions in same setting; therefore if resistant β-lactam strains are found in aquatic environment, they present a health risk to the public using the water (Jacobson *et al*., 1995).

Resistance to sulfamethazole was high (44%) in Figure 4.8 and when compared to virgin land, it was (52.8%) in virgin land from Table 4.1 which indicates that the virgin land with no apparent human or industrial activities also harbored highly resistant strains. This is similar to results from a related study in Poland that reported resistance of 94.4% among integron-harbouring *E. coli* from upstream water (Koczura *et al*., 2012). Amoxillin/clavulanic acid resistance was 46.2% in general and when compared to virgin land was a high of 81.1% in industrial area which was significantly high while compared with other sites with ($P<0.001$). Reports on bacteria resistant to amoxicillin/clavulanic acid in clinical settings emerged as early as 1987 and majority of these were isolated from patients who were receiving this antibiotic combination as a first line treatment for respiratory infections (Lefton-Guibout & Heym 2000). Resistance to this antimicrobial is worrying because amoxicillin/clavulanic is in the World Health Organization list of agents that are frequently used for empiric treatment of many infections. It is therefore possible that

isolates showing resistance to this antibiotic combination have a clinical background (Castillo *et al*., 2013; Hawkey *et al*., 2009; Khan et al., 2016).

In this study, 65.4% of *E. coli* isolates showed resistance to three or more groups of antimicrobials screened as shown in Table 4.2and 4.8. Majority of the isolates were resistant to four to five groups of antimicrobials (54.7%) from Table 4.2, which were found more in industrial zone and significantly different from other sites ($P \leq 0.001$). Presence of highly MDR strains in the water system could be interpreted to mean that Athi River is a significant reservoir for MDR strains. Related studies conducted in China and Oregon identified similar river systems as a significant risk factors for highly MDR strains (Ali *et al*., 2012; Hamelin *et al*., 2007; Tao *et al.*, 2010). Other studies have similarly identified highly resistant strains from river sections polluted with industrial effluent (Lupo *et al*., 2012; Kummerer 2004; Suzuki *et al*., 2012). Similar to these findings, a related study showed that high MDR strains are more common from water ways contaminated with runoffs from land occupied by animal and human, and those with urban and industrial sewerage (Khan *et al.,* 2016). More so, the study showed that sections of river that showed no apparent human and agricultural activities had no *E. coli* isolates which were fully susceptible to antimicrobials.

Although no experiments were done in this study to link the MDR isolates to infections, the high antimicrobial resistance pattern observed highly suggest that some of these strains may have a clinical background where they are subjected to strong selection pressure due to heavy use of antimicrobials and eventually find their way to the environment through human activities.

The proportion of ESBL producing *E. coli* in the study was 2.2% (7 out of 318 isolates) from Figure 4.9. A study done in Switzerland has linked presence of ESBL strains in water to anthropogenic activities and intensive agriculture where they reported prevalence of 36.2% ESBL producers from De Boeck and Rivers (Zurfluh *et al*., 2013). There are other similar studies that have reported ESBLs in aquatic environment and found that their distribution across the sampling points were almost uniform with very little difference between sites (Tissera & Lee 2013). This was similar to this study as the 7 isolates out of 318 were distributed across sites.

The resistance genes that were isolated from this study were *bla*$_{TEM}$ (22%), *bla*$_{CTX-M}$ (33%) and *bla*$_{CMY}$ (28%) as shown in Figure 4.10. Evidence has shown that antibiotic resistant bacteria and antibiotic resistance genes (ARGs) are ubiquitous in natural environments, including sites considered pristine. This finding is similar to this study because AGR genes were found in virgin land. The distribution of genes were more during wet season ($P<0.004$) and near the sewage treatment plant with the proximity of the plant strongly influencing the local antimicrobial resistance genes and this was similar to a study done in Havana, Cuba on almendares River (Knapp *et al*., 2012). The study argued that ARG (antimicrobial resistance genes) conditions differed dramatically between the dry and wet season in a tropical river like the Almendares River. In the dry season, significant spatial localization in ARG developed due to lower flow rates, increased local influence of outfalls, and reduced sediment transport. However, dry reason sampling allowed one to identify major inputs that influence stream ARG. In contrast, ARG transport rate downstream was much greater in the wet-season. Further, water column ARG were more equally distributed along the river during the wet-season. This implied the potential for increased human exposure occurred almost everywhere on the river during the wet season. Furthermore, a study done in China supported the theory of bacterial resistance being strongly influenced by discharge of waste water (Su *et al*., 2012).

However another study done in Denmark found that there was no difference in distribution of antimicrobial resistance genes across effluents of waste water treatment plants (WWTP), waste water and surface water and concluded that many unknown factors may influence the biological purification processes in conventional WWTPs and the evaluation of their relationship to ARG removal or selection which required more complex case studies (Laht *et al.*, 2014). Both *bla*$_{TEM}$ and *bla*$_{SHV}$ genes have been reported mostly from clinical samples and from environmental samples like farm animals and estuarine waters. Interestingly, majority of the isolates in this study were positive for *bla*$_{CTX-M}$ while none were positive for *bla*$_{SHV}$. The abundance of *bla*$_{CTX-M}$ in aquatic environments has been reported in other studies done in Switzerland and Malaysia (Zurfluh *et al*., 2013; Tissera & Lee 2013).

Enteroaggregative *E. coli* (EAEC) had the highest prevalence (11%) in this study as shown in Figure 4.17. Although no study in Kenya has analyzed prevalence of

diarrheagenic *E. coli* in river water, our findings concur with others done in Kenya using clinical isolates. A recent study in Kenya demonstrated EAEC (8.9%), as the most frequent followed by ETEC (1.2%) and EIEC (0.6%) (Sang *et al*., 2012). A similar study on shigatoxigenic *E. coli* from in Maasailand, Kenya, revealed a different scenario with ETEC (29.8%), EHEC (24% , EAEC (14.2%) and EPEC (3.5%) being identified (Sang *et al*., 2012).

Pathogenic *E. coli* has also been isolated in other studies from river water (Ndlovu *et al*., 2015). Presence of pathogenic *E. coli* in water creates a potential risk for infections in humans and animals especially if the water is used for irrigation, drinking and for recreational purposes (Hamelin *et al*., 2007; Koczura *et al*., 2012; Kummerer 2009). Increase in presence of multi-drug resistant pathogenic *E. coli* in water that was seen in San Pedro River in Mexico (Castillo *et al*., 2013) as well as in India where surface, municipal and ground water were collected (Varghese & Roymon 2013).

It is likely that multiple exposure pathways are involved in transmitting the MDR pathogenic *E. coli* to humans and river water play a significant role as it is highly contaminated. While the presence of virulence genes (VGs) in *E. coli* isolates alone is insufficient to determine pathogenicity, the presence of diarrheagenic *E. coli* pathotypes in high frequency after the rain period ($P<0.001$) could lead to increased health risks if untreated rain water were to be used for nonpotable purposes and recreational activities.

The prevalence of integron 1 was 36% in this study as shown in Figure 4.23. The *int 1* gene was found mostly near sewage treatment plant as well as after the treatment plant. This could be attributed by the effluent of the treatment plant becoming a reservoir of *intI* gene. This phenomenon was described by a previous study done in Poland (Koczura *et al*., 2012) where the number of integron I genes were higher downstream the discharge of WWTPS effluent. The wet season reported more *int1* genes than dry season ($P<0.001$) which could be explained by increased sedimentation as the water volume increased and moved more vigorously along the river bed that led to more genes from the sediments.

Analysis of antibiotic resistance profiles of *int1* positive *E. coli* showed that majority of them was multi resistant which is typical of bacteria harbouring integrons. These

isolates expressed a broader antimicrobial resistance ranges which was explained by greater diversity of the gene cassette content of class 1 integrons that was also observed in previous study (Koczura *et al*., 2012). Integron 1 in *E. coli* from aquatic environment has also been reported in previous study done in Kenya using water from different sources (47.4%, 18/38) (Njugu, 2011) which was higher compared to this study. In China, 41% of *E. coli* from Minjiang River harboured *int* 1(Chen *et al.*, 2011). The study also confirmed that isolates that carried integron 1 were more likely to be resistant to ampicillin and cephalosporins than those who didn't carry the integron.

CHAPTER SIX

CONCLUSIONS AND RECOMMEDATIONS

6.1 Conclusions

From the study, Athi River had a high occurence of *E. coli* isolates that is an indication of water pollution. The total number of CFUs was higher near the sewage effluent site ($5.0x10^5$, mean $9.5x10^3$) than human settlements ($8.8x\ 10^4$, mean $1.6x10^3$). This is likely due to high volume of sewage flow at this area compared to near the human settlements sampled .The standard guidelines from WHO stipulates that coliforms in water should not exceed 10^3 per 100 ml of water that is used for irrigation of crops eaten raw, sports fields and public parks. This guideline helped to conclude that Athi River is highly a polluted water system (WHO, 2006).

E. coli isolated from most sections were also highly resistant. The prevalence of MDR isolates in Athi River was also high especially in industrial zone. These findings are similar to those reported in clinical settings and therefore it can be concluded that anthropogenic activities along water bodies can play a role in the contamination of water with potential clinical isolates which can help spread antimicrobial resistance to the environmental strains.

Proportion of ESBL producing *E. coli* was low (2.2%) indicating that isolates from this River do not produce as much extended spectrum β lactamases as reported by other studies done in Africa.

Furthermore, the isolates from this study harbored resistance genes, virulence genes and integron class 1 genes. Resistance genes, virulence genes and integron class 1 were more near the sewage effluent during rainy than dry season. Isolates positive for integron 1 gene had broader resistance to multiple classes of antimicrobials which indicated that this River was a potential reservoir of resistance genes, virulence genes and integron 1.

The sewage treatment plant played a key role in contamination of the river and isolation of resistance and virulence genes.

6.2 Recommendations

Based on the findings of this study, the following recommendations are made

- Athi River should be decontaminated especially near the sewage treatment plant to reduce water pollution as well as proper waste handling along the river body.
- A prospective study to investigate the direct link of river isolates and infections in Machakos County should be carried out.
- Better treatment and quality control of the sewage treatment plant should be implemented.
- A surveillance system should be placed to monitor antimicrobial resistance in environment.
- There should be awareness carried out to inform general public on increasing antimicrobial resistance in the environment and this surveillance data on resistance should be availed to policy makers such as ministry of health who can use the information to advice on treatment options on patients coming from this region.

REFERENCES

Abe, C. M., Knutton, S., Pedroso, M. Z., Freymüller, E., & Gomes, T. A. (2001). An enteroaggregative *Escherichia coli* strain of serotype O111: H12 damages and invades cultured T84 cells and human colonic mucosa. *FEMS microbiology letters, 203*(2), 199-205.

Abraham, W. R. (2010). Megacities as sources for pathogenic bacteria in rivers and their fate downstream. *International journal of microbiology*, 2011.

Ali, H., Bacha, K., & Ketema, T. (2011). Bacteriological quality and antimicrobial susceptibility of some isolates of well water used for drinking in jimma town, southwest ethiopia. *Ethiopian Journal of Education and Sciences, 6*(2), 95-108.

Amábile-Cuevas, C. F., Arredondo-García, J. L., Cruz, A., & Rosas, I. (2010). Fluoroquinolone resistance in clinical and environmental isolates of *Escherichia coli* in Mexico City. *Journal of applied microbiology, 108*(1), 158-162.

Amaya, E., Reyes, D., Paniagua, M., Calderón, S., Rashid, M. U., Colque, P., ... & Nord, C. E. (2012). Antibiotic resistance patterns of *Escherichia coli* isolates from different aquatic environmental sources in Leon, Nicaragua. *Clinical Microbiology and Infection, 18*(9), E347-E354.

Amine, A. E. K. (2013). Extended Spectrum Beta-Lactamase Producing Bacteria in Waste Water Alexandria, *Egypt. International Journal of Bioscience, Biochemistry and Bioinformatics, 3*(6), 605.

Aranda, K. R., Fabbricotti, S. H., Fagundes-Neto, U., & Scaletsky, I. C. (2007). Single multiplex assay to identify simultaneously enteropathogenic, enteroaggregative, enterotoxigenic, enteroinvasive and Shiga toxin-producing *Escherichia coli* strains in Brazilian children. *FEMS microbiology letters, 267*(2), 145-150.

Arıkan, B., & Aygan, A. (2009). Resistance variations of third generation of Cephalosporins in some of the Enterobacteriaceae members in hospital sewage. *International Journal of Agriculture and Biology, 11*(1), 93-96.

Arslan, H., Azap, Ö. K., Ergönül, Ö., Timurkaynak, F., & Urinary Tract Infection Study Group. (2005). Risk factors for ciprofloxacin resistance among

Escherichia coli strains isolated from community-acquired urinary tract infections in Turkey. *Journal of Antimicrobial Chemotherapy, 56*(5), 914-918.

Baker-Austin, C., Wright, M. S., Stepanauskas, R., & McArthur, J. V. (2006). Co-selection of antibiotic and metal resistance. *Trends in microbiology, 14*(4), 176-182.

Bennett, P. M. (2008). Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *British journal of pharmacology, 153*(S1), S347-S357.

Blaak, H., de Kruijf, P., Hamidjaja, R. A., van Hoek, A. H., de Roda Husman, A. M., & Schets, F. M. (2014). Prevalence and characteristics of ESBL-producing E. coli in Dutch recreational waters influenced by wastewater treatment plants. *Veterinary microbiology, 171*(3), 448-459.

Castillo, F. Y. R., González, F. J. A., Garneau, P., Díaz, F. M., Barrera, A. L. G., & Harel, J. (2013). Presence of multi-drug resistant pathogenic *Escherichia coli* in the San Pedro River located in the State of Aguascalientes, Mexico. *Frontiers in microbiology*, 4, 1-16

Chakraborty, R., Kumar, A., Bhowal, S. S., Mandal, A. K., Tiwary, B. K., & Mukherjee, S. (2013). Diverse gene cassettes in class 1 integrons of facultative oligotrophic bacteria of river Mahananda, West Bengal, India. *PloS one, 8*(8), e71753.

Chen, B., Zheng, W., Yu, Y., Huang, W., Zheng, S., Zhang, Y., ... & Topp, E. (2011). Class 1 integrons, selected virulence genes, and antibiotic resistance in *Escherichia coli* isolates from the Minjiang River, Fujian Province, China. *Applied and environmental microbiology, 77*(1), 148-155.

Clermont, O., Dhanji, H., Upton, M., Gibreel, T., Fox, A., Boyd, D., ... & Frank, T. (2009). Rapid detection of the O25b-ST131 clone of *Escherichia coli* encompassing the CTX-M-15-producing strains. *Journal of antimicrobial chemotherapy, 64*(2), 274-277.

Cooke, M. D. (1976). Antibiotic resistance among coliform and fecal coliform bacteria isolated from the freshwater mussel Hydridella menziesii. *Antimicrobial agents and chemotherapy, 9*(6), 885-888.

Coutinho, F. H., Pinto, L. H., Vieira, R. P., Martins, O. B., Salloto, G. R. B., Santoro, D. D. O., ... & Cardoso, A. M. (2013). Antibiotic resistance in aquatic environments of Rio de Janeiro, Brazil. *InTech*.

Dakic, I., Lilakos, D., Svabic-Vlahovic, M., Christofilopoulou, S., Poggas, N., & Charvalos, E. (2007). Integron-associated antimicrobial resistance in isolates of members of Enterobacteriaceae causing community-acquired urinary infections. *Microbial Ecology in Health and Disease, 19*(3), 201-206.

De Boeck, H., Miwanda, B., Lunguya-Metila, O., Muyembe-Tamfum, J. J., Stobberingh, E., Glupczynski, Y., & Jacobs, J. (2012). ESBL-positive enterobacteria isolates in drinking water. *Emerging infectious diseases, 18*(6), 1019-1021.

Dhanji, H., Murphy, N. M., Akhigbe, C., Doumith, M., Hope, R., Livermore, D. M., & Woodford, N. (2011). Isolation of fluoroquinolone-resistant O25b: H4-ST131 *Escherichia coli* with CTX-M-14 extended-spectrum β-lactamase from UK river water. *Journal of antimicrobial chemotherapy, 66*(3), 512-516.

Doughari, H. J., Ndakidemi, P. A., Human, I. S., & Benade, S. (2011). Virulence factors and antibiotic susceptibility among verotoxic non O157: H7 *Escherichia coli* isolates obtained from water and wastewater samples in Cape Town, South Africa. African *Journal of Biotechnology, 10*(64), 14160-14168.

Fanuncio, L. J. C., & Nuñeza, O. M. (2014). Antibiotic sensitivity profile of *Escherichia coli* in water samples from water hand pumps in selected areas in Iligan City, Philippines. *Advances in Environmental Sciences, 6*(1).

Felicitas, N., Romanus, I. I., Azubuike, A. C., Eze, A. T., Collins, O. N., & Chidiebube, N. A. (2010). Presence of antibiotic resistant coliforms in sachet water sold in some parts of South Eastern Nigeria. Journal of Microbiology and Antimicrobials, 2(5), 51-54.

Gillings, M. R., Gaze, W. H., Pruden, A., Smalla, K., Tiedje, J. M., & Zhu, Y. G. (2015). Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *The ISME journal, 9*(6), 1269-1279.

Guion, C. E., Ochoa, T. J., Walker, C. M., Barletta, F., & Cleary, T. G. (2008). Detection of diarrheagenic *Escherichia coli* by use of melting-curve analysis

and real-time multiplex PCR. *Journal of clinical microbiology, 46*(5), 1752-1757.

Hall, R. M., & Collis, C. M. (1995). Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. *Molecular microbiology, 15*(4), 593-600.

Hamelin, K., Bruant, G., El-Shaarawi, A., Hill, S., Edge, T. A., Fairbrother, J., ... & Brousseau, R. (2007). Occurrence of virulence and antimicrobial resistance genes in *Escherichia coli* isolates from different aquatic ecosystems within the St. Clair River and Detroit River areas. *Applied and environmental microbiology, 73*(2), 477-484.

Hawkey, P. M., & Jones, A. M. (2009). The changing epidemiology of resistance. Journal of *Antimicrobial Chemotherapy, 64*(suppl 1), i3-i10.

Holt, K. E., Wertheim, H., Zadoks, R. N., Baker, S., Whitehouse, C. A., Dance, D., ... & Brisse, S. (2015). Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. *Proceedings of the National Academy of Sciences, 112*(27), E3574-E3581.

Hooper, D. C. (2001). Emerging mechanisms of fluoroquinolone resistance. *Emerging infectious diseases, 7*(2), 337.

Jacobson, K. L., Cohen, S. H., Inciardi, J. F., King, J. H., Lippert, W. E., Iglesias, T., & VanCouwenberghe, C. J. (1995). The relationship between antecedent antibiotic use and resistance to extended-spectrum cephalosporins in group I β-lactamase-producing organisms. *Clinical infectious diseases, 21*(5), 1107-1113.

Jacoby, G. A. (2009). AmpC β-lactamases. *Clinical microbiology reviews, 22*(1), 161-182.

Jacoby, G. A., Walsh, K. E., & Walker, V. J. (2006). Identification of extended-spectrum, AmpC, and carbapenem-hydrolyzing β-lactamases in *Escherichia coli* and Klebsiella pneumoniae by disk tests. *Journal of clinical microbiology, 44*(6), 1971-1976.

Jafari, A., Aslani, M. M., & Bouzari, S. (2012). Escherichia coli: a brief review of diarrheagenic pathotypes and their role in diarrheal diseases in Iran. *Iranian journal of microbiology, 4*(3), 102-17.

Jung, S. H. (2014). Stratified Fisher's exact test and its sample size calculation. *Biometrical Journal, 56*(1), 129-140.

Kaper, J. B., Nataro, J. P., & Mobley, H. L. (2004). Pathogenic escherichia coli. *Nature Reviews Microbiology, 2*(2), 123-140.

Karisik, E., Ellington, M. J., Pike, R., Warren, R. E., Livermore, D. M., & Woodford, N. (2006). Molecular characterization of plasmids encoding CTX-M-15 β-lactamases from *Escherichia coli* strains in the United Kingdom. *Journal of antimicrobial chemotherapy, 58*(3), 665-668.

Kariuki, S., Revathi, G., Corkill, J., Kiiru, J., Mwituria, J., Mirza, N., & Hart, C. A. (2007). *Escherichia coli* from community-acquired urinary tract infections resistant to fluoroquinolones and extended-spectrum beta-lactams. *The Journal of Infection in Developing Countries, 1*(03), 257-262.

Khan, S., Knapp, C. W., & Beattie, T. K. (2016). Antibiotic Resistant Bacteria Found in Municipal Drinking Water. Environmental Processes, 1-12.

Kiiru, J., Kariuki, S., Goddeeris, B. M., & Butaye, P. (2012). Analysis of β-lactamase phenotypes and carriage of selected β-lactamase genes among *Escherichia coli* strains obtained from Kenyan patients during an 18-year period. *BMC microbiology, 12*(1), 1.

Knapp, C. W., Lima, L., Olivares-Rieumont, S., Bowen, E., Werner, D., & Graham, D. W. (2012). Seasonal variations in antibiotic resistance gene transport in the Almendares River, Havana, Cuba. *Frontiers in microbiology*, 3, 396.

Koczura, R., Mokracka, J., Jabłońska, L., Gozdecka, E., Kubek, M., & Kaznowski, A. (2012). Antimicrobial resistance of integron-harboring *Escherichia coli* isolates from clinical samples, wastewater treatment plant and river water. *Science of the Total Environment*, 414, 680-685.

KONG, K. F., Schneper, L., & Mathee, K. (2010). Beta-lactam antibiotics: from antibiosis to resistance and bacteriology. *Apmis, 118*(1), 1-36.

Kümmerer, K. (2004). Resistance in the environment. *Journal of Antimicrobial Chemotherapy, 54*(2), 311-320.

Kümmerer, K. (2009). Antibiotics in the aquatic environment–a review–part I. Chemosphere, 75(4), 417-434.

Kümmerer, K. (2009). Antibiotics in the aquatic environment–a review–part II. *Chemosphere, 75*(4), 435-441.

Laht, M., Karkman, A., Voolaid, V., Ritz, C., Tenson, T., Virta, M., & Kisand, V. (2014). Abundances of tetracycline, sulphonamide and beta-lactam antibiotic resistance genes in conventional wastewater treatment plants (WWTPs) with different waste load. *PloS one, 9*(8), e103705.

Leflon-Guibout, V., Speldooren, V., Heym, B., & Nicolas-Chanoine, M. H. (2000). Epidemiological Survey of Amoxicillin-Clavulanate Resistance and Corresponding Molecular Mechanisms in Escherichia coliIsolates in France: New Genetic Features of bla_{TEM} Genes. *Antimicrobial agents and chemotherapy, 44*(10), 2709-2714.

Leonard, A. F., Zhang, L., Balfour, A. J., Garside, R., & Gaze, W. H. (2015). Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters. *Environment international*, 82, 92-100.

Liu, L., Phanikumar, M. S., Molloy, S. L., Whitman, R. L., Shively, D. A., Nevers, M. B., ... & Rose, J. B. (2006). Modeling the transport and inactivation of E. coli and enterococci in the near-shore region of Lake Michigan. Environmental science & technology, 40(16), 5022-5028.

Livermore, D. M. (1995). beta-Lactamases in laboratory and clinical resistance. *Clinical microbiology reviews*, 8(4), 557-584.

Lupo, A., Coyne, S., & Berendonk, T. U. (2012). Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies. Analyzing possible intersections in the resistome among human, animal and environment matrices, 116.

Mansan-Almeida, R., Pereira, A. L., & Giugliano, L. G. (2013). Diffusely adherent *Escherichia coli* strains isolated from children and adults constitute two different populations. *BMC microbiology, 13*(1), 1.

Mbithi, J. N., Musyoki, A. M., Abednego, M., Maingi, J. M., & Suleiman, M. A. (2013). Water-borne bacterial pathogens in surface waters of Nairobi River and health implication to communities downstream Athi River.

Mellies, J. L., Navarro-Garcia, F., Okeke, I., Frederickson, J., Nataro, J. P., & Kaper, J. B. (2001). espC pathogenicity island of enteropathogenic *Escherichia coli* encodes an enterotoxin. *Infection and immunity, 69*(1), 315-324.

Mohamudha, P. R., Harish, B. N., & Parija, S. C. (2010). AmpC beta lactamases among gram negative clinical isolates from a tertiary hospital, South India. *Brazilian Journal of Microbiology, 41*(3), 596-602.

Nataro, J. P., & Kaper, J. B. (1998). Diarrheagenic escherichia coli. *Clinical microbiology reviews, 11*(1), 142-201.

Ndlovu, T., Le Roux, M., Khan, W., & Khan, S. (2015). Co-detection of virulent *Escherichia coli* genes in surface water sources. *PloS one, 10*(2), e0116808.

Nemergut, D. R., Robeson, M. S., Kysela, R. F., Martin, A. P., Schmidt, S. K., & Knight, R. (2008). Insights and inferences about integron evolution from genomic data. *BMC genomics, 9*(1), 261.

Njugu, P. M. (2013). Antibiotic Susceptibility Patterns and Detection of Genes Responsible for Resistance of Klebsiella species and *Escherichia coli* Isolated from Environmental Sources around Nairobi, Kenya (Doctoral dissertation).

Páll, E., Niculae, M., Kiss, T., Şandru, C. D., & Spînu, M. (2013). Human impact on the microbiological water quality of the rivers. *Journal of medical microbiology, 62*(11), 1635-1640.

Paterson, D. L., & Bonomo, R. A. (2005). Extended-spectrum β-lactamases: a clinical update. *Clinical microbiology reviews, 18*(4), 657-686.

Peter-Getzlaff, S., Polsfuss, S., Poledica, M., Hombach, M., Giger, J., Böttger, E. C., ... & Bloemberg, G. V. (2011). Detection of AmpC beta-lactamase in Escherichia coli: comparison of three phenotypic confirmation assays and genetic analysis. *Journal of clinical microbiology*, JCM-00091.

Pitout, J. D. D., Thomson, K. S., Hanson, N. D., Ehrhardt, A. F., Moland, E. S., & Sanders, C. C. (1998). β-Lactamases responsible for resistance to expanded-spectrum cephalosporins in *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* isolates recovered in South Africa. *Antimicrobial agents and chemotherapy, 42*(6), 1350-1354.

Rashid, M., Rakib, M. M., & Hasan, B. (2015). Antimicrobial-resistant and ESBL-producing *Escherichia coli* in different ecological niches in Bangladesh. *Infection ecology & epidemiology*, 5.

Rosser, S. J., & Young, H. K. (1999). Identification and characterization of class 1 integrons in bacteria from an aquatic environment. *Journal of Antimicrobial Chemotherapy, 44*(1), 11-18.

Rutgersson, C., Fick, J., Marathe, N., Kristiansson, E., Janzon, A., Angelin, M., ... & Larsson, D. J. (2014). Fluoroquinolones and qnr genes in sediment, water, soil, and human fecal flora in an environment polluted by manufacturing discharges. *Environmental science & technology, 48*(14), 7825-7832.

Sang, W. K., Boga, H. I., Waiyaki, P. G., Schnabel, D., Wamae, N. C., & Kariuki, S. M. (2011). Prevalence and genetic characteristics of Shigatoxigenic *Escherichia coli* from patients with diarrhoea in Maasailand, Kenya. *The Journal of Infection in Developing Countries, 6*(02), 102-108.

Sang, W. K., Oundo, V., & Schnabel, D. (2012). Prevalence and antibiotic resistance of bacterial pathogens isolated from childhood diarrhoea in four provinces of Kenya. *The Journal of Infection in Developing Countries, 6*(07), 572-578.

Schwartz, T., Kohnen, W., Jansen, B., & Obst, U. (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS microbiology ecology, 43*(3), 325-335.

Sekowska, A., Hryniewicz, W. A. L. E. R. I. A., Gniadkowski, M. A. R. E. K., Deptuła, A. L. E. K. S. A. N. D. E. R., Kusza, K. R. Z. Y. S. Z. T. O. F., & Gospodarek, E. U. G. E. N. I. A. (2010). Antimicrobial susceptibility of metallo-beta-lactamase positive and negative *Klebsiella pneumoniae* strains isolated from intensive care unit patients. *Pol J Microbiol, 59*(1), 67-69.

Shehane, S. D., Harwood, V. J., Whitlock, J. E., & Rose, J. B. (2005). The influence of rainfall on the incidence of microbial faecal indicators and the dominant sources of faecal pollution in a Florida river. Journal of Applied Microbiology, 98(5), 1127-1136.

Sirot, D., De Champs, C., Chanal, C., Labia, R., Darfeuille-Michaud, A., Perroux, R., & Sirot, J. (1991). Translocation of antibiotic resistance determinants including an extended-spectrum beta-lactamase between conjugative plasmids of *Klebsiella pneumoniae* and *Escherichia coli*. *Antimicrobial agents and chemotherapy, 35*(8), 1576-1581.

Smith, R. D., & Coast, J. (2002). Antimicrobial resistance: a global response. Bulletin of the *World Health Organization, 80*(2), 126-133.

Sobieszczańska, B. A., Duda-Madej, A. B., Turniak, M. B., Franiczek, R., Kasprzykowska, U., Duda, A. K., ... & Iwańczak, B. (2011). Invasive properties, adhesion patterns and phylogroup profiles among *Escherichia coli*

strains isolated from children with inflammatory bowel disease. *Advances in clinical and experimental medicine: official organ Wroclaw Medical University, 21*(5), 591-599.

Sreeshma, P., Champa, H., Padmaraj, S. R., & Subbannayya, K. (2015). An Insight into Antibiogram and β-Lactamase Production of *Pseudomonas aeruginosa* Isolated from River Water and Soil. *International Journal of Current Microbiology and Applied Sciences*, 4, 82-87.

Storberg, V. (2014). ESBL-producing Enterobacteriaceae in Africa–a non-systematic literature review of research published 2008–2012. *Infection ecology & epidemiology*, 4.

Su, H. C., Ying, G. G., Tao, R., Zhang, R. Q., Zhao, J. L., & Liu, Y. S. (2012). Class 1 and 2 integrons, sul resistance genes and antibiotic resistance in *Escherichia coli* isolated from Dongjiang River, South China. *Environmental Pollution*, 169, 42-49.

Suzuki, S., & Hoa, P. P. (2007). Distribution of quinolones, sulfonamides, tetracyclines in aquatic environment and antibiotic resistance in Indochina. Role and prevalence of antibiosis and the related resistance genes in the environment, 45.

Tacão, M., Correia, A., & Henriques, I. (2012). Resistance to broad-spectrum antibiotics in aquatic systems: anthropogenic activities modulate the dissemination of $bla_{\text{CTX-M-like}}$ genes. *Applied and environmental microbiology, 78*(12), 4134-4140.

Tao, R., Ying, G. G., Su, H. C., Zhou, H. W., & Sidhu, J. P. (2010). Detection of antibiotic resistance and tetracycline resistance genes in Enterobacteriaceae isolated from the Pearl rivers in South China. *Environmental Pollution, 158*(6), 2101-2109.

Tissera, S., & Lee, S. M. (2013). Isolation of Extended Spectrum ß-lactamase (ESBL) Producing Bacteria from Urban Surface Waters in Malaysia.

Varghese, M., & Roymon, M. G. (2013). Studies on antibiotic resistance profiles of thermotolerant *Escherichia coli* and multiple antibiotic resistance index of different water sources. *Recent Research in Science and Technology*, 5(2).

Versalovic, J., Koeuth, T., & Lupski, R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to finerpriting of bacterial enomes. *Nucleic acids research, 19*(24), 6823-6831.

Vidal, M., Kruger, E., Durán, C., Lagos, R., Levine, M., Prado, V., ... & Vidal, R. (2005). Single multiplex PCR assay to identify simultaneously the six categories of diarrheagenic *Escherichia coli* associated with enteric infections. *Journal of clinical microbiology, 43*(10), 5362-5365.

World Health Organization. (2006). Guidelines for the Safe Use of Wastewater, Excreta and Greywater: Excreta and greywater use in agriculture (Vol. 4). World Health Organization.

Xi, C., Zhang, Y., Marrs, C. F., Ye, W., Simon, C., Foxman, B., & Nriagu, J. (2009). Prevalence of antibiotic resistance in drinking water treatment and distribution systems. *Applied and environmental microbiology, 75*(17), 5714-5718.

Zhang, H., Zhou, Y., Guo, S., & Chang, W. (2015). Multidrug resistance found in extended-spectrum beta-lactamase-producing Enterobacteriaceae from rural water reservoirs in Guantao, China. *Frontiers in microbiology,* 6, 267.

Zurfluh, K., Hächler, H., Nüesch-Inderbinen, M., & Stephan, R. (2013). Characteristics of extended-spectrum β-lactamase-and carbapenemase-producing Enterobacteriaceae isolates from rivers and lakes in Switzerland. *Applied and environmental microbiology, 79*(9), 3021-3026.

APPEDICES

Appendix 1: Antibiotics used

Beta lactams Amoxicillin/clavulanic acid (AMC 20/10ug)

Ampicillin (AMP 10ug) (moderate-spectrum),

aztreonam(ATM,30ug)(monobactam)

cefoxitin(FOX,30ug)(2nd generation cephem)

cefotaxime (CTX,30ug)(broad spectrum 3rd generation),

ceftazidime(CAZ, 30ug)(3rd generation cephem)

cefpodoxime (CPD, 10ug)(3rd generation cepham)

cefepime (FEP, 30 ug)(4th generation cepham)

Aminoglycosides: gentamicin(Cn,10ug), streptomycin(S,10ug)

Tetracycline (Te,30ug),

Chloramphenicol (C, 30ug)

Nucleic acid inhibitors: Sulfamethoxazole (SMX, 50 ug), Trimethoprim (W ,5ug)

Fluoroquinolones: Ciprofloxacin (Cip, 5 ug) and Nalidixic acid (NA,30ug).

Appendix 2: Bacteria media preparation

MACCONKEY AGAR

Formula

Gelatin peptone 17.0

Bile salts No: 3 1.5g

Lactose 10.0g

Neutral red 0.03g

Sodium chloride 5.0g

Peptone mixture 3.0g

Bacteriological agar 13.5g

Preparation

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

Use: For selection of enteric bacteria.

MUELLER HINTON AGAR.

Formula

Beef infusion 300.0g

Acid hydrolysate of casein 17.5g

Starch 1.5g

Agar 17.0g

Preparation

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

Use: For sensitivity testing.

TRYPTIC SOY BROTH

Formula

Pancreatic digest of casein 17.0g Papaic digest of soy meal 3.0g Sodium chloride 5.0g Dipotassium phosphate 2.5g Dextrose 2.5g

Preparation

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

Use: General purpose broth media.

Appendix 3: Biochemical tests

1. Urease Test.

The urease test helps in the identification of certain species of Enterobacteraceae. Urease enzyme hydrolyses substrate urea into ammonia, water, and carbon dioxide.

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

Procedure

Streak the surface of a urea agar slant with a portion of a well isolated colony or inoculate slant with 1 to 2 drops from an overnight brain heart infusion broth culture.

Leave the cap on loosely and incubate tube at 35-37ºC in ambient air for 48 hours to 7 days.

Expected results

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

Negative: No color change in media.

Quality control

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

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

2. Citrate utilization test

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

Method

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

Incubate at 35-37ºC for upto 7 days.

Observe for development of blue color, denoting alkalinization.

Expected Results

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

The color change of the indicator is due to acid or alkali production by the test

organism as it grows on the medium. Growth usually results in the bromothymol

blue indicator, turning from green to blue.

Quality control

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

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

3. Indole test

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

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

Method

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

Incubate at 35ºC in ambient air for 24- 48 hours

Add 0.5 ml.of Kovacs reagent

Expected results

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

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

Salmonella Tphimurium and *Salmonella enteritidis* are indole negative

4. Triple Sugar Iron (TSI)

Principle

TSI is used to determine whether a gram negative bacilli utilizes glucose and lactose or sucrose fermentatively and forms hydrogen sulfide (H_2S).TSI contains 10 parts lactose: 10 parts sucrose: 1 part glucose and peptone. Phenol red and ferrous sulfate serve as indicators of acidification and (H_2S) formation respectively. When glucose is utilized by a fermentative organism, the entire media becomes acidic (yellow) in 18 to 12 hours. The butt remains acidic after the recommended 18 to 24 hours incubation period because of the presence of organic acids resulting from the fermentation of glucose under anaerobic conditions in the butt of the tube. The slant however reverts to the alkaline (red) state because of oxidation of the fermentation products under aerobic conditions in the slant. This change is a result of the formation of carbon dioxide and water and the oxidation of peptones in the medium to alkaline amines. When in addition to glucose, lactose and/or sucrose are fermented, the large amount of fermentation products formed on the slant will more than neutralize the alkaline amines and render the slant acidic(yellow), provided the

reaction is read in 18 to 24 hours. Reactions in TSI should not be read beyond 24 hours of incubation, because aerobic oxidation of the fermentation products from lactose and/or sucrose does not proceed and the slant will eventually revert to the alkaline state. The formation of carbon dioxide and water (hydrogen gas) is indicated by the presence of bubbles or cracks in the agar or by separation in the tube. The production of H_2S requires an acidic environment and is manifested by blackening of the butt of the medium.

Method

With a straight inoculation needle, touch the top of a well isolated colony.

Inoculate TSI by first stabbing through the center of the medium the bottom of the tube and then streaking the surface of the agar slant.

Leave the cap on loosely and incubate the tube at 35º - 37ºC in ambient air for 18 -24 hours.

Expected Results

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

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

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

Note: A black precipitate in the butt indicates production of ferrous sulfide and H_2S gas (H_2S +).

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

Quality control

Escherichia coli

K/A H_2S+: *Salmonella typhi, Salmonella typhimurium*

K/NC: *Pseudomonas aeruginosa*

S.typhimurium produces H_2S while *Salmonella enteritidis* does not

5. TE (Tris hydromethyl amino methane EDTA (ethylene diamine tetraacetic acid)
For 100 ml TE

10 mM Tris-HCI pH 8.0 1 mL 1 M Tris-HCI, pH 8.0

1 mM EDTA pH 8.0 0.2 ml 0.5M EDTA pH 8.0

Q.S to 100 ml with H_2O

Filter sterilize, store at RT

Appendix 4: Clinical and laboratory standards institute (CLSI)

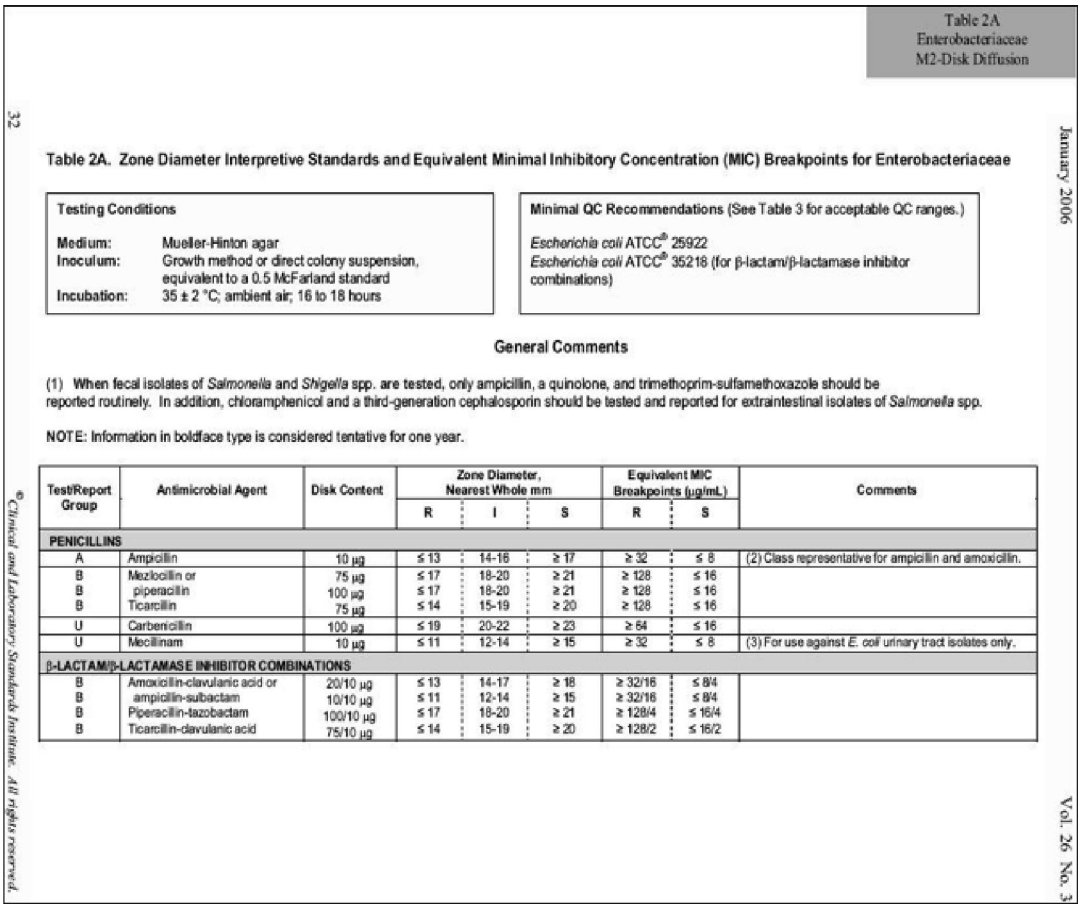


Table 2A. Zone Diameter Interpretive Standards and Equivalent Minimal Inhibitory Concentration (MIC) Breakpoints for Enterobacteriaceae

Testing Conditions

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

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

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

General Comments

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

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

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

Table 2A. (Continued)

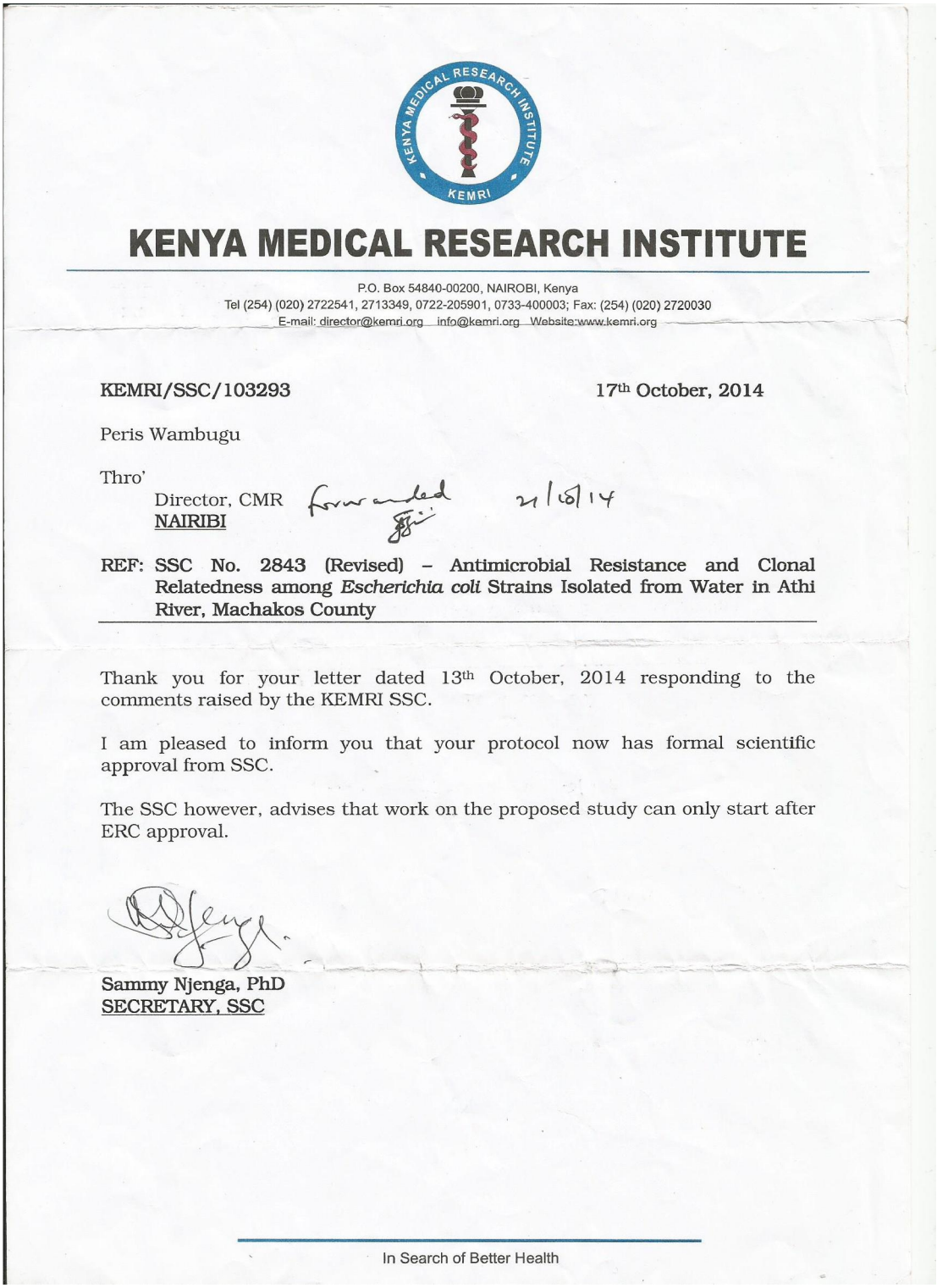
Test/Report Group	Antimicrobial Agent	Disk Content	Zone Diameter, Nearest Whole mm			Equivalent MIC Breakpoints (µg/mL)		Comments
			R	I	S	R	S	
AMINOGLYCOSIDES								(14) **WARNING:** For *Salmonella* spp. and *Shigella* spp., aminoglycosides may appear active *in vitro*, but are not effective clinically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

Table 2A
Enterobacteriaceae
M2-Disk Diffusion

Appendix 5: Tris-borate Electrophoresis buffer (TBE)

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

Appendix 6: Approval Letter by Scientific Steering Committee –KEMRI



KENYA MEDICAL RESEARCH INSTITUTE

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

KEMRI/SSC/103293 17th October, 2014

Peris Wambugu

Thro'
Director, CMR forwarded 21/10/14
NAIRIBI

REF: SSC No. 2843 (Revised) – Antimicrobial Resistance and Clonal Relatedness among *Escherichia coli* Strains Isolated from Water in Athi River, Machakos County

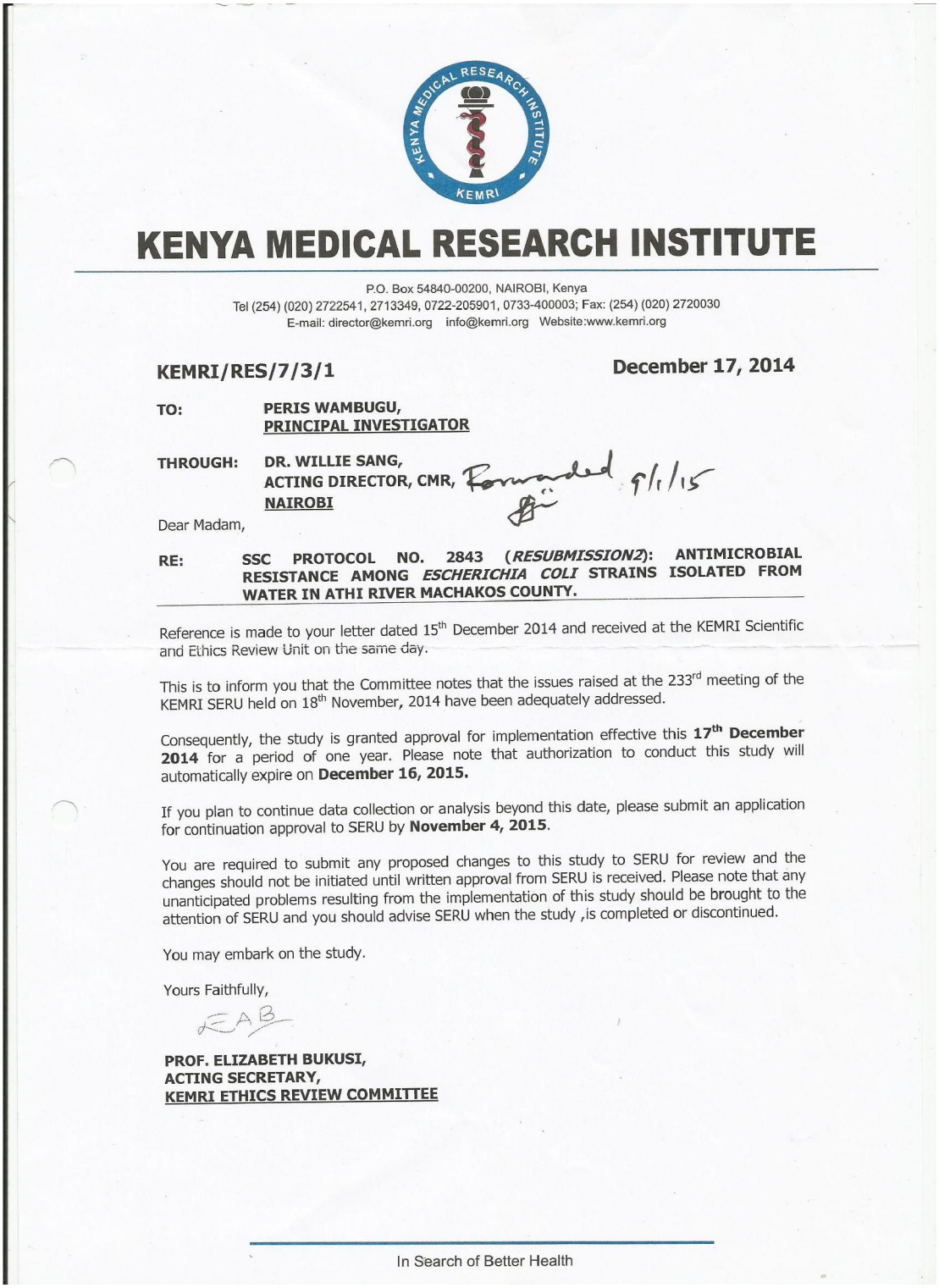
Thank you for your letter dated 13th October, 2014 responding to the comments raised by the KEMRI SSC.

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

Appendix 7: Approval Letter by Ethical Review Committee – KEMRI



KENYA MEDICAL RESEARCH INSTITUTE

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

KEMRI/RES/7/3/1 **December 17, 2014**

TO: **PERIS WAMBUGU,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. WILLIE SANG,**
ACTING DIRECTOR, CMR, Forwarded 9/1/15
NAIROBI

Dear Madam,

RE: **SSC PROTOCOL NO. 2843 (*RESUBMISSION2*): ANTIMICROBIAL RESISTANCE AMONG *ESCHERICHIA COLI* STRAINS ISOLATED FROM WATER IN ATHI RIVER MACHAKOS COUNTY.**

Reference is made to your letter dated 15th December 2014 and received at the KEMRI Scientific and Ethics Review Unit on the same day.

This is to inform you that the Committee notes that the issues raised at the 233rd meeting of the KEMRI SERU held on 18th November, 2014 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this **17th December 2014** for a period of one year. Please note that authorization to conduct this study will automatically expire on **December 16, 2015.**

If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **November 4, 2015**.

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

You may embark on the study.

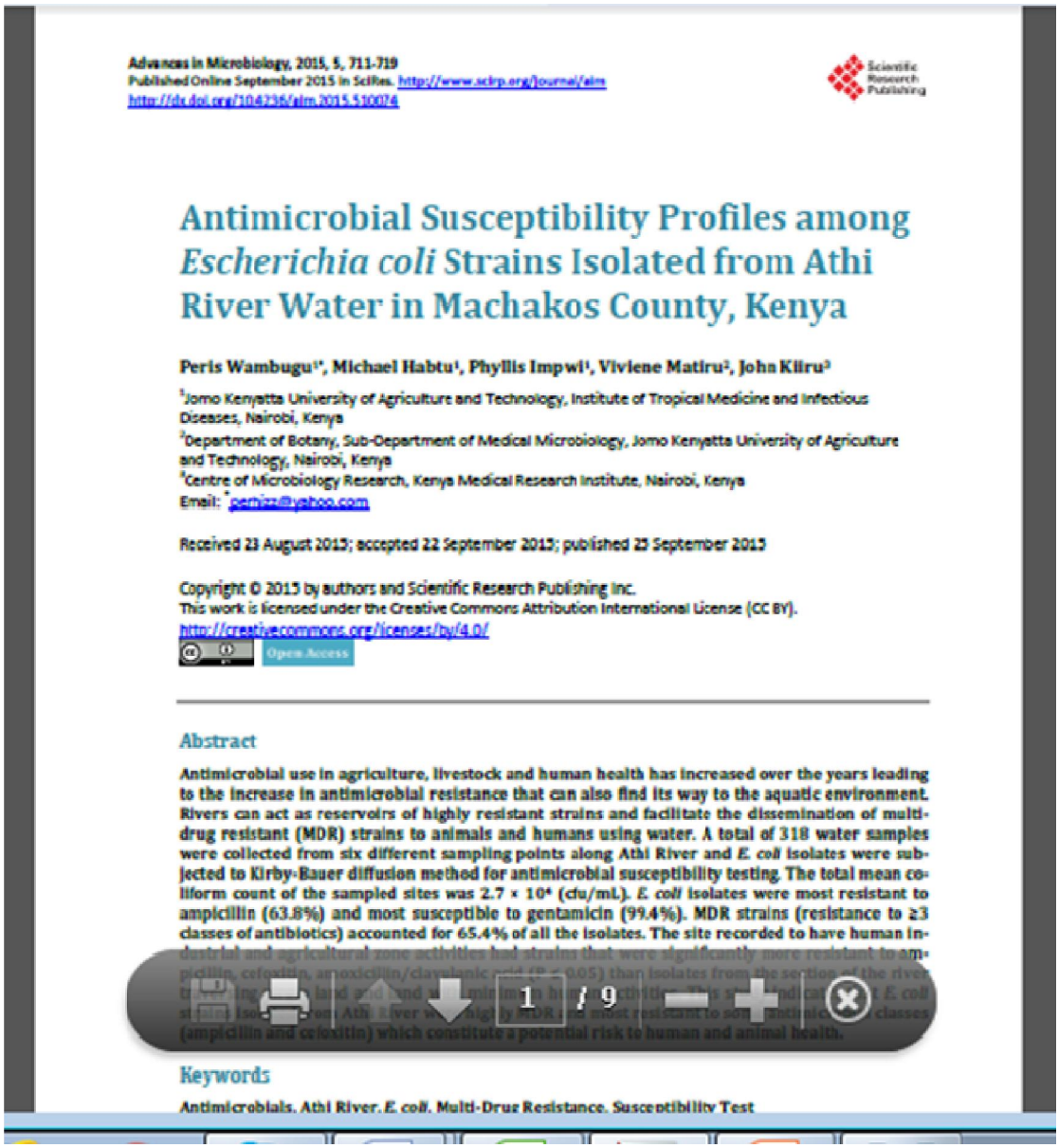
Yours Faithfully,

EAB

PROF. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

In Search of Better Health

Appendix 8: Publication



Advances in Microbiology, 2015, 5, 711-719
Published Online September 2015 in SciRes. http://www.scirp.org/journal/aim
http://dx.doi.org/10.4236/aim.2015.510074

Antimicrobial Susceptibility Profiles among *Escherichia coli* Strains Isolated from Athi River Water in Machakos County, Kenya

Peris Wambugu[1*], Michael Habtu[1], Phyllis Impwi[1], Viviene Matiru[2], John Kiiru[3]

[1]Jomo Kenyatta University of Agriculture and Technology, Institute of Tropical Medicine and Infectious Diseases, Nairobi, Kenya
[2]Department of Botany, Sub-Department of Medical Microbiology, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya
[3]Centre of Microbiology Research, Kenya Medical Research Institute, Nairobi, Kenya
Email: *perizz@yahoo.com

Received 23 August 2015; accepted 22 September 2015; published 25 September 2015

Abstract

Antimicrobial use in agriculture, livestock and human health has increased over the years leading to the increase in antimicrobial resistance that can also find its way to the aquatic environment. Rivers can act as reservoirs of highly resistant strains and facilitate the dissemination of multi-drug resistant (MDR) strains to animals and humans using water. A total of 318 water samples were collected from six different sampling points along Athi River and *E. coli* isolates were subjected to Kirby-Bauer diffusion method for antimicrobial susceptibility testing. The total mean coliform count of the sampled sites was 2.7×10^4 (cfu/mL). *E. coli* isolates were most resistant to ampicillin (63.8%) and most susceptible to gentamicin (99.4%). MDR strains (resistance to ≥3 classes of antibiotics) accounted for 65.4% of all the isolates. The site recorded to have human industrial and agricultural zone activities had strains that were significantly more resistant to ampicillin, cefoxitin, amoxicillin/clavulanic acid ($P < 0.05$) than isolates from the section of the river [illegible] and [illegible] activities. [illegible] *E. coli* strains isolated from Athi River were highly MDR and most resistant to some antibiotic classes (ampicillin and cefoxitin) which constitute a potential risk to human and animal health.

Keywords

Antimicrobials, Athi River, *E. coli*, Multi-Drug Resistance, Susceptibility Test