MOLECULAR IDENTIFICATION, VIRULENCE CHARACTERIZATION AND ANTIMICROBIAL RESISTANCE PROFILES OF *ESCHERICHIA COLI* IN MILK INTENDED FOR HUMAN CONSUMPTION IN ISIOLO COUNTY, NORTHERN KENYA

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology, University of Nairobi

2020

DECLARATION

I declare that this thesis is my original work, and to the best of my knowledge has not been submitted or examined for the award of degree in any other higher institution of learning.

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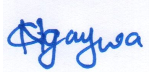

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DEDICATION

To my husband Rodgers and our two children, Amanda and Eddah, with love and gratitude for the support and encouragement. God bless you.

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ABBREVIATONS AND ACRONYMS

AMR	Antimicrobial resistance
ATCC	American Type Culture Collection
AST	Antimicrobial Susceptibility Testing
BLAST	Basic Local Alignment Search Tool
BPW	Buffered Peptone Water
CATs	Chloramphenicol Acetyltransferases
CFUs	Colony Forming Units
CTX-M	Cefotaxime-Munich
CLSI	Clinical Laboratory Standards Institute
DAEC	Diffusely adherent *Escherichia coli*
DHPS	Dihydropteroate Synthase
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
ELISA	Enzyme-Linked Immunosorbent Assay
EMBA	Eosin Methylene Blue Agar
ESBL	Extended Spectrum Beta Lactamase
Eae	Attaching and effacing

EhxA Enterohaemolysin A

ExPEC Extra intestinal Pathogenic *Escherichia coli*

FDA Food and Drug Administration

FQ Fluoroquinolone

GC Guanine Cytosine

Gb3 Globotriaosylceramides

HUS Hemolytic Uremic Syndrome

HC Hemolytic Colitis

HGT Horizontal Gene Transfer

JSAR Japan Infectious Agents Surveillance Reports

Kbp Kilo base pairs

LEE Locus of Enterocyte Effacement

LIM Lysine Indole Motility

Ltg Lytic transglycosylase

MS Mass Spectrometry

MALDI-TOF Matrix-Associated Laser Desorption and Ionization time of Flight

MIC Minimum Inhibitory Concentration

MHA Mueller Hinton Agar

MDR Multidrug resistance

MLST Multi-locus sequence typing

MLVA Multi locus variable number tandem repeat analysis

NCBI National Center for Biotechnology Information

NSFC Nonsorbital Fermenting Colonies

Nr	Nucleotide
ORF	Open Reading Frame
PBPs	Penicillin Binding Proteins
PCR	Polymerase Chain Reaction
PFGE	Pulse field gel electrophoresis
QRDRs	Quinolone resistance – determining regions
RAJ	Rectal-anal junction
RNA	Ribonucleic Acid
RT	Reverse transcriptase
RAPD	Randomly Amplified Polymorphic DNA
SIM	Sulfide Indole Motility
SBA	Sheep Blood Agar
SNPs	Single Nucleotide Polymorphisms
Stxs	Shiga toxins
SMAC	Sorbitol MacConkey Agar
STEC	Shiga toxin- producing *Escherichia coli*
SHV	Sulfhydryl variable
TEM	Temoniera
TSI	Triple Iron Sugar
mTSB	Tryptic Soy Broth
WGS	Whole Genome Sequencing
WHO	World Health Organization

ABSTRACT

The Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a widely studied foodborne pathogen which has an adverse effect on human health. The threat is aggravated by the fact that there are reported resistance of EHEC O157:H7 to antimicrobial agents. This study used lytic transglycosylase gene to identify *Escherichia coli* in milk of livestock for human consumption. In addition, the virulence factors (*stx*1, *stx*2 and *eae*) and antibiotic resistance profiles including their capacity to produce extended-spectrum β-lactamases (ESBLs) were determined.

Three hundred and four (304) milk samples were obtained from lactating animals in Isiolo County, Northern Kenya. *Escherichia coli* was isolated using Eosin Methylene Blue Agar (EMBA) and Sorbitol MacConkey agar (SMAC), and identified using biochemical tests (Triple Sugar Iron, Lysine Indole Motility and Citrate). The isolates were then confirmed using Polymerase Chain Reaction (PCR) and sequencing. Additionally, antimicrobial resistance profiles of the isolates to 11 antimicrobial agents were evaluated by disc diffusion method on Mueller Hinton Agar (MHA). Furthermore, the isolates were evaluated for antimicrobial genetic determinants conferring the resistance phenotypes to beta-lactams and tetracycline.

Overall, colonies suggestive of *E. coli* were isolated in 42 (13.8%) milk samples including 19(8.8%) from household and 23(31.08%) from individual animal. Also, all the 42 isolates were confirmed as *E. coli* by PCR. Also, *stx*1, *stx*2 and *eae* genes were detected in 85.7% (36), 57.1% (24) and 90.4% (38) isolates respectively and both *stx*1 and *stx*2 in 47.6% (20) isolates.

This study revealed that 95% (40) of the isolates were resistant to at least one of the tested antimicrobials. Furthermore, Multidrug resistance (MDR) was detected in 14.28% (6) of the isolates. This study established that milk consumed in Isiolo County is contaminated with genes with the potential to produce enterotoxins and antimicrobial resistant *E. coli* strains.

CHAPTER ONE

1.0 INTRODUCTION

1.1. Background

Escherichia coli occupies the lower intestinal tract of healthy animals and humans as commensal (Fratamico et al., 2016). However, some strains are pathogenic and cause both intestinal and extra-intestinal (ExPEC) infections in humans and even death in some cases (Messele et al., 2019; Nobili et al., 2017). Enterohemorrhagic *E. coli* (EHEC) O157: H7, a subset of shiga toxin producing *E. coli* (STEC), is the most common serotype among the six human pathogenic *E. coli* that is responsible for foodborne illnesses in human (Farrokh et al., 2012; Estrada-garcia et al., 2013). Enterohemorrhagic *E. coli* O157:H7 is approximated to result in 73,000 illnesses as well as 2,200 hospitalizations, and 61 deaths each year in America (Deisingh & Thompson, 2004; Bedasa et al., 2018). *Escherichia coli* foodborne illness results from ingestion of raw food contaminated with toxin-producing bacteria (Farrokh et al., 2012). Infection in human can present a range of symptoms including abdominal cramps, diarrhea and Hemolytic Uremic Syndrome (HUS) (Smith et al., 2014). In most cases, the symptoms begin with non-bloody diarrhea that is self-limiting and progress to bloody diarrhea in 1–3 days in some patients (Iweriebor et al., 2015). However, only 5–10% of patients with bloody diarrhea can have the disease progressing to HUS (Farrokh et al., 2012). Among those at increased risk of developing HUS are children and the elderly (Deisingh & Thompson, 2004; Farrokh et al., 2012).

The invention and use of antibiotics in management of microbial infections has changed the field of medicine, with overuse by humans and in food-producing animals resulting in a range of clinical challenges with regard to therapeutics (Lobanovska & Pilla, 2017). Selective pressure brought about by overuse of antibiotics is considered a major contributor to the development and continued spread of traits such as drug-resistance between commensal and pathogenic *E.*

coli. (Shin et al., 2014). Antimicrobial resistance (AMR) is also complicated by the emergence of multidrug resistance (MDR) strains, which are generally associated with the interaction of a number of mechanisms that confer resistance to a range of antimicrobials agents (Davies & Davies, 2010). Nearly, all classes of antimicrobials, including sulfonamides (sulfamethoxazole-trimethoprim), penicillins (ampicillin), tetracyclines (tetracycline), aminoglycosides (kanamycin) and cephalosporins (cephalexin) used in both veterinary and human medicine are affected by antimicrobial resistance (Hao et al., 2016).

Prolonged use of a particular antibiotic is linked to the emergence and maintenance of certain resistance traits in bacterial strains (Chang et al., 2015). This problem has been reported for beta-lactams and tetracyclines, which have been used widely for the treatment of *E. coli* infections in animals and humans. *Escherichia coli* isolates known to be resistant to beta-lactams such as penicillins have the capability to inactivate the drugs (Poirel et al., 2018). The bacterial infection is more prevalent as a result of prolonged use of the drugs (Aarts, 2011; Davies & Davies, 2010). Despite efforts to develop penicillin based antibiotics, which are resistant to β-lactamases-degradation, many bacterial strains have continued to acquire varied resistance traits to enhance their survival (Bush & Jacoby, 2010; Davies & Davies, 2010). Currently, there are nearly 1000 different types of β-lactamases encoded by novel gene classes (Bush & Jacoby, 2010). Usually, the resistant traits are encoded by certain genes that are found within mobile genetic elements such as bacterial plasmids and transposons and can be transferred amongst bacterial isolates.

Horizontal gene transfer (HGT) is believed to play a significant role in both development and transmission of genes encoding resistance phenotypes to β-lactam antibiotics (Davies & Davies, 2010; Nüesch-Inderbinen & Stephan, 2016; Wintersdorff et al., 2016). Mutations in genes

encoding β-lactamase enzymes have modified their catalytic activities resulting in increase in their antibiotic resistance spectra (Palzkill, 2018). *Escherichia coli* isolates that express the extended-spectrum beta-lactamases (ESBLs) phenotypes are generally known to be resistant a range of beta-lactam antibiotics and are therefore difficult to control in clinical set-up. The beta lactamases include the narrow-spectrum beta-lactamases such as TEM-1, SHV-1 and TEM-2, and the new extended-spectrum beta-lactamase such as CTX-M (Odenthal et al., 2016; Poirel et al., 2018). Tetracycline resistant *E. coli* isolates also harbor a range of genetic determinants such as *tet*(B), and *tet*(C), which are responsible for the resistance phenotypes.

1.2. Statement of the problem

Studies worldwide have documented increasing incidence of contamination of processed and raw milk with antimicrobial resistant *E. coli* strains (Tabaran et al., 2017; Sudda et al., 2016; Ranjbar et al., 2018; Ombarak et al., 2018). Some of these resistant strains harbor multidrug resistant phenotypes and this poses serious public health concerns (Ombarak et al., 2018; Sudda et al., 2016). In Northern Kenya, raw milk from camels, goats, sheep, and cattle serve as a major source of nutrient for humans, especially young children and women (Dror & Allen, 2011). For example, pastoral communities in Northern Kenya consume raw milk since it is perceived to have a higher nutritive value and medicinal properties compared to boiled milk (Wanjohi et al., 2013).

The problem with consumption of raw milk is that it can serve as a transmission route for antimicrobial resistant *E. coli.* Inspite of this concern, only a few studies on the presence of antimicrobial resistant *E. coli* in milk have been done in Kenya. Therefore, the extent of risk of contamination of raw milk with antimicrobial resistant *E. coli* generally remains unknown yet this information is important in mitigating the spread of antimicrobial resistant isolates along the milk value chain.

1.3. Justification

Raw milk can serve as a suitable medium for growth of foodborne microorganisms such as *E. coli* which can be transmitted to humans. In Northern Kenya raw milk is consumed by the pastoral communities, however, raw milk can serve as a good medium for transmission of antimicrobial resistant *E. coli*. This study was done to assess the potential health hazards caused by *E. coli* with emphasis on the virulence factors and antimicrobial resistant determinants

responsible for resistance phenotypes. Therefore, the data generated has provided useful information that can help in informing policy makers on intervention strategies to reduce contamination and spread of antimicrobial resistance in milk value chain, which will eventually help promote food safety and food security in the study area.

Furthermore, the primers pairs generated during this study can be employed in further studies involving antimicrobial resistant *E. coli* infections hence contribute to infection control, reduced mortality and economical loss in livestock sector.

1.4. General objective

To identify and characterize the phenotypes, virulence factors and antimicrobial resistance profiles of *E. coli* isolated from raw milk intended for human consumption in Isiolo County.

1.4.1. Specific objectives

1. To identify the phenotypes of *E. coli* isolated from milk intended for human consumption.
2. To characterize the virulence factors associated with pathogenicity of *E. coli* isolated from milk.
3. To characterize the phenotypic antimicrobial resistant profiles of milk-borne *E. coli.*
4. To determine the genetic basis of phenotypic antimicrobial resistant profiles of *E. coli.*

1.4.2. Hypothesis

Milk consumed in Isiolo County is contaminated with toxin producing and Antimicrobial resistant *Escherichia coli* strains.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. *Escherichia coli*

Escherichia coli is a gram negative, flagellate, non sporing and rod shaped bacteria that belongs to the Enterobacteriaceae family (Croxen et al., 2013; Mathusa et al., 2010). They reside harmlessly in the gastrointestinal tract of humans and animals and are beneficial to the host for producing vitamins B and K (Fang et al., 2017; Farrokh et al., 2012). *Escherichia coli* are also found residing in the environment, water and food (Shii & Adowsky, 2008; Croxen & Finlay, 2010; Kabiru et al., 2015). *Escherichia coli* are classified as coliform bacteria. The existence of *E. coli* in food or water implies faecal contamination due to uncleanliness and careless handling (Altalhi & Hassan, 2009; Tabaran et al., 2017). It also implies that other enteric pathogens may be present. *Escherichia coli* is serotyped based on the somatic (O), flagella (H) and capsular (K) antigens (Gyles, 2007). More than 186 different O antigen and 53 H antigen serogroups are currently recognized, a combination of which defines a serotype (Fratamico et al., 2016).

2.1.1. Pathogenic *Escherichia coli*

Escherichia coli inhabits the large intestines of healthy humans and animals as commensal, although, some strains are pathogenic and cause both extra-intestinal (ExPEC) and intestinal infections in humans and even death in some cases (Fratamico et al., 2016; Nobili et al., 2017). These strains have acquired certain virulence factors and evolved to be pathogens (Ho et al., 2013; Kaper et al., 2004). Extra-intestinal *E. coli* infections include urinary tract infections, septicemia and meningitis in newborns (Breland et al., 2017). Intestinal *E. coli* induces foodborne illnesses when host ingests contaminated food or water via fecal-oral route (Croxen & Finlay, 2010). Pathogenicity varies depending on the virulence traits acquired via transfer of

plasmids (Altalhi & Hassan, 2009). Most pathogenic isolates share a number of virulence strategies (Croxen & Finlay, 2010; Breland et al., 2017).

Six categories of pathogenic *E. coli* responsible for intestinal illness in human include Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), Shiga toxin-producing *E. coli* (STEC) or Enterohemorrhagic *E. coli* (EHEC) and Diffusely adherent *E. coli* (DAEC) (Dell'Orco et al., 2019). Enterotoxigenic *E. coli* causes the infantile and travelers' diarrhea among populations living in under-developed countries or regions with poor sanitation. Enteroinvasive *E. coli* resemble Shigella dysentery in their disease causing mechanisms, and the symptoms include dysentery-like diarrhea with fever. Enteropathogenic *E. coli* induces watery diarrhea similar to ETEC and it usually occurs in infants. Enteroaggregative *E. coli* resemble ETEC strains through bacterial adherence to the intestinal mucosa causing non-bloody diarrhea without being invasive or resulting in inflammation (Kaper et al., 2004). Shiga toxin-producing *E. coli* are represented by STEC O157:H7, also known as EHEC O157:H7, which causes Hemorrhagic colitis (bloody diarrhea) and hemolytic uremic syndrome (HUS) in humans (Estrada-garcia et al., 2013; Fratamico et al., 2016).

2.1.2. Shiga toxin producing *Escherichia coli*

Shiga toxin-producing *E. coli* is a major food-borne human pathogen responsible for a range of mild to severe infections in humans (Elhadidy et al., 2015). Shiga toxin-producing *E. coli* causes bloody diarrhea and HUS in humans, with HUS being the leading cause of kidney failure in children (Mora et al., 2005; Gyles, 2007). Domestic animals including cattle, goats, and sheep are the main reservoirs of STEC and are a potential source of infection to humans (Farrokh et

al., 2012; Pinaka et al., 2013). In cattle, STEC has been shown to causes sub-clinical mastitis resulting in elevated somatic cell count with no gross changes on the udder or in milk production (Lira et al., 2004; Jamali et al., 2018). Transmission of these bacteria occurs during milking where contaminated milk from infected quarters comes into contact with uninfected quarters thus infection of teat canals (Farrokh et al., 2012).

Over 400 serotypes of STEC have been identified worldwide from humans, foods, cattle and within the environment (Farrokh et al., 2012). However, the "Big 7" serogroups including O103, O26, O45, O145, O157, O111 and O121 have been commonly associated with human illness (Mathusa et al., 2010). While 20-50% of STEC infections have been attributed to non-O157 serogroups, EHEC O157:H7 has been the major cause of severe human infection (Mathusa et al., 2010) hence the main focus of most studies (Croxen et al., 2013; Jajarmi et al., 2017).

2.1.3. Enterohemorrhagic *Escherichia coli* O157:H7

The foodborne pathogen EHEC O157:H7 was recognized in 1982 and associated with a bloody diarrhea outbreak within the United States of America (Carvalho et al., 2014). It is estimated that, an infectious dose of 10–100 colony-forming units (CFUs) are required to cause disease (Smith et al., 2014; Mikhail et al., 2017). Due to this low infectious dose, it is paramount to prevent human infection early enough in order to prevent outbreaks (Saeedi et al., 2017). Symptoms include non-bloody diarrhoea, bloody diarrhoea and HUS (Smith et al., 2014). Infection occurs due to consumption of raw or undercooked foods contaminated with animal waste (Saeedi et al., 2017; Ivbade et al., 2014).

Cattle are the main reservoir of EHEC O157: H7 (Deisingh & Thompson, 2004; Croxen et al., 2013), and animal products such as meat and dairy products have been linked to outbreaks

(Farrokh et al., 2012). In animals, the organism resides in their intestinal tract harmlessly (Croxen & Finlay, 2010). However, in human, it can cause infection or death (Smith et al., 2014). Epidemiological studies have linked its ancestral origin to the less virulent and non-toxigenic strains of *E. coli* O55:H7 (Saeedi et al., 2017). Its chromosome size is 5.5 Mb and the genome contains 4.1 Mb sequence at the backbone. The 4.1 Mb is conserved within *E. coli* strains while 1.4 Mb is specific to *E. coli* O157:H7. Furthermore, EHEC O157:H7 poses a putative extra chromosomal DNA (pO157), which is also responsible for its virulence (Ji et al., 2010). This plasmid is highly conserved and has additional characteristics of being non-conjugative with its size ranging from 92 to 104 Kb (Croxen & Finlay, 2010). The pO157 has 100 open reading frames in which 35 of this ORF have been found to encode protein responsible for the *E. coli* O157:H7 pathogenicity (Ho et al., 2013).

2.1.4. Geographical distribution of EHEC O157:H7

Shiga toxin-producing *E. coli* O157:H7 is widely distributed in Africa, Western Europe, the Far East, North America, South America, Japan, Central America, the Middle East and Australia (Saeedi et al., 2017; Farrokh et al., 2012). This distribution is due to lack of effective methods to control colonization of the animal reservoirs (Croxen & Finlay, 2010; Ji et al., 2010).

2.1.5. Virulence factors in EHEC O157:H7 infections

Virulence factors for EHEC O157:H7 infection are encoded by genes on mobile genetic elements such as pathogenicity Islands and plasmids (Ji et al., 2010). The major virulence factors of EHEC are intimin and the shiga toxins (Oporto et al., 2008; Lee et al., 2011). Two subgroups of shiga toxins namely *Stx*1 and *Stx*2 have been identified. Studies have shown that Stx2 is more

toxic and prevalent in hemorrhagic colitis and HUS than Stx1 (Croxen & Finlay, 2010). Shiga toxin consists of two subunits namely subunit A (A1) and B (B5) (Estrada-garcia et al., 2013). Its receptors are the globotriaosylceramides (Gb3s) which are found on both the Paneth and Epithelial cells of human intestines and kidney (Saeedi et al., 2017; Croxen & Finlay, 2010).

The subunit B is associated with the formation of pentamer which are bound by Gb3 while subunit A (with an RNA N-glycosidase activity) is bound by the 28S rRNA (60s ribosomal subunit) where it removes one adenine residue, thus inhibiting protein synthesis and initiating apoptosis in host (Croxen & Finlay, 2010). The effect of Shiga toxin is felt in human when it is released by the EHEC O157:H7 and bound by the endothelial cell which expresses Gb3 (Saeedi et al., 2017; Estrada-garcia et al., 2013).

The binding enables the toxin to enter the bloodstream through absorption and get disseminated to other body organs. There exists a variation in tissues and cells that express Gb3 within hosts. Cattle lack such type of receptors and this is why EHEC O157:H7 establishment in cattle is usually referred to as asymptomatic (Saeedi et al., 2017; Croxen & Finlay, 2010). As opposed to colonization in humans, EHEC O157:H7 is associated with the natural colonization of cattle recto-anal junction mucosa (RAJ) and gastrointestinal tract (Croxen & Finlay, 2010). Cattle, therefore, serve as good reservoirs and it is estimated that 1% to 50% healthy cattle usually carry and shed the pathogen when they defecate (Ji et al., 2010).

Intimin is important in ensuring intimate adherence between the bacteria and the mammalian epithelial cells (Saeedi et al., 2017). This attachment leads to the formation of a distinct lesion or attaching and effacing (A/E) lesions on infected mammalian cells. The *eae* gene plays a crucial role in adhesion by encoding intimin. Although *eae* gene is regarded as a marker for virulence, its presence in a particular strain of STEC does not indicate its pathogenicity. This

gene is located within the locus of enterocyte effacement (LEE) which is also known to be located in the Pathogenicity Island of EHEC O157:H7 (Croxen & Finlay, 2010).

Enterohaemolysin also plays a significant role during the initial stages of EHEC O157:H7 infection. It is found on a 60MDa plasmid and is encoded by *ehxA* gene. Other pathogenic factors not so common including catalase peroxidase, enterotoxin, adhesion and extracellular serine protease have also been documented (Colello et al., 2015).

2.1.6. Transmission of *E. coli* to Humans

Various modes of transmission of EHEC O157:H7 have been identified, including airborne (Ji et al., 2010), food-borne (Rangel et al., 2005) and person to person transmission (Saeedi et al., 2017). However, the most common one is the food-borne, while food contaminated with animal waste serves as the source of infection (Saeedi et al., 2017; Gyles, 2007). Foods associated with transmission include sausages, raw milk, beef, lettuce, apple juice, radish sprouts, and melon (Ji et al., 2010; Farrokh et al., 2012; Saeedi et al., 2017; Messele et al., 2019). The main reservoirs of EHEC O157:H7 infection are cattle (Farrokh et al., 2012; Pinaka et al., 2013), sheep (La Ragione et al., 2009), goats, seagulls (Naylor et al., 2005; Wetzel & Le Jeune, 2006), pigs (Parma et al., 2000) dogs and rabbits.

2.1.7. Transmission of *E. coli* through milk

The occurrence of *E. coli* in the dairy food chain may either be due to milk contamination or rare clinical infections (mastitis) of livestock (Blum et al., 2017; Farrokh et al., 2012). Within the intestines of dairy animals, *E. coli* exists harmlessly and is usually excreted in faeces which eventually soils the teats of the lactating animals (Gomes et al., 2016). Occasionally, the bacteria

persist in teat canals and subsequently in mammary glands and are usually contagious. Milking sessions are the key point at which bacteria are spread from infected teat quarter to uninfected ones. Milking containers not properly cleaned and maintained also serve as a reservoir for *E. coli* infections. Further, factors including hygiene of the milker, dirty milking towels, dirty environment (cattle yard, milking parlour), improper cleaning of teats and mammary glands before milking, milking salve/jelly and teat dips are potential sources of contamination of milk (Farrokh et al., 2012).

2.1.8. EHEC O157:H7 infections in humans

Konowalchuk et al. (1977) demonstrated that certain strains of *E. coli* were able to produce a toxin that could cause a cytotoxic effect on Vero cells (Parsons et al., 2016). Enterohemorrhagic *E. coli* O157:H7 was the initial STEC serogroup identified in 1982 and linked with a bloody diarrhea outbreak in humans in the United States (Hunt, 2010). Numerous studies later on, revealed that EHEC O157:H7 was transmitted through ingestion of contaminated animal food products and cattle were the main reservoir hosts and are key in human disease (Saeedi et al., 2017; Mathusa et al., 2010).

In 2010, the World Health Organization (WHO) approximated the burden of foodborne disease globally to be 600 million illnesses, of which an estimated 0.7-2.5 million severe cases were due to STEC with a death rate of 128 annually resulting from diarrhoea related diseases (Havelaar et al., 2015). Also, STEC was ranked fourth most important cause of acute foodborne illnesses globally amounting to 2.8 million cases every year (Majowicz et al., 2015).

Over 350 STEC outbreaks were documented in the US alone between 1982 and 2002, and these were attributed to EHEC O157:H7. Minced beef was recognized to be responsible for 61% of

the cases (Rangel et al., 2005). In the US, minced beef, dairy food products and vegetables were the most frequently incriminated foods in STEC outbreaks. Gould et al. (2013) documented increased incidence in STEC other than STEC O157 from 0.12 - 0.95 for every 100,000 people in the United States, while STEC O157 decreased from 2.5 - 0.95 for every 100,000 for the period 2000 – 2010. Over 900 cases of STEC are documented yearly, with STEC O26 and O157 being the major causes of serious human disease in the United Kingdom particularly in young children (Byrne et al., 2014). Conversely, atypical O104:H4 STEC serotype was implicated in a STEC outbreak in Germany, where 3816 people were affected comprising 845 cases of HUS and 54 death (Askar et al., 2011). Between 2000 and 2010, the rate of STEC human infections increased slowly in Australia, with 58% of the cases being attributed to STEC O157, while non O157 STEC (O26 and O111) accounting for 13.7% and 11.1% infections respectively in humans (Vally et al., 2012). Further, the total incidence of STEC infections for the entire decade amounted to 0.4 per 100,000 persons (Vally et al., 2012).

In 2014, in Japan, an outbreak of STEC affected 78 people, out of which 24 were admitted in hospital, 4 of which developed HUS. Enterohemorrhagic *E. coli* O157:H7 was implicated in this outbreak (Furukawa et al., 2018). The outbreak was linked to consumption of uncooked minced beef/cutlets that were sold in a supermarket chain. Also, the Japan Infectious Agents Surveillance Reports (JIASR) reported that STEC serotypes O26, O157, O121, O111 and O103 were isolated at several health centres and public health facilities between the years 2005 and 2010 from human cases. Kanayama et al. (2015) reported a steady rise in outbreaks linked to STEC in daycare facilities in Japan between the years 2010 - 2013. The outbreaks were ascribed to STEC O111, O26, O157, O103, O145 and O121.

Although the problem of *E. coli* O157:H7 in animals and humans is worldwide, data on *E. coli*

food poisoning is mostly available in urbanized countries. However, there is limited information on *E. coli* food poisoning in Africa including in Kenya. Arimi et al. (2005), documented that the occurrence of *E. coli* O157:H7 was 0.8% in milk from consumer households in Kenya. Two years later, Kangethe et al. (2007) reported a 0% prevalence of *E. coli* O157:H7 in household milk and 5.2% in cattle stool samples.

2.1.9. Isolation of *E. coli* and EHEC O157:H7 from milk

Different selective and differential culture media have been used to isolate *E. coli* and EHEC O157:H7 from milk. In a study done in Egypt, raw milk samples were enriched in Tryptic Soy Broth (TSB) prior to culture on Eosin Methylene Blue (EMB) agar. Colonies with greenish metallic sheen after overnight incubation were further streaked on Tryptic Soy Agar (TSA) and incubated overnight, followed by sub-culture on nutrient agar slants. *Escherichia coli* was then identified using Lysine Indole Motility (LIM), Triple Sugar Iron agar (TSI), Sulfide Indole Motility (SIM) and Simmons Citrate Tests (Ombarak et al., 2016). Another study done in 2014 used tryptic soy broth for pre-enrichment, followed by inoculation on modified tryptic soy broth (mTSB) supplemented with novobiocin for selective enrichment for 18 hours at 37°C. Later, the cultures were streaked onto Sorbitol MacConkey agar containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (SMAC-BCIG) agar plates supplemented with tellurite and cefixime and incubated for 18 hours at 37°C for selective isolation of *E. coli* O157. Pale yellow or straw colour colonies indicating non-sorbitol fermenter on SMAC-BCIG plates were presumably identified as suspected *E. coli* O157:H7 colonies. Five colonies were picked from each plate and tested for catalase and oxidase production. Catalase positive and oxidase negative colonies were then subjected to further biochemical testing for presence of Gram-negative bacilli using

commercially available test kit (Ivbade et al., 2014).

Presently, new methods have been documented for identification of *E. coli* and other pathogens in foods including Matrix-Associated Laser Desorption and Ionization time of Flight (MALDI-TOF), whole genome sequencing and protein separation techniques (Kelley, 2017; Jadhav et al., 2018). Matrix-associated laser desorption and ionization time of Flight technique was employed in studies of Ntuli et al. (2016) for accurately identifying *E. coli* and other microbes in milk samples in South Africa. The study identified over 50% of Enterobacteriaceae to species level of which 36% of the microbes were *E. coli*. VITEK MS and MALDI Biotyper machines have been commonly used in microbial identification (Sloan et al., 2017). Under this protocol, microbial cultures are treated with a highly concentrated solvent and centrifuged. The extracted bacteria are then mixed with matrices and loaded on the MALDI plates to facilitate detection by mass spectrometry. Laser energy is then used to shot the sample spots which in turn generates mass spectra, which are then compared with the stored mass fingerprints of already confirmed bacteria in the database. Identification of the bacteria relies on the set in the database that gives the best match with the obtained spectra (Sloan et al., 2017). Matrix-Associated Laser Desorption and Ionization time of Flight is considered as one of the most superior bacteria identification platform since it produces results in just a few seconds after loading of the samples. The test requires smaller amount of reagents and involves limited steps for performing the technique (Sloan et al., 2017). These recent advances in protein separation technologies, mass spectrometry and bioinformatics have resulted in improvements in current phenotypic typing schemes (Zhou et al., 2017).

2.1.10. Serotyping of EHEC O157:H7

Serotyping technique has been commonly used to classify *E. coli* based on antibody-antigen agglutination reaction (Fratamico et al., 2016). *Escherichia coli* serotyping system consists of 188 O-serogroups namely O (1-188), with serogroups O (31, 47, 67, 72, 94 and 122) missing from the scheme (Gyles, 2007; Fratamico et al., 2016). Additionally, STEC serotyping also comprises flagellar (H) antigen typing. About 53 H antigens designated 1-56 have been identified, with numbers 13, 22, and 50 missing (Fratamico et al., 2016; Banjo et al., 2018). Conventional detection of O157 and H7 antigens involves the use of latex agglutination reagents with commercially available O157 and H7 antisera. Detection of H7 antigen entails subculturing of the isolates on blood agar medium, followed by an overnight incubation at 37°C. A sweep of the growth on blood agar plate is then reacted with specific H7-antisera.

Serotyping is, however, costly and can only be done in reference laboratories (Debroy et al., 2011). To overcome challenges associated with conventional serotyping, genetic subtyping techniques have been developed, and are usually based on detection of specific O and H antigens from bacterial cultures. While -field gel pulsed electrophoresis (PFGE) was seen as the standard method, Multi locus variable number tandem repeat analysis (MLVA) and Multi-locus sequence typing (MLST) have also been adopted for typing *E. coli* (Pérez-losada et al., 2017). Examination of the arrangement of bands obtained by randomly amplified polymorphic DNA (RAPD), together with PFGE has contributed in defining diversity in the genetic make-up among *E. coli*. Genetic typing techniques can also be used together with PCR to establish similarity, virulence traits, O and H serotypes among *E. coli* (Farrokh et al., 2012).

Currently, new molecular techniques have been developed for characterizing pathogenic *E. coli* (Fratamico et al., 2016; Banjo et al., 2018). Among the techniques are the DNA based

microarrays, PCR and DNA sequencing (Lacher et al., 2016; Geue et al., 2014). Various microarrays methods are usually deployed in detection of pathogenic strains, key among them is the spotting of O-group-specific genes such as *wzx* or *wzy* (Liu & Fratamico, 2006). The technique was used by Lacher et al. (2016) in identifying *E. coli* through specific typing of their H- and O- groups using a Food and Drug Administration - *E. coli* identification (FDA-ECID). Food and Drug Administration- *E. coli* identification chip technique is considered as one of the most advanced microarray detection technique since it was designed with consideration of genome of over 250 *E. coli* hence incorporating more *E. coli* genes (Lacher et al., 2016). Other microarrays techniques used are designed based on antibody and detect important serogroups which are non O157 STEC (Hegde et al., 2013).

2.1.11. Molecular detection of virulence genes by PCR

Polymerase chain reaction technique was developed in 1980s and has changed the field of molecular biology by aiding in rapid identification of pathogens. Polymerase chain reaction based techniques are widely used in detection of STEC, together with the associated virulence traits in food and beverages since they are extremely sensitive, specific and fast. The technique has the ability to detect STEC from complex samples as well as samples with low number of *E. coli* cells (La Ragione & Newell, 2018). The continued use of PCR - based technique has led to the generation of stx sequences that are continually used in designing oligonucleotide targeting pathogenic *E. coli* (La Ragione & Newell, 2018).

For suitable design of oligonucleotide primers, the melting temperature for the primer pair sets should be similar, the GC content should be balanced and at the same time avoid the formation of hair pin and self-complementary structures (Parsons et al., 2016). To design efficient

oligonucleotide primers, it is important to select the correct software tool. One of the most commonly used tools is the primer blast that is available at http://www.ncbi.nlm.nih.gov/tools/primer-blast (Ye et al., 2012). It permits the use of FASTA files, accession numbers or primer from other sources as input. Primer blast can be used both for designing primers and also for checking the specificity of already designed primers. Primer 3 is another software tool, which can be used to design oligonucleotide primers, however, unlike primer blast, it doesn't perform target specific analysis thus forcing user to test for primer specificity using other additional tools, which can be time consuming (Ye et al., 2012).

Currently, PCR protocols that are able to detect specific species have been developed (Pansare et al., 2017). Likewise, a number of simplex and multiplex PCR assays that allow for detection of stx genes from foods have been documented (Karns et al., 2007; Madic et al., 2011). Other PCR assays including reverse transcriptase (RT-PCR), nested PCR and PCR-ELISA (Hu et al., 2018; La Ragione & Newell, 2018) that offer advantages of specificity and sensitivity have also been adopted for detection of virulence factors.

Deoxyribonucleic acid (DNA) hybridization is another technique used to detect *E. coli* virulence. Among the earliest DNA hybridization techniques that were developed includes the technique which utilizes radioactive labeled DNA probes (Weagant et al., 2016; Anjum et al., 2014). The labeling by radioactive material is highly recommended by key bacteriological analytical manual which is developed by FDA because it produces high recovery rates of STEC from contaminated foods (FDA, 2011). Despite their usage in detection of Shiga toxin, radio labeled probes had previously shown some weakness such as having short half-life. Other disadvantages included handling and disposal, which if not taken carefully may result in environmental pollution. The technique is also quite expensive in that it only requires

recruitment of qualified laboratory personnel. The infrastructure for shiga toxin detection is done using the radioactive probes and requires well equipped biosecurity measures for safety. Currently, the disadvantages that are associated with radioactive labeled probes have been overcome by introduction of non-radioactive labels. Some of the major probe labels that have so far been availed for use include biotin and digoxigenin (La Ragione & Newell, 2018). The label probes can easily detect *stx*1 or *stx*2 genes within *E. coli* strains that have been isolated from targeted food product or surrounding environment using Trypticase soy agar. Other probes are much more sensitive and can only detect specific genes such as enterohaemolysin (*ehxA* or *Hly*) and *eae* A gene in O157:H7 and less likely in other STEC. The sensitive technique takes a lot of time (approximately 72 hours) for the results to be obtained and, therefore, not highly recommended for routine diagnosis. It should therefore be used for analysis of haplotypes that are in circulation in a given country.

2.2. Lytic transglycosylase gene as a molecular marker

Escherichia coli secrete six membrane-bound lytic transglycosylase (ltg) enzymes (MltA –MltD and EmtA) and one soluble lytic transglycosylase (Slt70) (Scheurwater et al., 2008). A good number of these lytic transglycosylases exhibit exo-lytic activity accompanied by the release of 1, 6-anhydroMurNAc from one end of the glycan strand. Conversely, EmtA exhibit endo-specific activity, breaking the glycosidic bonds within the peptidoglycan strand, producing shorter strands with the 1,6-anhydromuramic acid specific to LT lysis (Scheurwater et al., 2008). Lytic transglycosylases (LTs) are produced by a number of bacteria species including *Pseudomonas aeruginosa, Escherichia coli, Clostridium difficile* and *Neisseria gonorrhea*. They are organized into four distinct families (1 - 4) based on amino acid sequences alignments

and consensus motifs (Scheurwater et al., 2008). They appear to be ubiquitous amongst bacteria and individual species often produce multiple LTs from different families thus exhibiting functional redundancy. For instance, *E. coli* produces six distinct LTs consisting of Slt70 (Family 1A), MltC (Family 1C), MltD (Family 1D), EmtA (Family 1E), MltA (Family 2), MltB (Family3) (Scheurwater et al., 2008). They cleave glycosidic bond within peptidoglycan sacculus making space for bacterial cells to grow.

The LTs are also key in recycling of released 1, 6 anhydromuropeptidases in *E. coli* and in high concentration they induce β-lactamase production. Lytic transglycosylases have also been associated with pathogenesis (Scheurwater et al., 2008).

2.3. Use of antimicrobial agents in livestock

In livestock, antimicrobials are mainly used for prevention and treatment of disease and also as growth enhancers (Odeyemi, 2016). Ideally, the use of antimicrobials in livestock should be based both on the animal factors (age, body weight and immune status) and drug properties (drug toxicity, pharmacodynamics, tissue distribution and pharmacokinetics) (Economou & Gousia, 2015). However, in cases where several animals are sick, farmers choose to treat the entire flock with antibiotics to prevent the spread of the disease (Economou & Gousia, 2015).

Despite the banning of antimicrobials that are used as growth promoters in most developed countries, developing countries still use such drugs for various reasons. For example, stimulation of livestock immunity, vitamin synthesis from intestinal stimulation, reduced competition between host and gastrointestinal microflora, modification of bacterial metabolism in the rumen and growth inhibition of harmful bacteria. One major drawback of using antibiotics as growth promoters is that only small doses of the drugs are given that are not sufficient enough

to kill all the harmful bacteria thereby increasing the chances of emergence of resistance strains (Compassion in World Farming, 2011). Among the commonly used antibiotics by farmers in Kenya includes streptomycin, vancomycin, erythromycin, tetracyclines, Ciprofloxacin, Amoxicillin, Ampicillin, and Cephalexin (Lamuka et al., 2017). Despite the recent efforts to reduce the use of antibiotics in animals, it is estimated that antibiotic consumption in food animals will increase drastically in the coming years (Van Boeckel et al., 2015).

Diseases such as pneumonia in cattle are commonly treated using oxytetracycline, while macrolides and florfenicol are considered to be the second choice, with cephalosporins being administered as the last choice (Economou & Gousia, 2015). In feeds, antibiotics are used in targeting conditions such as liver abscesses, respiratory diseases, aflatoxins and increase animal growth (Economou & Gousia, 2015). Animal conditions such as mastitis are treated mostly using antibiotics with narrow spectrum such as the β-lactam antibiotics when targeting Streptococci, penicillin when the target bacterium is *Staphylococci* and tetracycline, aminoglycoside or penicillin when targeting *E. coli* (Economou & Gousia, 2015; Poirel et al., 2018). Some farmers use antibiotics targeting mastitis for prevention purposes. In this case, the drug is administered in non-lactating cows (Economou & Gousia, 2015). In other ruminants such as sheep and goats, amoxicillin, clavulanic acid/amoxicillin combination, erythromycin, penicillin G and oxytetracycline are among the antibiotics that are widely used (Economou & Gousia, 2015). Despite the benefits of using antibiotics, problems such as the emergence of resistant bacterial strains have been a major occurrence (Poirel et al., 2018).

2.4. Causes of antimicrobial resistance in *E. coli*

Antimicrobial agents have been broadly used in animals and humans for years (Laxminarayan et al., 2016). Antimicrobial resistance results primarily due to the interaction of antimicrobial agent with the organism leading to either death or inhibition of susceptible organism while selecting the resistant ones (Economou & Gousia, 2015). The resistance determinants in the selected organism multiply in the host and disseminates to other hosts. While antimicrobial resistance occurs naturally, most resistance results from overuse and uncontrolled use of various antibiotics (Aarts, 2011; Koo & Woo, 2011; Chirila et al., 2017). Numerous studies have linked antibiotic usage to the emergence and dissemination of resistant bacteria strains (Levy & Marshall, 2004; Davies & Davies, 2010; Messele et al., 2019). The resistant bacteria and the resistant genes can be inherited by members of the same species or can be transferred to other bacterial species via plasmids (Economou & Gousia, 2015). Among the key methods used by bacteria to transfer resistant traits to other strains includes the transfer of resistance gene horizontally also known as Horizontal gene transfer (Chirila et al., 2017; Messele et al., 2019). Other reasons behind the emergence of resistance strains include the coselection of resistance and virulence genes due to overuse of antibiotics that displace the normal flora and adaptive mutations (Chirila et al., 2017). Regardless of the efforts that have been made in sensitization of farmers on proper use of antibiotics in animals, there is continued misuse of antibiotics across the African continent. The majority of these countries do not control antibiotic usage since they are readily available over the counter even without prescription (Lamuka et al., 2017).

Lack of regulations has therefore made it easy and convenient to access antibiotics and increased their haphazard usage. The inappropriate antibiotic prescription has basically supported the modification of bacterial genome hence altering the gene expression, causing mutagenesis and

HGT. Gene expression that is induced by antibiotics, increases bacterial virulence while HGT and the increased mutagenesis facilitates spread and emergence of resistance. Administration of low doses of a particular antibiotic has also been shown to cause resistance by causing strain diversification. Within *E. coli*, there are various genes that confer resistance and can be transferred from one strain to the other.

2.5. Genetic determinants for Antimicrobial resistance in *E. coli*

2.5.1. Resistance to Beta lactams

The alteration in bacteria to confer resistance involves modification of virulence factors such as the biofilms and efflux (Szmolka & Nagy, 2013). The noted resistances to Beta-lactam antibiotics have been linked to various mechanisms such as mutations, reduction, and alterations of proteins that bind penicillin (PBPs) (Economou & Gousia, 2015). *Escherichia coli* resistance to β-lactam antibiotics is attributed to the production of β-lactamases that break down the β-lactam ring of these antibiotics (Economou & Gousia, 2015). Usually, the resistant traits are encoded by certain genes that are found in mobile genetic elements such as bacterial plasmids and transposons and can be transferred between bacterial isolates.

A study that was done in Germany reported a 4.5% prevalence of β-lactamase encoding genes among milk borne *E. coli* (Eisenberger et al., 2018). Also, another study in Thailand documented a high prevalence of CTX-M β-lactamase encoding gene among Enterobacteriaceae (Sasaki et al., 2010). The plasmid-mediated beta-lactamase genes (blaCTX-M-2, blaCMY-2 and blaCTX-M-14) were identified to be present within different resistance strains with blaCTX-M-2 and blaCTX-M-14 being clonally related (Sato et al., 2014). The genes are located within the transmissible plasmid. These results indicate the role of antimicrobial selection pressure in the

upsurge of the resistant strain of *E. coli.*

Sato et al. (2014) established the existence of *E. coli* cefazoline resistance strains in cattle which were treated with ceftiofur. Further discovery revealed that the resistant strains had mutations in the promoter region of *amp*C. The research reaffirmed the existence of Cephalosporin-resistant strain with an extended spectrum. *Escherichia coli* resistant to ciprofloxacin have also been reported to be due to mutations in par and *gyr*A genes.

2.5.2. Resistance to tetracyclines

The alteration in bacteria to confer resistance to tetracycline involves modification of virulence factors such as the efflux pump for detoxification, porin proteins, enzymatic inactivation, ribosomal protection (Koo & Woo, 2011). However, the main resistance to tetracycline in *Escherichia* spp. has been attributed to the efflux system that is coded for by the *tet* genes (A-E, G, L, J, and Y) (Koo & Woo, 2011; Poirel et al., 2018). Most *E. coli* isolated from animals harbor *tet*(B), *tet*(A) and *tet*(C) genes, which are responsible for the resistance phenotypes (Poirel et al., 2018). Other tet genes such as *tet*(W), *tet*(O), *tet*(M) t*et*(Q) and *tet*(X) provide protection to ribosomes and also modify the antibiotic upon expression (Poirel et al., 2018).

2.5.3. Resistance to Aminoglycosides

Resistance occurs by various mechanisms including alteration of the drug target site, mutational alteration of 16S RNA and ribosomal proteins, prevention of drug entry and enzymatic inactivation of the drugs (Poirel et al., 2018). For example, alterations in the sites targeted by gentamicin, amikacin and tobramycin can occur through methylation of A1408 and G1405 residues of 16S RNA. Conversely, enzymatic inactivation is normally due to acetyltransferases,

phosphotransferases and nucleotidyltransferases that modify the drugs hindering them from binding to the target site.

The most common acetyltransferases in *E. coli* are AAC(3)-II/IV and AAC(6)-1b (Ramirez et al., 2011). They act by catalyzing the addition of an acetyl group to positions 1 - 3/6 of an amine group of the aminoglycoside (Shi et al., 2013). On the other hand, ANT (2″ and 3″) are the most commonly found nucleotidyltransferases in Gram negative bacteria in general. Among the phosphotransferases, are the globally distributed APH6 (Ia and Id) that are encoded by the *str* (A and B) genes.

2.5.4. Resistance to Quinolones and Fluoroquinolones

The major resistance mechanism through which *E. coli* develop resistance to Fluoroquinolones (FQ) involves mutational alterations in the chromosomes of the key target enzymes namely DNA gyrase and Topoisomerase IV (Karczmarczyk et al., 2011; Redgrave et al., 2014). These alterations primarily involves mutations located in the quinolone resistance-determining region (QRDRs) of the gyrA gene (Poirel et al., 2018; Redgrave et al., 2014). Although DNA gyrase is the primary target of resistance, alterations in *Par*C and *ParE* of topoisomerase IV are secondary targets. While alterations in the gyrA are adequate to cause resistance to quinolones, mutations within *par*C and *gyr*A are vital for Fluoroquinolones resistance to occur (Poirel et al., 2018). Plasmid-borne resistance to quinolones have also been documented (Cattoir & Nordmann, 2009). For example, the Qnr-like proteins prevents binding of quinolone to target DNA, modification of enrofloxacin and ciprofloxacin by acetyltransferases enzyme (AAC (6′)-Ib-cr) and possession of QepA and OqxAB efflux pumps (Karczmarczyk et al., 2011; Redgrave et al., 2014).

2.5.5. Resistance to Sulfamethoxazole/Trimethoprim-potentiated sulfonamides

Sulfonamides compete with p-amino-benzoic acid, its structural analog, for binding to dihydropteroate synthase (DHPS) enzyme, thus preventing the formation of dihydrofolic acid (Poirel et al., 2018). In *E. coli*, resistance occurs following the acquisition of dihydropteroate synthase (DHPS) genes by the bacteria (Koo et al., 2015). Resistance to sulfonamides is encoded by the plasmid mediated Sul genes namely Sul1, Sul2 and Sul3 genes (Poirel et al., 2018). Among the three genes, sul1 is the most predominant gene in *E. coli* isolates from animals. It is a conserved gene and is usually associated with other resistance genes especially those situated on class 1 integrons gene cassettes. Similarly, sul2 gene are extensively distributed among *E. coli* of different animal species worldwide (Kikuvi et al., 2007; Wu et al., 2010). The sul2 gene is often linked to resistance to streptomycin particularly strA and strB. The plasmid mediated sul3 gene has been associated with other resistance genes, for example, the mef (B) gene which is responsible for macrolide resistance (Liu et al., 2009).

Likewise, several mechanisms of resistance to trimethoprim exists in *E. coli*. They include efflux pumps, development of permeability barriers, regulation and mutational changes in the target enzymes, existence of naturally insensitive target dihydrofolate reductase and the acquisition of enzymes responsible for drug-resistance (Koo et al., 2015; Poirel et al., 2018). A number of dfr genes including dfr (A and B) have been identified by various researchers responsible for trimethoprim resistance in other Enterobacteriaceae family. While dfrA genes code for proteins with amino acids sizes ranging from 152 to 189, the dfrB genes code for proteins with 78 amino acids only. And like the sul genes, most of the dfr genes are located on

class 1 or class 2 integrons gene cassettes (Poirel et al., 2018).

2.5.6. Resistance to phenicols

Bacterial resistance to phenicols is conferred by a number of mechanisms. The major mechanisms entails inactivation of chloramphenicol by the enzyme chloramphenicol acetyltransferases (CATs) which is encoded by one or more of the cat genes. Secondly, by the non-enzymatic chloramphenicol resistance genes including FLOR or CMLA that encodes efflux pumps. Lastly, by methylation of the drug target sites by a methylase enzyme mediated by cfr gene (Poirel et al., 2018). Other mechanisms of chloramphenicol resistance including inactivation by phosphotransferases and permeability barriers have been reported (Schwarz et al., 2004).

2.6. Phenotypic methods of AMR detection

The main phenotypic methods used for in vitro susceptibility testing of bacteria includes agar dilution, disc diffusion and broth dilution methods (Balouiri et al., 2016). Agar dilution method entails inclusion of different concentration of the antimicrobial agents into a nutritious agar media, followed by inoculation of a standardized inoculum on agar plate. Broth dilution (macro- or micro-), involves making of serial dilutions of an antimicrobial agent in 2ml broth medium (microdilution) or in 96 well microtiter plate using smaller volumes (microdilution). Growth is assessed after incubation for 16-20 hrs and the minimum inhibitory concentration (MIC) value is read.

The Agar disc diffusion is the most commonly used method for determining phenotypic antimicrobial resistance due to its low cost, efficiency, simplicity and the ease to interpret the

results (Balouiri et al., 2016). To begin with, Muller-Hinton agar plates are evenly seeded with a standardized inoculum of the isolate of interest, followed by impregnation of the plates with commercially obtained paper disks containing a standard amount of the test antimicrobial and then incubated overnight. Typically, the test antimicrobial agent begins to diffuse from the disk into the agar inhibiting growth of the test bacteria. The diameter zones of growth inhibition are then measured in mm and compared to a standard interpretation chart used to categorize the isolate as susceptible, intermediate or resistant. Unlike the dilution methods, MIC measurement cannot be determined from this qualitative test. Other commercially available diffusion tests including the antimicrobial gradient method (E-test), agar plug and agar well have been developed. For instance, E-test incorporates both dilution and diffusion principles in being able to establish MIC values as well. It entails streaking the organism on agar plate prior to positioning of an antimicrobial incorporated commercial strip on the surface. This method determines the MIC values of antifungals, antibiotics and antimycobacterials at the point where the strip and the growth inhibition forms an ellipse. Although E-test is simple and sensitive, it cannot be routinely used due to its high cost.

2.7. Genotypic methods of AMR detection

Molecular techniques are used to determine mechanism for resistance and genes responsible for phenotypic resistance. Resistance traits are genetically encoded and test for particular genes responsible for the observed phenotypes. PCR is one of the most commonly used molecular technique for detecting targeted genes. It entails denaturation of template DNA, annealing of primers to the sequence and extension which is aided by Taq polymerase (Anjum et al., 2017). The other method is DNA hybridization which is based on the fact that specific DNA

pyrimidines pair with specific DNA purines.

Other new methods including whole-genome sequencing (WGS) and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) have also emerged (Ambardar et al., 2016; Kelley, 2017). Whole-genome sequencing involves generation of large amount of data on sequences as compared to previous techniques such as Sanger sequencing (Endimiani & Jacobs, 2016). Ion Torrent, Genome analyzer and GS Junior machines have been used in high throughput sequencing of pathogenic bacteria genomes (Liu et al., 2012). The machines output relatively consist of sequence reads of up to 400bp. The short reads are disadvantageous for microbial gene detection since most genes sequences are beyond 400bp. Another major drawback is the sequencing error rates that might be encountered in a single sequence read. The error rate places traditional Sanger sequence technique as better tool compared to the new techniques (Liu et al., 2012). Conversely, MALDI-TOF MS technology offers advantage of being able to detect single nucleotide polymorphisms (SNPs) within genes responsible for phenotypic resistance (Tuite et al., 2014). Compared to conventional PCR, MALDI-TOF MS is fast, relatively cheap and consistent.

2.8. Multidrug resistant *E. coli*

Multidrug resistance (MDR) in *E. coli* isolates from food animals and animal food products has been documented for different antimicrobial classes worldwide (Solomakos et al., 2009; Shin et al., 2014; Nobili et al., 2017; Messele et al., 2019). Ombarak et al. (2018) reported that *E. coli* strains from cheese and raw milk were resistant to tetracyclines, ampicillin, streptomycin and sulfamethoxazole-trimethoprim at levels between 11.3% and 27.5%, whereas resistance to nalidixic acid and ciprofloxacin were very low. Additionally, high resistance to tetracycline up

to (100%), ampicillin (100%), sulphonamides (93%), streptomycin (86%), sulfamethoxazole/trimethoprim (50%), florfenicol (50%) and chloramphenicol (50%) has been reported from various food samples of animal origin among *E. coli* isolates globally (Nagy et al., 2015).

Few research has been done on AMR in *E. coli* and STEC in Africa (Onono et al., 2010). Multidrug resistant *E. coli* isolates from the environment, humans and animals has been documented against several classes of veterinary and human antimicrobial agents (Njie & Carlos, 2008; Iweriebor et al., 2015). For instance, Njie & Carlos, (2008) using *E. coli* O157 strains isolated from cattle, human and pig faecal samples reported that the isolates were resistant to a range of antimicrobials including tetracycline, sulphamethoxazole and erythromycin at levels ranging from 52.6% to 92.1%. In addition, Iweriebor et al. (2015) documented that *E. coli* O157 isolates that were obtained from faecal samples of dairy cattle in South Africa were resistant to a range of antimicrobials. In their research, the greatest resistance levels were documented for tetracycline.

2.9. Transmission of antimicrobial resistant *E. coli* through milk

Raw milk has been reported to be one of the main foods that are most contaminated by resistant strains of bacteria due to persistent use of antimicrobial agents (Messele et al., 2019). In livestock, the continuous use of various antimicrobial agents results from the fear of losing livestock due to diseases like bovine mastitis (Wanjohi et al., 2013). Raw milk can also be contaminated with resistant microbial strains that are present in the animal feeds hence serving as an effective route of spreading the resistant strains from farm animals to humans (Economou & Gousia, 2015). Milk regarded safe for human consumption should be free from antibiotic-

resistant strains and antibiotic residues. Raw milk is considered to be a suitable media for growth of microorganisms; hence it harbors variable microorganisms that are considered as significant source of food borne pathogens (Farrokh et al., 2012).

CHAPTER THREE

3.0 METHODOLOGY

3.1. Study area

This study was part of a larger ongoing study, titled “Accelerated Value Chain Development–Livestock component (AVCD-L)” implemented by ILRI and other partners, in which milk samples were collected from Isiolo County, Northern Kenya. Four out of six wards namely Kinna, Merti, Burat and Oldonyiro were randomly chosen using systematic sampling method along transects defined by feeder roads. A sampling frame for the households was created from records kept by the county government authorities. Every fifth household that kept animals of interest such as goats, cattle, and sheep were recruited.

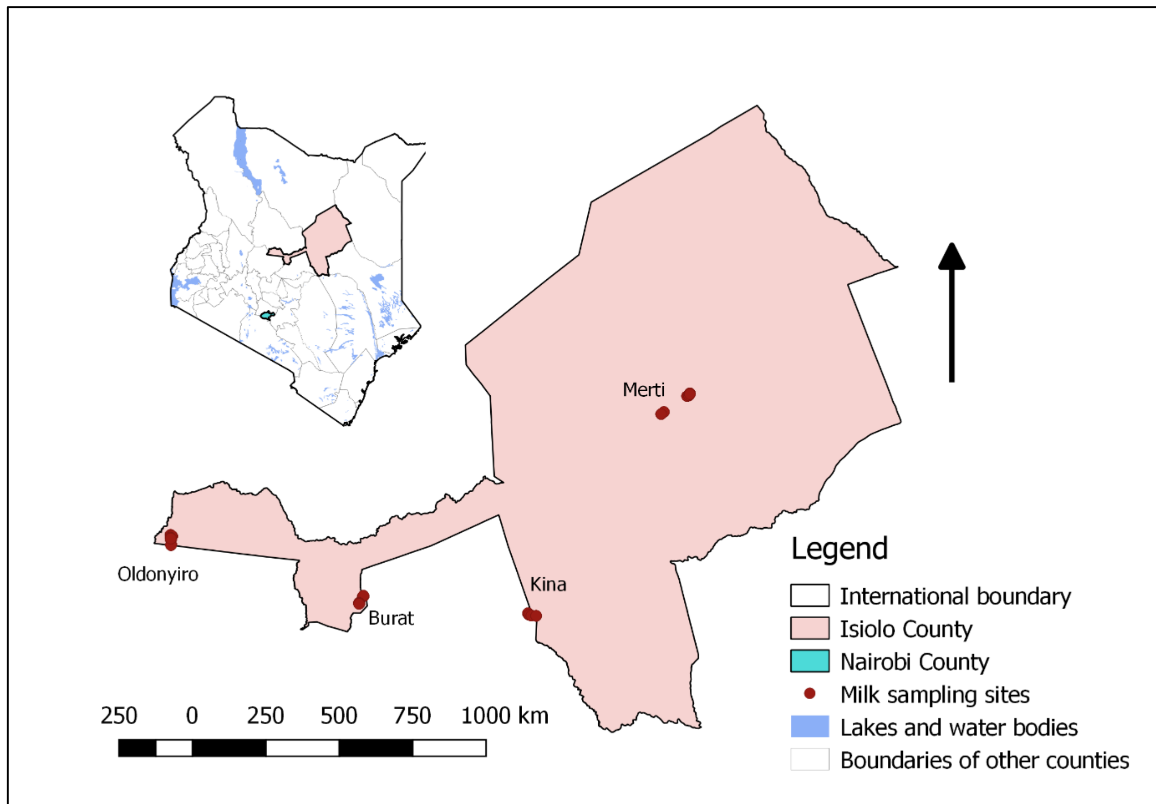

Figure 3.1: Map of Isiolo County showing the sampling points highlighted in dots.

3.2. Milk sample collection

Three hundred and four (304) milk samples were collected from seventy-six households in Isiolo. Within each household, samples from three randomly selected lactating animals (cattle, sheep and goats) and one pooled sample (often from domestic containers) were obtained. Initially, teats and udders were cleaned with cotton swabs moisten with 70% ethyl alcohol. Then, three streams of the first milk were discarded, and about 50ml was collected in sterile sampling bottles. Additionally, a questionnaire was administered to capture history of antimicrobial usage and milk processing before consumption. Households were geo-referenced using Garmin ETrex hand held GPS units. A sample identification system that could link a milk sample to a household was used. Samples were then taken to the laboratory at the Centre for Biotechnology and Bioinformatics, University of Nairobi on ice packs for analysis.

3.3. Sample enrichment and isolation of *E. coli*

Milk samples were enriched using Buffered Peptone Water (BPW) media. An amount of 10g powdered BPW was dissolved in 500 ml of purified water. Then, 4ml of this broth was aliquoted into different test tubes and sterilized at 121°C for 15 minutes by autoclaving. A volume of 1 ml of each milk sample was mixed in 4 ml of sterile buffered peptone water (BPW) to make a dilution of 1:10 and incubated in a Memmert incubator at 37˚C for 24hr. The *E. coli* isolates were confirmed phenotypically by bacterial culture on Eosin Methylene Blue agar and Sorbitol MacConkey agar (SMAC) and biochemical tests (triple sugar iron test, lysine indole motility test and citrate test) followed by DNA extraction.

3.3.1. Eosin Methylene Blue Agar culture

An amount of 37.5g Eosin Methylene Blue Agar (Oxoid) was dissolved in 1000 ml of purified water. This mixture was boiled and autoclaved to sterilize at 121°C for 15 minutes. The media was cooled to 60°C then mixed well by shaking to suspend the precipitate and oxidize methylene blue. A total volume of 20 ml of the media was dispensed into the petri dishes and left to solidify for subsequent inoculation. Using sterile disposable wire loops, a loop-full of the buffered peptone water broth was picked and streaked on the surface of the Eosin Methylene Blue Agar plates. The inoculated plates were then incubated in a Memmert incubator overnight at 37°C. Thereafter, single discreet colonies suggestive to be *E. coli* based on their green metallic sheen color were picked from EMBA plates for biochemical characterization.

3.3.2. Sorbitol MacConkey Agar culture

An amount of 37.5 g of powdered Sorbitol MacConkey Agar (Oxoid) was dissolved in a 1 litre of purified water. The media was boiled then sterilized by autoclaving at 121°C for 15 minutes, thereafter cooled to 60°C and 20 ml of the media aseptically dispensed in each petri-dishes and left to solidify. Using sterile disposable wire loops, a loop-full of the buffered peptone water broth was picked and streaked on the surface of the Sorbitol MacConkey Agar plates. The inoculated plates were then incubated in a Memmert incubator overnight at 37°C. Thereafter, a small portion of Nonsorbital fermenting colonies (NSFC) suggestive of *E. coli* O157:H7 for their colorlessness were picked from SMAC plates using a plastic stick and tested for the presence of O157 and H7 antigens using RIMTM latex kit (O157:H7).

3.3.3. Triple sugar Iron test

An amount of 32.2 g of powdered Triple sugar Iron agar (Himedia) was dissolved in 500 ml of purified water. The media was boiled and about 3ml was dispensed into test tubes. The media was then sterilized at 121°C for 15 minutes by autoclaving. The media was cooled in slanted positions to allow for the formation of deep butts. Using sterile disposable wire loop, a single colony was picked from the EMBA plate and emulsified in 2µl of sterile distilled water in an Eppendorf tube. A sterile straight loop was dipped into the mixture and used to stab the formed butt and slant. The inoculated tubes were then incubated aerobically over night at 37^{0}C. Triple Sugar Iron agar was examined for color change from red to yellow due to acid production, no blackening in the medium and the presence of cracks in the medium.

3.3.4. Lysine Indole Motility test

An amount of 36.52 g of powdered Motility Indole Lysine agar (Himedia) was dissolved in 1000 ml of purified water. The broth was boiled and about 5 ml distributed into McCartney bottles. The media was then sterilized for 15 minutes by autoclaving at 121°C. The bottles were then cooled in upright positions. Using the already emulsified distilled water suspension containing the bacteria in the Eppendorf tube, a sterile straight loop was dipped into the mixture and inoculated in the broth medium. The inoculated bottles were then incubated overnight at 37°C. Lysine Indole Motility broth was observed for diffuse growth and purple color formation in the medium due to lysine carboxylation. A drop of Kovac's reagent was then dispensed on the surface of the overnight broth culture and observed immediately for presence of a red ring on the surface.

3.3.5. Citrate test

An amount of 24.2 g of powdered citrate agar (Himedia) was dissolved in a 1000 ml of purified water. The media was boiled and about 3ml was distributed into Bijou bottles. The media was then sterilized for 15 minutes by autoclaving at 121°C. The media was cooled in upright positions. Using the already emulsified distilled water suspension containing the isolate, a sterile straight loop was dipped into the mixture and used to inoculate the media. The inoculated bottles were then incubated over night at 37°C. Citrate media were observed for no color change in the Citrate Agar. Additionally, Gram staining technique was done on all the isolates.

3.4. Serotyping of *E. coli*

A drop of control and test latex (O157 and H7) were dispensed onto a labelled tile. Thereafter, a small portion of Nonsorbital fermenting colonies (NSFC) were picked from SMAC plate using a plastic stick and emulsified in the control and test latex on the tile, then mixed for one minute. Following agglutination with O157 and not with H7 antibodies, the one isolate was subcultured on sheep blood agar.

3.4.1. Culture on Blood Agar

An amount of 20 g powdered base (Oxoid) was dissolved into 500 ml of purified water. This mixture was sterilized for 15 minutes by autoclaving at 121°C. Afterwards, the media was kept in a 55°C water bath and then 7% sterile sheep blood was added and mixed well to make the Sheep blood agar (SBA). About 20 ml of the media was distributed into the petri dishes and left to set for subsequent inoculation. Using a heat sterilized disposable wipe loop, a discrete pale colony was selected aseptically from SMAC media and streaked on the SBA plates and then

incubated over night at 37°C. Afterwards, the isolate was serotyped again using H7 antibodies.

3.5. Extraction of *E. coli* DNA

Pure *E. coli* cultures were revived from skimmed milk by inoculation on Mueller Hinton Agar (Oxoid). Bacterial DNA was extracted using Invitrogen DNA Kits (DNAeasy, USA) using the manufacturer's protocol. First, the bacteria were harvested from an overnight culture and suspended in 200 µL of PureLink™ genomic lysis buffer for subsequent lysis at 55˚C for 30 minutes. Thereafter, twenty (20) µL of Proteinase K was added to the lysed mixture and then incubated at 55˚C for 30 minutes. The digested mixture was treated with 200 µL of 96-100% ethanol and then briefly centrifuged in order to bind the bacterial DNA to silica-gel-membrane. To remove inhibitors of PCR, two washing steps were performed and pure DNA bound to the column was eluted with elution buffer. The recovered DNA was kept at -20˚C pending further analysis. The DNA quantity and purity was measured spectrophotometrically at 260-280 nm, with NanoDrop ND-1000 full spectrum UV-Vis spectrophotometer.

3.6. Primer design for *E. coli* and virulence genes identification

The complete coding sequences of *E. coli* (VCYJ01000008.1), *stx*1 (AB035142.1), *stx*2 (AB071845.1) and *eae* (Z11541.1) were retrieved from GenBank (Hamabata, 2002; Pacheco & Sperandio, 2012) on NCBI and queried against NCBI nucleotide (nr) database using BLASTx algorithm. Primer blast tool at NCBI (www.ncbi.nlm. nih.gov/tools/primer-blast/), was then used to design the primers from the selected biomarkers. The designed primers (Table 3.1) were submitted to Macrogen Company, Korea for synthesis. The synthesized primers were used for PCR assay.

3.7. Confirmation of *E. coli* and virulence genes by PCR and sequencing

Polymerase Chain Reaction (PCR) was carried out to confirm the *E. coli* (*ltg*) and virulence factors (*stx*1, *stx*2 and *eae*) using primers designed in this study (Table 3.1). The PCR reaction was done using a 25µl PCR mixture containing 1µl of DNA template, 12.5µl of 1X BiolabsTM Dream Taq master mix, 1µl each of reverse and forward (*ltg*/*stx*1/*stx*2/*eae*) primers and 9.5µl of nuclease free water. The PCR was done in a Veriti thermal cycler (Applied Biosystems, USA). The thermo-cycling conditions consisted: denaturation at 95°C for 2 min, 30 amplification cycles at 95°C for 30 sec, annealing at (56°C for 50 sec, 62°C for 50 sec, 54°C for 50 sec and 58°C for 60 sec), extension at 72°C for 40 sec and a final extension at 72°C for 5 min. Amplified products were then analyzed by electrophoresis on a 1.3% agarose gel at 80V for 45 min, stained with ethidium bromide, and the images were captured using gel documentation imaging system (Bio-Rad, England). A 100bp DNA ladder (Biolabs, New England) was used as a molecular size marker. American Type Culture Collection (ATCC) 25922 *E. coli* strain and purified water were used as controls. The PCR-products were extracted from the gels using QIAquickTM gel extraction kit. The purified *E. coli* DNA was sequenced with the same primers used for PCR. The isolates were confirmed by BLAST analysis accessed through the GenBank database of the NCBI.

Table 3.1: Primers designed and used in identification of *E. coli* and virulence genes

Gene	Primer	Oligonucleotide sequence (5′- 3′)	Amplicon size (bp)	Annealing temperature
Ltg	F	TTCATCGGCATTCTGGCACT	829	56
Ltg	R	CATCGACGGCGCGTAAAAAT		
Stx1	F	TGAGATCTCGGGAAAAGCGT	443	62
Stx1	R	TGCTGACAATGGCGTTTACC		
Stx2	F	CGTGTCGCCCTTATTCCCTT	500	54
Stx2	R	CTCCGGTTCCCAACGATCAA		
Eae	F	CCGTCACGCTGTTGTTAGGA	608	58
Eae	R	TTCATCGCCACGTTATCGCT		

3.8. Phenotypic antimicrobial resistant profiles

Antimicrobial susceptibility testing (AST) was done using the Kirby-Bauer disc diffusion method on Mueller Hinton agar with an inoculum equivalent to 0.5 McFarland standards. The inoculums for AST were prepared by making direct saline suspension of colonies obtained from an overnight bacterial growth on Mueller Hinton Agar (MHA) plate. The suspensions were adjusted to achieve a turbidity corresponding to 0.5 McFarland standards. A visual comparison was made for each inoculums prepared to a 0.5 McFarland standard tube. A sterile cotton swab was dipped into the adjusted suspension within 15 minutes and streaked over a dried surface of the Mueller-Hinton agar plate. The antimicrobial impregnated disks were dispensed onto the surface of the inoculated agar plates, pressed down gently and incubated at 37°C for 18 hours. The following antimicrobials were tested: tetracycline, streptomycin, amoxicillin/clavulanic acid, cefotaxime, ceftazidime, ampicillin, chloramphenicol, ciprofloxacin, nalidixic acid, kanamycin, trimethoprim and kanamycin. Finally, the zone diameters of inhibition were measured using a ruler to the nearest whole millimeter. For interpretation, these mean diameter zones of inhibition were compared with standard break points for *Escherichia coli* for each tested antibiotic using Clinical Laboratory Standard Institute guidelines (CLSI, 2014). The antimicrobial susceptibility was scored as susceptible, intermediate or resistant. The overall results were tabulated on an antibiogram. American Type Culture Collection 25922 *E. coli* strain was used for quality-control (CLSI, 2014).

3.9. Primer design for AMR genes

The complete coding sequences of *E. coli* bla_{TEM} (AB201242.1), $bla_{CTX\text{-}M}$ (KM211691.1), bla_{SHV} (NG049989.1) *tet*B (HQ018801.1) and *tet*C (HQ018801.1) were retrieved from

GenBank (Ziebell et al., 2011; Strauß et al., 2015) and queried against NCBI nucleotide (nr) database using BLASTn algorithm. Primer blast tool of the NCBI was then used to design the primers from the selected biomarkers. Primers targeting bla_{TEM}, bla_{CTX-M}, bla_{SHV}, *tet*B and *tet*C genes were designed and used for the confirmation of antimicrobial resistant determinants. The designed primers (Table 3.2) were submitted to Macrogen Company, Korea for synthesis. The synthesized primers were used for PCR assay.

3.10. Detection of antibiotic resistant genes by PCR

The initially designed primers (*tet*B, *tet*C, bla_{TEM}, bla_{SHV}, and bla_{CTX-M}) were used to optimize PCR assay conditions respectively. A total reaction volume of 25 µl contained: 1 µl of DNA, 1µl of both forward and reverse primers, 12.5 µl of BiolabsTM Taq master mix and 9.5 µl of nuclease free water. Veriti™ Thermal Cycler (Applied Biosystems™) was used for optimization of the different annealing temperature for each gene by a gradient PCR method using the conditions listed in table 3.2. The resulting PCR products were detected by agarose gel electrophoresis.

Table 3.2 : List of primers used in detection of resistance determinants

Gene	Primer	Oligonucleotide sequence (5′-3′)	Amplicon size (bp)	Annealing temperature
TetB	F	ACCACCTCAGCTTCTCAACG	586	56
TetB	R	GTAAAGCGATCCCACCACCA		
TetC	F	TGAGATCTCGGGAAAAGCGT	308	62
TetC	R	TGCTGACAATGGCGTTTACC		
SHV	F	GCTGGAGCGAAAGATCCACT	568	54
SHV	R	CCCGGCGATTTGCTGATTTC		
TEM	F	CGTGTCGCCCTTATTCCCTT	480	62
TEM	R	CTCCGGTTCCCAACGATCAA		
CTX-M	F	CCGTCACGCTGTTGTTAGGA	370	58
CTX-M	R	TTCATCGCCACGTTATCGCT		

3.11. Visualization of PCR products

A 1.3% agarose gel was prepared by weighing 0.6g of agarose powder and dissolved in 40 ml of 1 x TAE buffer, followed by boiling. The mixture was allowed to cool and Ethidium Bromide added and dispensed in casting tray to polymerize. A 10µl PCR product was loaded into the gel wells. A 100bp DNA ladder was loaded alongside the samples. Electrophoresis was carried out at 70 volts for 45 minutes and the images of the gels captured, analyzed and recorded using UV trans-illuminator (BIORAD).

3.12. Cleaning of the PCR product

The QIAquick Gel Extraction Kit was used to clean the PCR products in accordance to the instructions of the manufacturer. The DNA fragments were cut from the agarose gel and weighed in an autoclaved 1.5 ml Eppendorf tube. Into the tube, three volumes of QG buffer was dispensed for every 100mg of the gel, with each tube comprising 0.4g of the gel-DNA fragment. Then, the mixture was incubated for 10 minutes in a 50℃ water bath while occasionally vortexing the tubes to help in dissolving the agarose gel. Afterwards, 100µl of isopropanol was added for every 1 volume of gel and mixed. This was followed by binding of DNA where the content of Eppendorf tube was placed into the provided QIAquick column. The tube was spun (13,000rpm) for 1 min then flow-through poured and same tube fitted back to the spin column. An amount of 500µl of QG buffer was added into the QIA quick column and spun (13,000rpm) for 1 minute, flow-through was discarded then tube placed back to the QIAquick column. This was followed by a washing step whereby 750 µl of PE buffer was pipetted to the QIAquick column and spun (13,000rpm) for 1minute after 10 minutes. The flow-through was poured and the same tube fixed back onto the QIAquick column followed by spinning (13,000rpm) of the

column for 1 minute to remove the residual buffer. The QIAquick column was put into a sterile 1.5ml Eppendorf tube. To recover the DNA, 50 µl of EB buffer was pipetted to the center of QIAquick membrane and the tube spun (13,000rpm) for 1minute. The recovered DNA was confirmed by running it on agarose gel.

3.13. Sequencing of PCR products

The cleaned PCR products were sequenced using both forward and reverse primers. Sanger sequencing technique was used, where the sequencing reactions were carried out using ABI BigDye® Terminator v3.1 Cycle Sequencing Kit in the DNA Master cycler pro 384 (Applied Biosystems). The Bioedit software was used to edit and assemble the chromatogram peaks obtained (Hall, 1999), and the consensus sequences obtained. The sequences were then trimmed using the Gene Runner software V6.5.51 (Hastings Software, Inc.) to remove the stop codons and the ORF finder to find the open reading frame. The sequences were then queried against their nucleotide homologs in the NCBI database and then submitted to NCBI database through Bankit (Pirovano et al., 2017) and accession number assigned to them.

3.14. Data analysis

The raw data was cleaned in Access (Microsoft Inc.) and merged with results data. The data was then imported into STATA software version 13 and descriptive statistics conducted. Chi square tests were performed to evaluate the incidence.

CHAPTER FOUR

4.0 RESULTS

4.1. *Escherichia coli* isolated from raw milk

Overally, *Escherichia coli* was detected in 42(13.8%) samples including 19(8.8%) from household and 23(31.08%) from individual animal (Table 4.1). The colonies were characterized by metallic green colour on EMBA and pink colour on SMAC (Fig 4.1) indicating that the colonies were *E. coli* and non O157 STEC strains. Also, all the 42 fermented lactose and changed TSI medium from red to yellow with production of gas, and showed diffuse growth of the organism throughout the medium coupled with formation of a red colour after the addition of Kovac's reagent to an overnight bacteria culture on Lysine Indole Motility agar in peptone water. Furthermore all the isolates showed no colour change on simmon citrate agar after an overnight incubation of the organism. The biochemical tests indicated that all the 42 isolates were *E. coli* and Gram staining confirmed that they were gram-negative rods.

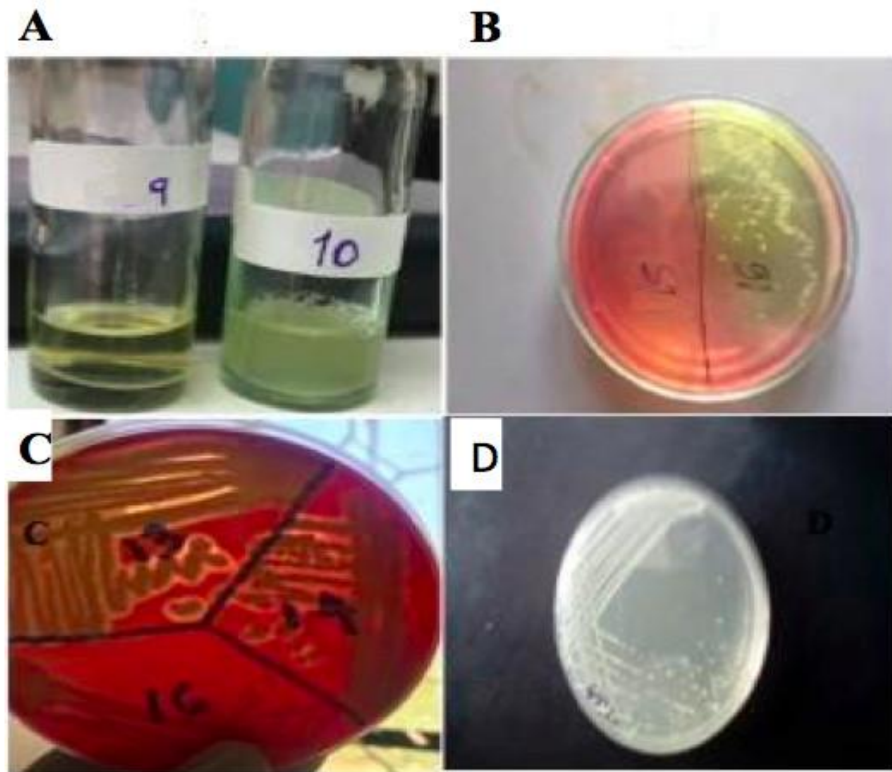

Figure 4.1: Isolation of *E. coli* by culture on EMBA and SMAC.

(A) Bacterial revived in BPW, sample 9 remains clear hence no microbial growth while sample 10 shows turbidity hence microbial growth, (B) Shows pale colonies suggestive of suspected EHEC O157:H7 colonies on SMAC culture, (C) Shows metallic sheen colonies of suspected *E. coli* growth on EMBA culture, (D) Shows suspected colonies of *E. coli* on MHA.

Table 4.1: Number of *E. coli* Isolated and identified from raw milk

Source	Number positive (%)
Overall	42 (13.8)
From animal (mammary gland)	19 (8.7)
From household (pooled)	23 (31.0)

Total no. of samples (Animal =228, pooled =76), p=0.0002)

Pooled milk samples were significantly (p=0.0002) contaminated by *E. coli* as compared to individual lactating animals.

4.2. *Escherichia coli* and virulence genes detected by sequencing

Molecular markers targeting the lytic transglycosylase gene amplified the fragment yielding reliable bands corresponding to 829bp. All the 42 samples screened for the presence of *ltg* gene were positive. The band positions are shown in figure 4.2 below. Furthermore *stx*1, *stx*2 and *eae* primers amplified the respective genes yielding reliable bands corresponding to 443bp, 500bp and 608bp respectively (Figure 4.4). The sequenced PCR products of representative samples were confirmed to be *E. coli* by BLASTn and BLASTx tools (Figure 4.3). BLASTn analysis revealed that the sequences were homologous to the ltg gene of *E. coli* available in the GenBank of the NCBI. The sequence identities for these homologous were 100%. Furthermore, Blastx analysis revealed that the translated amino acid sequence was homologous to the *E. coli* lytic transglycosylase protein revealing a 100% sequence identity. Therefore, these results confirmed that the 42 isolates obtained from the milk samples were *E. coli.* The sequences are available in GenBank database with accession numbers MH818568-MH818570 and MH818570.

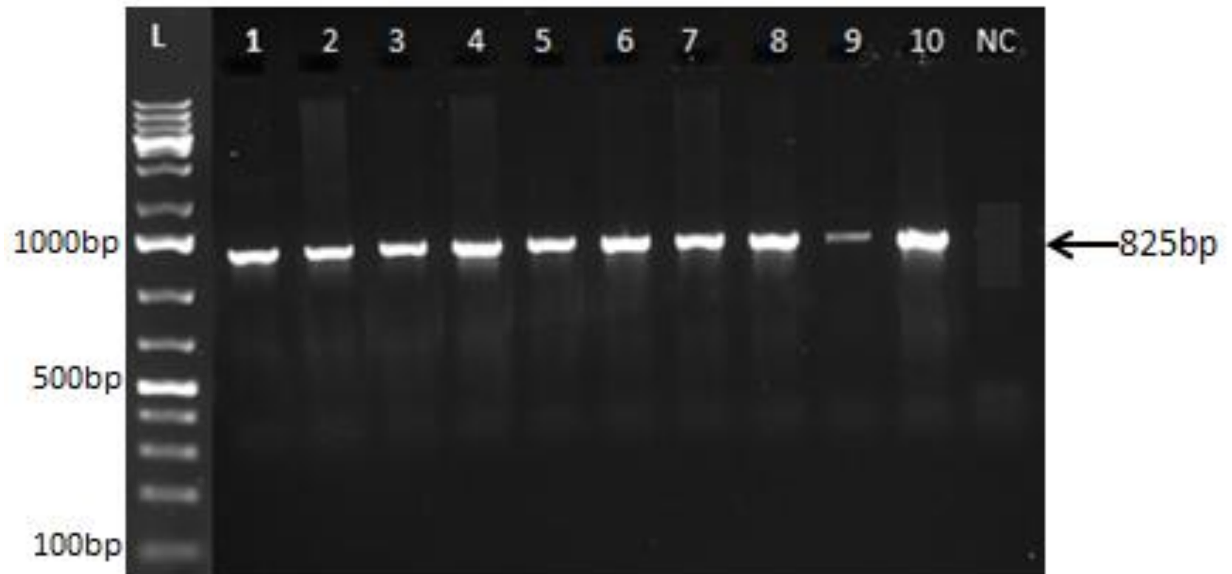

Figure 4.2: Conventional PCR amplification of *ltg* gene.

Lane L is the DNA Ladder (100bp), Lane NC is the negative control, and the numbered lanes (1-10) are test samples hence the possibility of *E. coli* presence. The arrow shows the position of amplified gene at 825 bp

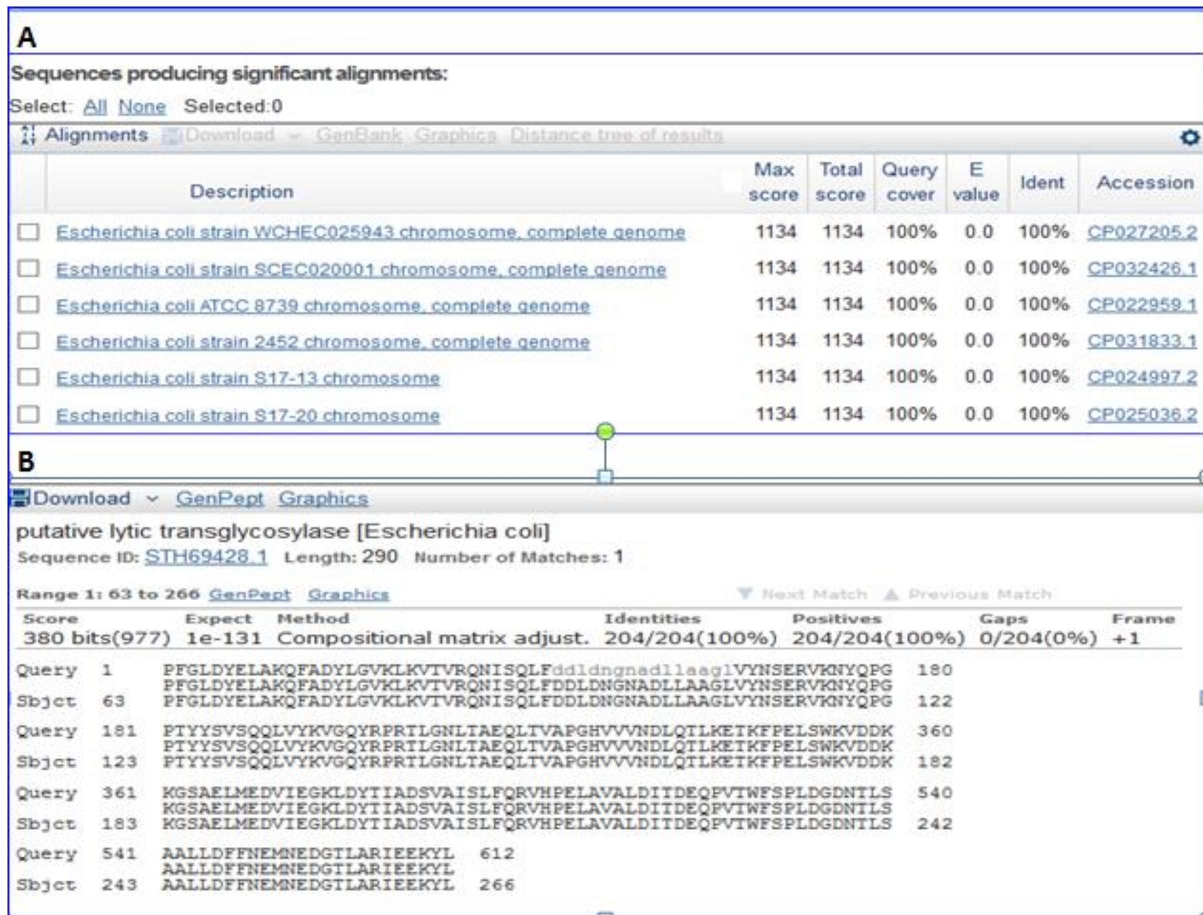

Figure 4.3: Blastn and Blastx results of sequenced lytic transglycosylase gene.

(A) BLASTn results showing nucleotide identities of 100% to the sequenced amplicons. (B) BLASTx result showing amino acid sequences of the homologue in the GenBank aligned with the sequences obtained in this study.

4.3. Virulence genes detected by PCR

Among the 42 *E. coli* isolates recovered from raw milk, 47.6% (20/42) carried both *stx*1 and *stx*2 genes, 85.7% (36/42) carried *stx*1 only and 57.1% (24/42) carried *stx*2 only. The *eae* gene was detected in 90.4% (38/42) of isolates as shown in Table 4.2 below.

Table 4.2: Distribution of virulence genes in milk-borne *E. coli*

*Stx*1	*Stx*2	*eae*	No. of isolates (%)
+	-	+	36 (85.7)
-	+	+	24 (57.1)
+	+	+	20 (47.6)

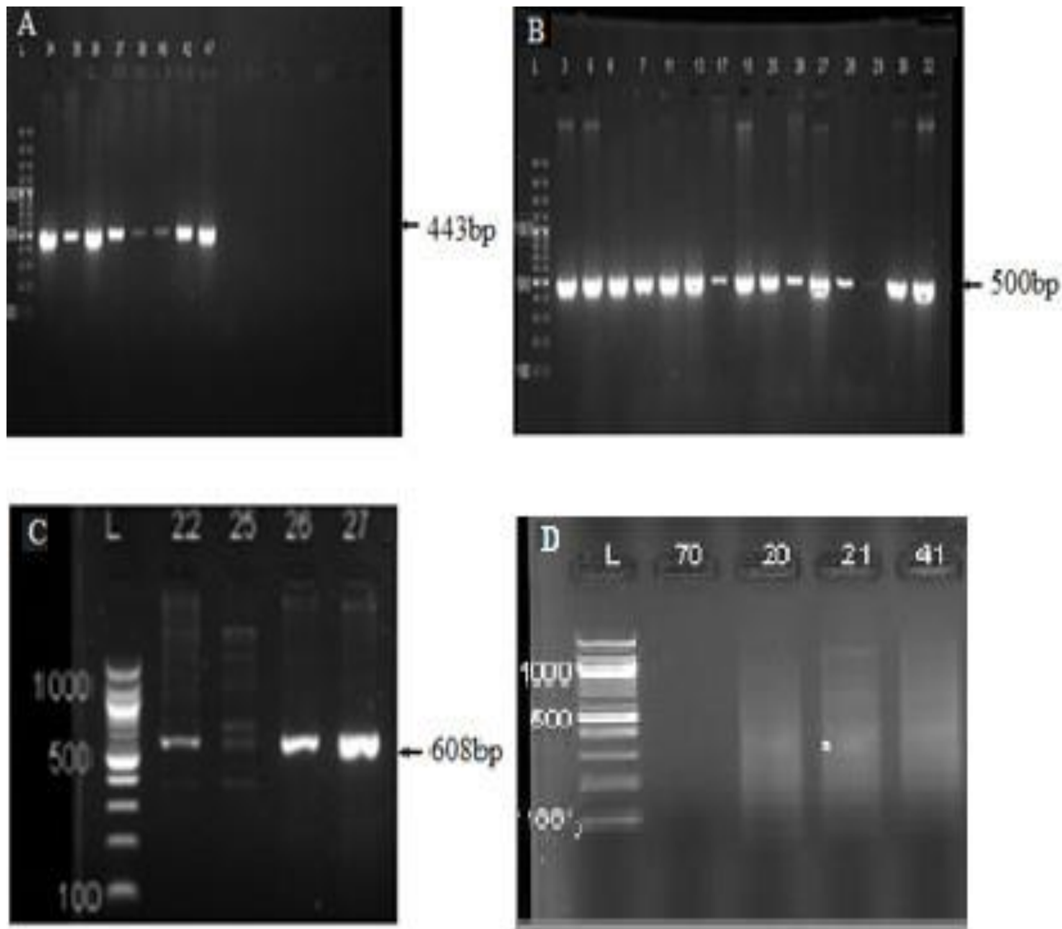

Figure 4.4: Gel images of PCR-amplified *stxs* and *eae*.

(A) *stx*1 gene yielding bands 443bp, (B) *stx*2 gene yielding bands of approximately 500bp, (C) *eae* gene yielding bands of approximately 608bp, (D) Lack of amplification of *stx*1 gene. Lane L is the DNA Ladder (100bp), numbered lanes are the test samples; Lane P is the positive control and Lanes with no bands are samples negative for *stx*1, *stx*2 and *eae* genes and the lanes with bands show presence of *stx*1, *stx*2 and *eae* genes. The arrow shows the positions of amplified genes.

4.4. Antimicrobial usage by the pastoralists

Questionnaire data revealed that tetracyclines (65.5%), penicillins (15.6%) and aminoglycosides (16%) were the most commonly used antibiotic classes. The other classes of antimicrobials that were used by the pastoralists included macrolides (3%) and sulphonamides (0.3%).

4.5. Antimicrobial resistant *E. coli* phenotypes

Overall, 40(95%) *E. coli* isolates were resistant to at least one of the antibiotics including tetracycline, ampicillin, streptomycin, amoxicillin/clavulanic, cefotaxime, cephalexin, chloramphenicol, kanamycin and ceftazidime. Of these, 55% were resistant to tetracycline, 48% to ampicillin, 29% to streptomycin, 19% to amoxicillin/clavulanic, 14% to cefotaxime, 12% to cephalexin, 12% to chloramphenicol, 10% to kanamycin and 7% to ceftazidime. However, all the isolate were susceptible to nalidixic acid and ciprofloxacin (Table 4.3). Only 2(5%) *E. coli* isolates were susceptible to all the antibiotics.

Furthermore, resistance was significantly higher (p=0.0008) in pooled milk samples (22.97%, 95% CI; 14.88-33.75%) as compared to individual lactating animals (10.64% 95% CI: 7.20-15.47%) as shown in table 4.4. Conversely 6(14.28%) isolates were MDR phenotypes, with all the MDR isolates being resistant to ampicillin, tetracycline and cefotaxime/chloramphenicol. Three (3) of the MDR isolates were resistant to cefotaxime suggesting that they are ESBL producers.

Table 4.3: Proportions of antibiotic-resistant *E. coli* in milk of Isiolo County

Antimicrobial Agent	Susceptible n(%)	Intermediate n(%)	Resistant n (%)
Tetracycline	19 (45)	0 (0)	23 (55)
Ampicillin	21 (50)	1 (2)	20 (48)
Streptomycin	27 (64)	3 (7)	12 (29)
Amoxicillin/Clavullanic	33 (79)	1 (2)	8 (19)
Cephalexin	37 (88)	0 (0)	5 (12)
Cefotaxime	36 (85)	0 (0)	6 (14)
Ceftazidime	35 (83)	4 (10)	3 (7)
Chloramphenicol	35 (83)	2 (5)	5 (12)
Kanamycin	34 (80)	4 (10)	4 (10)
Ciprofloxacin	42 (100)	0 (0)	0 (0)
Nalidixic acid	42 (100)	0 (0)	0 (0)
Multidrug Resistant	36(85)	0 (0)	6 (15)

Table 4.4: Proportion of *E. coli* resistant isolates in pooled and individual animal milk

Source	No. of positive isolates (%)
From animal (mammary gland)	23 (10.64)
From household (pooled)	17 (22.9)

Total no. of samples (Animal =216, pooled =74), p=0.008)

4.6. Antimicrobial resistance genes detected in *E. coli*

Among the 42 *E. coli* isolates, 41(98%) were found to harbor at least one of the antimicrobial resistant genes including bla_{SHV}, bla_{TEM}, bla_{CTX-M} (Fig 4.5), *tet*(B) and *tet*(C) (Fig 4.6). For example, bla_{SHV} and bla_{TEM} were the most frequent genes both detected in 41 (98%) isolates, followed by the *tet*(B) detected in 73.8% of the isolates, *tet*(C) detected in 66.6% of the isolates, bla_{CTX-M} gene, detected in 7 (16.7%) isolates. Additionally, both *tet*(B) and *tet*(C) detected together in 35 (83.3%) of the isolates (Table 4.5).

Table 4.5: Distribution of antimicrobial resistance genes in raw milk

Antimicrobial agent	Resistance gene	No.of isolates detected n (%)
Tetracycline	*tet*(B)	31 (73.8)
	tet(C)	28 (66.6)
	tet(B)/*tet*(C)	35 (83.3)
Ampicillin	bla_{SHV}	41 (98)
	bla_{TEM}	41 (98)
Cefotaxime	bla_{CTX-M}	7 (16.7)

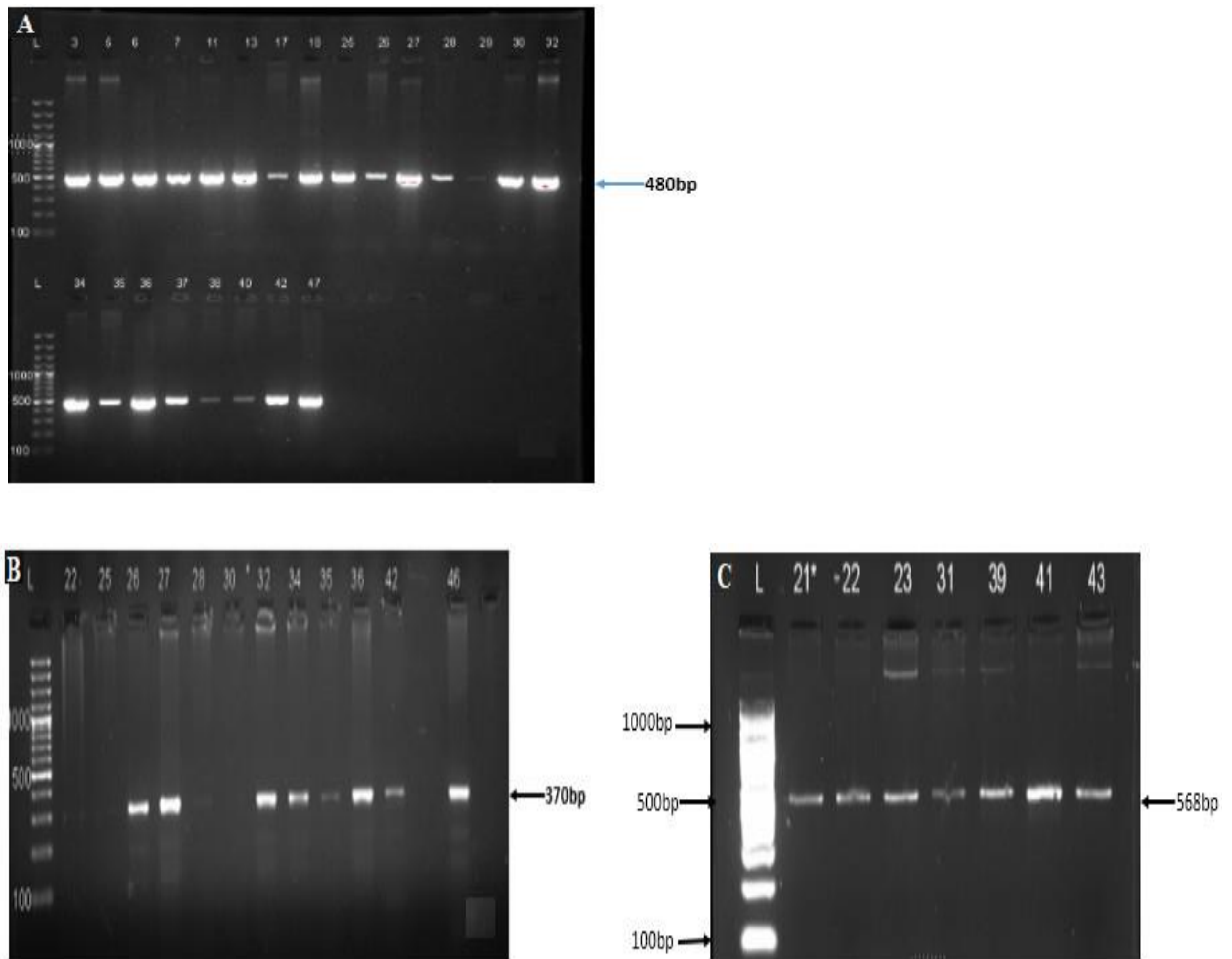

Figure 4.5 : Gel images of PCR-amplified TEM, CTX-M and SHV genes of suspect STEC.

(A) TEM gene yielding bands of approximately 480bp (B) CTX-M gene yielding bands of approximately 370bp, (C) SHV gene yielding bands of approximately 568bp. Lane L is the DNA Ladder (100bp), the numbered lanes are the test samples, Lane P is the positive control, Lanes with no bands are samples negative for the target genes and the lanes with bands show presence of TEM, CTX-M and SHV genes. The arrow shows the positions of amplified genes.

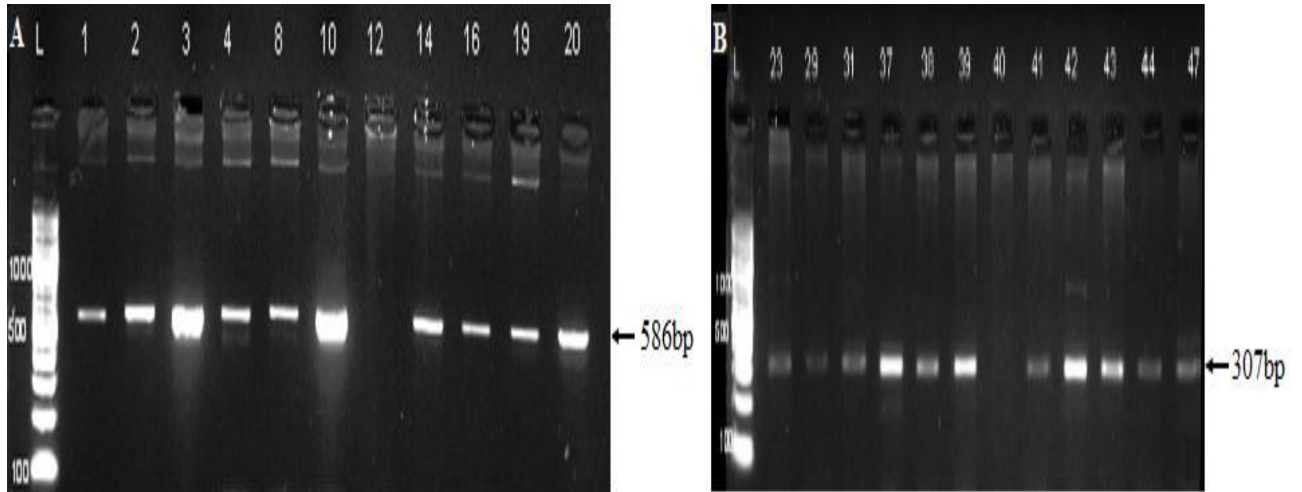

Figure 4.6 : Gel images of PCR-amplified TetB and TetC genes of *E. coli* isolates

(A) TetB gene yielding bands of approximately 586bp (B) TetC gene yielding bands of approximately 307bp, Lane L is the DNA Ladder (100bp), numbered lanes are the test samples. The lanes with no bands are samples negative for TetB and TetC.

4.7. Sequenced PCR products confirm the presence of AMR genes

Representative samples for bla$_{SHV}$, bla$_{TEM}$, bla$_{CTX-M}$, *tet*(B) and *tet*(C) genes were sequenced. BLASTn analysis showed that the sequences were homologous to bla$_{SHV}$ and bla$_{TEM}$ genes of *E. coli* and *Klebsiella pneumonia* available in the GenBank of the NCBI. The sequence identities for these homologous isolates were 100%. Blastx analysis also revealed that the translated amino acid sequence were homologous to SHV1 and TEM proteins revealing a 100% sequence identity (Figures 4.7 and 4.8). The SHV sequences are available in GenBank database with accession numbers MH744737 and MH818217. The detection of bla$_{SHV}$ gene and the associated SHV proteins indicate that the isolates are ESBL carriers. Furthermore, Blastx analysis of *tet*(B) and *tet*(C) genes also revealed translated amino acid sequence to TetA/B/C tetracycline efflux of *E. coli* with 100% amino acid identities (Figure 4.9A and B)

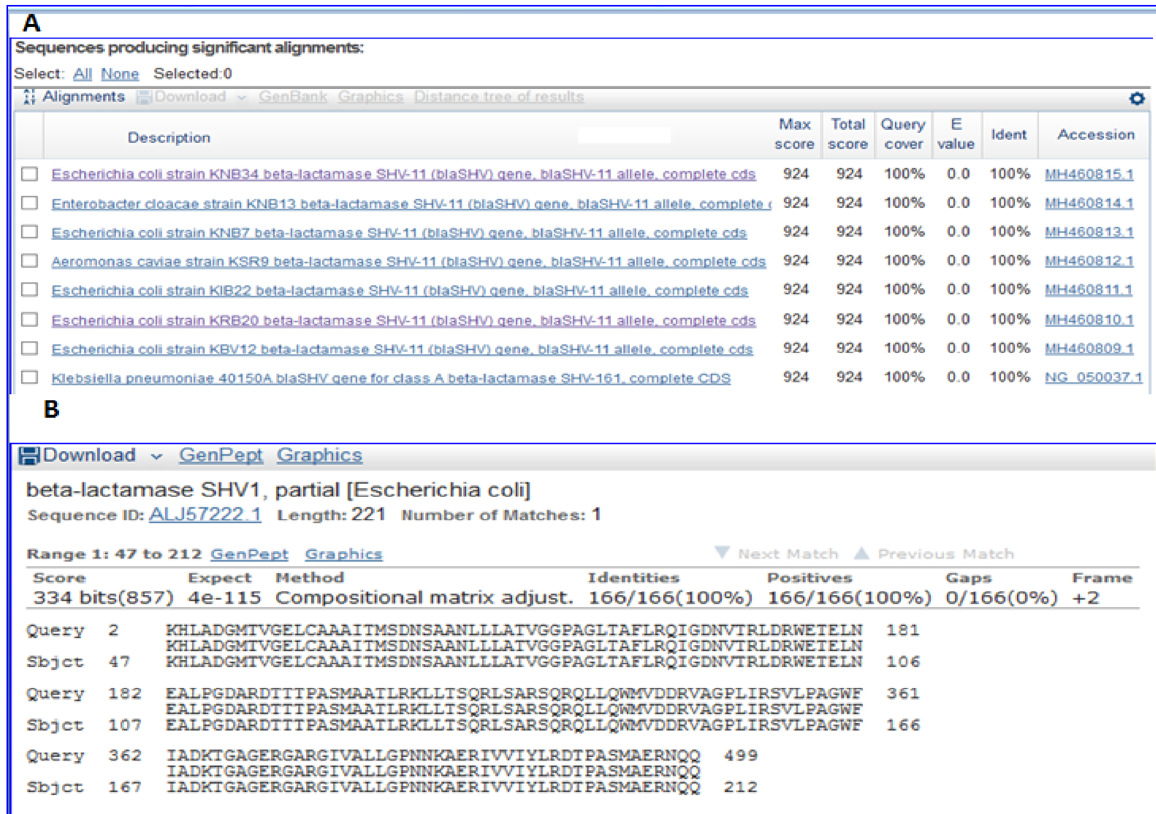

Figure 4.7 : BLASTn and BLASTx analysis of the sequenced SHV gene.

(A) BLASTn showing nucleotide identities of 100% to the sequenced amplicons (B) BLASTx result showing amino acid sequences of the homologue in the GenBank aligned with sequences obtained in this study.

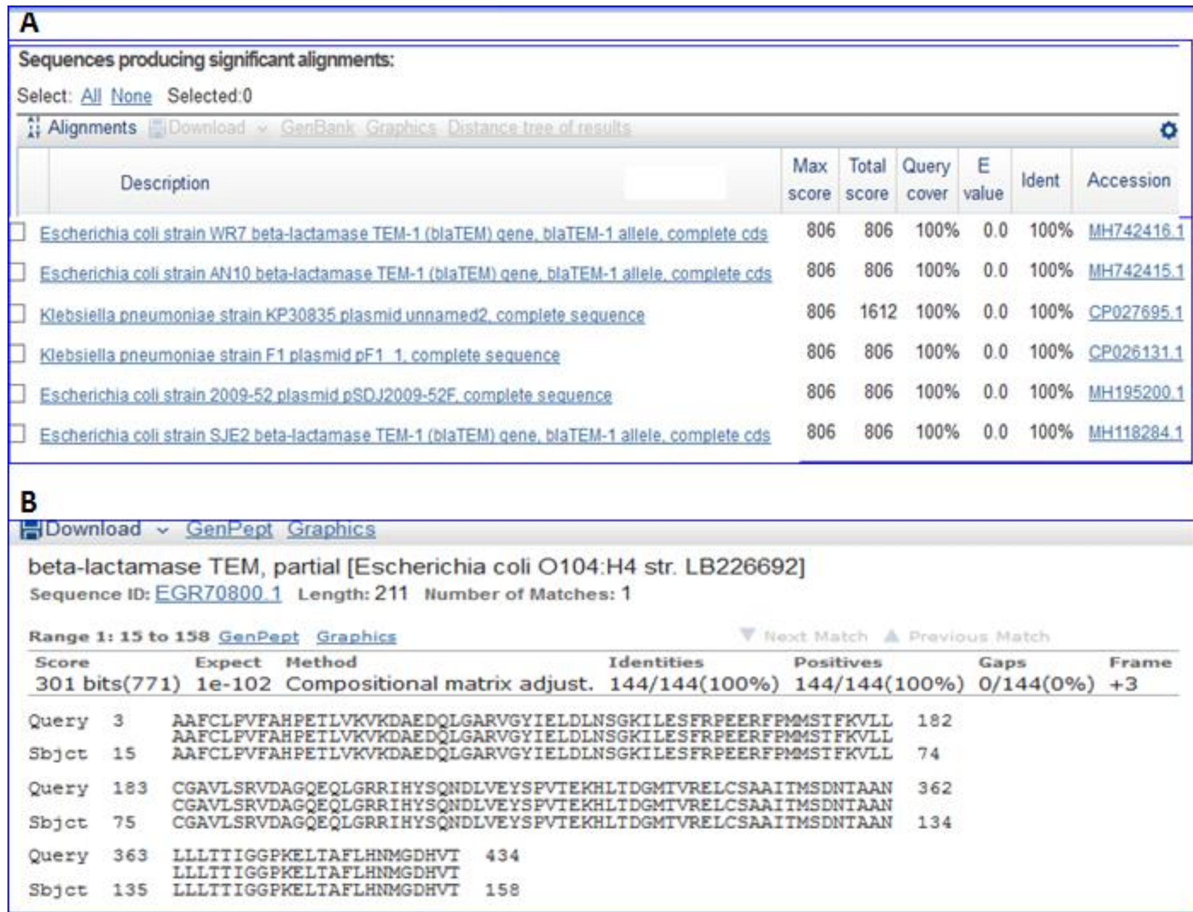

Figure 4.8: BLASTn and BLASTx analysis of the sequenced TEM gene.

(A) BLASTn showing nucleotide identities of 100% to the sequenced amplicons (B) BLASTx results showing amino acid sequences of the homologue in the GenBank aligned with sequences obtained in this study.

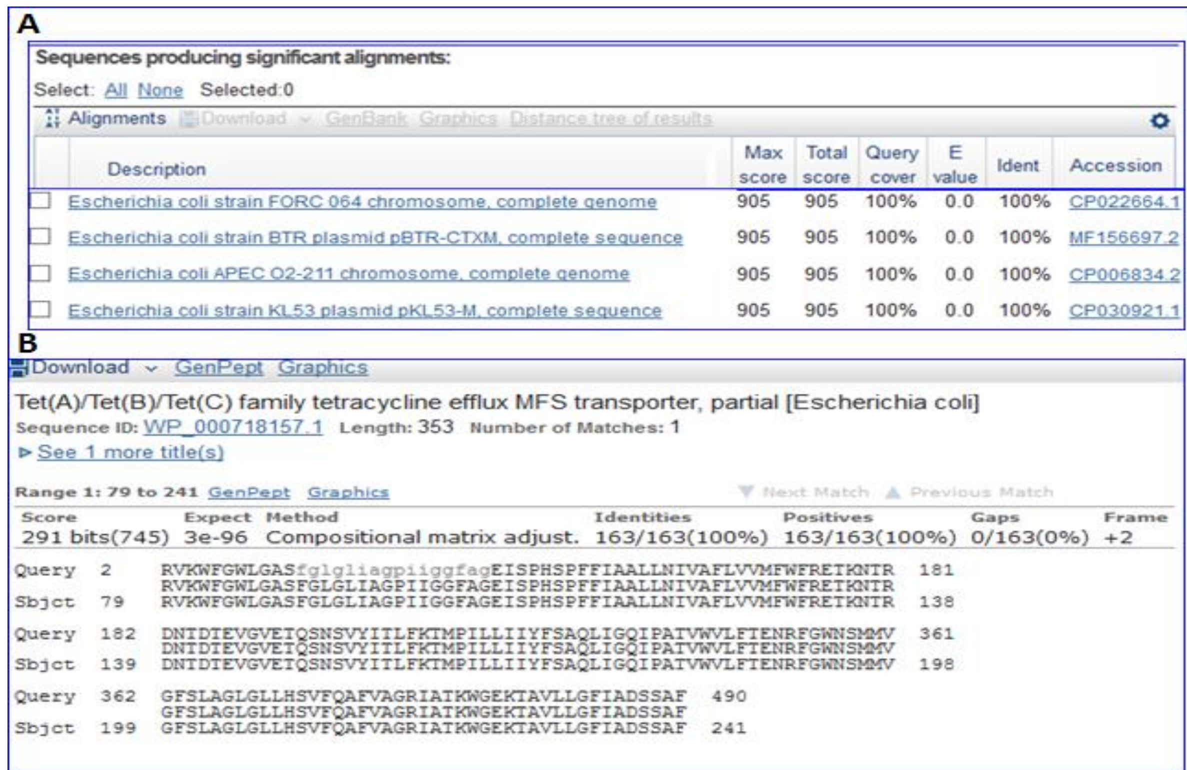

Figure 4.9 : BLASTn and BLASTx analysis of the sequenced Tet genes.

(A) BLASTn showing nucleotide identities of 100% to the sequenced amplicons (B) BLASTx result showing amino acid sequences of the homologue in the GenBank aligned with sequences obtained in this study.

CHAPTER FIVE

5.0 DISCUSSION

The emergence of antibiotic-resistant *E. coli* strains endangers the efficacy of most conventional antibiotics. The impact is more severe if the strains contaminate food products or food such as milk. Milk is one of the nutritious meals that is mostly consumed by the Kenyan population but also serves as a suitable medium for growth of numerous microorganisms (Wanjohi et al., 2013). Within the intestine of animals, *E. coli* exists as a normal flora. However, the recovery of *E. coli* in milk or dairy products indicate possible risk of contamination by other pathogenic strains such as *E. coli* O157:H7. The presence of pathogenic strains present challenges that may range from gastrointestinal disturbances to life threating conditions, and the state may worsen in cases where resistance to antibiotics is noted.

In this study, *E. coli* was isolated phenotypically on EMBA and SMAC medium, and identified through biochemical tests, PCR and sequencing. Phenotypic identification on EMBA revealed 42 (13.8%) of the isolates including 19(8.8%) from household and 23(31.08%) from individual animal with metallic green sheen colonies on the culture medium. The noted change in color on EMBA from brown to metallic green sheen showed that the isolates were *E. coli*. Furthermore, phenotypic identification on SMAC revealed 41 (13.4%) of the isolates with pink colonies indicating non O157:H7 STEC growth, and 1 (0.3%) isolate with pale/colorless suggesting EHEC O157:H7 presence. Also, the biochemical tests indicated that all the 42 isolates were *E. coli*. One of the isolate suspected to be EHEC O157:H7 was further confirmed by growth on Blood Agar culture medium, followed by serotyping using O157 and H7 antisera. However, the isolate did not react with either the O157 or H7 antisera, thus confirming its absence. The result is similar to other findings, which documented absence of EHEC O157:H7 in raw milk (Sancak et al., 2015). In addition, more accurate molecular techniques including PCR and gene

sequencing were used to confirm the presence of *E. coli* using ltg gene. Although lytic transglycosylase are known to be ubiquitous among bacteria (Dik et al., 2017), *ltg* gene accurately identified the isolates as *E. coli.*

Other studies have reported varying prevalences of *E. coli* in milk ranging from 7.1% (Messele et al., 2019) to 76.4% (Ombarak et al., 2016). This variation may be due to differences in geographical location, milking technique and culture method. Although poor hygiene in dairies and animal kraals can increase the risk of coliform mastitis resulting in the production of contaminated milk in lactating animals, this study could not conclusively confirm this claim. Again, contamination at the time of milking or storage could not be ruled out since the study revealed that pooled samples had higher *E. coli* contamination ($p = 0.0002$) than those obtained directly from lactating animals. Improving the hygienic conditions of the milking environment and/or utensils used for storage would decrease the prevalence of *E. coli* in milk.

The lack of detection of EHEC O157:H7 in this study should not be overinterpreted. Most researchers test only a few *E. coli* colonies on agar plates. In addition, EHEC O157:H7 isolates may be overshadowed by abundant commensal *E. coli*. In other countries, such as Nigeria, Egypt, Mexico and Ethiopia, EHEC O157:H7 have been detected in fresh milk and unpasteurized cheese (Mohammadi et al., 2013; Ivbade et al., 2014; Ombarak et al., 2016; Disassa et al., 2017; Bedasa et al., 2018).

A number of the *E. coli* isolates carried *stx*1, *stx*2 and *eae* genes with 95.7% of the isolates harboring *stx*2 gene. Previous studies have documented varying prevalence rates of *E. coli* harboring *stx* and *eae* genes in raw milk in different countries including Iran (Mohammadi et al., 2013), Romania (Tabaran et al., 2017), Egypt (Ombarak et al., 2016), Nigeria (Ivbade et al., 2014) and England (Byrne et al., 2014).

Antimicrobial resistance profiling showed that almost all (95%) *E. coli* isolates were resistant to at least one of the antibiotics tested, with only 2 isolates being susceptible to all the antibiotics. This is in agreement with a previous study done in Arusha city, which found resistant isolates in 87.5% of milk samples collected from zero grazed cows (Sudda et al., 2016). Other studies conducted in other countries further reaffirmed this finding (Hoang et al., 2017; Ranjbar et al., 2018). Further, isolates from pooled milk samples had significantly higher (p=0.008) rates of resistance compared to isolates from individual milk samples. Among the 40 resistant *E. coli* isolates, most of them were resistant to tetracycline, followed in a descending order by ampicillin and streptomycin. The trend of resistance to the three antibiotics (tetracycline, ampicillin, and streptomycin) has been documented in Egypt in *E. coli* recovered from cheese and raw milk (Ombarak et al., 2018).

Data on antimicrobial usage revealed that tetracyclines (65.5%), penicillins (15.6%) and aminoglycosides (16%) were the antibiotics of choice by livestock producers in the study area. This trend of antimicrobial usage explains the observed resistance pattern within this study. All the isolates were susceptible to ciprofloxacin and nalidixic acid, a result that is contrary to other research which has noted some degree of resistance to the two antibiotics (Ombarak et al., 2018). From the 40 resistant isolates, 14.28% were classified as multidrug resistant (MDR). They exhibited resistance to tetracycline, ampicillin and cefotaxime/chloramphenicol. The MDR results indicate that, with time, the antibiotics being used in the treatment of livestock will be less effective or totally unsuitable (Van Boeckel et al., 2015). Some of the factors that may have led to the development of this type of resistance pattern may include long-term exposure of livestock to various kinds of antibiotics, antibiotic concentration frequently used, type of antibiotics being used and the immune status of livestock being treated. Exposure of livestock

to low doses and over prolonged period are some of the major contributors to the emergence of resistance (Van Boeckel et al., 2015). The AMR *E. coli* isolates harbored genes conferring resistance phenotypes to tetracycline, ampicillin and third generation cephalosporin. From the study results, there is a clear indication that strains producing beta lactamase are predominant in raw milk in Northern Kenya. However, among the beta lactamase producing strains, those with bla_{SHV} and bla_{TEM} genes were the most prevalent (98%) as compared to 17% isolates found harboring bla_{CTX-M} gene. High occurrence of *E. coli* harboring bla_{TEM} genes in milk has also been reported by Ombarak et al. (2018) in which the gene was detected in 40 out of 42 ampicillin-resistant isolates as compared to other broad spectrum beta lactamases.

The presence of isolates carrying ESBL reaffirms the ability of these strains to have broad resistance to monobactams (aztreonam), penicillins and even third or fourth generation antibiotics such as cephalosporins (Rawat & Nair, 2010). Given that ESBL is plasmid mediated, there is a higher risk of resistance being transferred to other strains through a plasmid mediated mechanism (Chirila et al., 2017). More research is required to establish the source of AMR bacteria observed in the study. The areas used were in remote locations that were not connected with urban or health facilities, which are thought to harbor such isolates. Pastoralists in the area, however, use a lot of antibiotics, especially tetracyclines and penicillin-streptomycin combinations, to manage a wide range of infections including tick-borne parasites and bacterial infections. While there is no evidence that this practice is linked to increased exposure of humans to AMR bacteria, it certainly selects AMR isolates in livestock, which ultimately contaminate the local environments (Kappell et al., 2015). Although the lactating animals were not infected, and may not have produced AMR *E. coli* in milk, environmental contamination is also a possible critical pathway that might contribute to the presence of the AMR bacteria in

raw milk.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

1. Raw milk consumed in Northern Kenya harbor genes with the potential to produce enterotoxin.
2. *Escherichia coli* isolates from milk were highly resistant to tetracycline (55%) and ampicillin (48%) while ciprofloxacin and nalidixic acid showed the highest susceptibility of 0% each.
3. The *E. coli* isolated from raw milk harbor resistant determinants responsible for resistant phenotypes.
4. A number of the *E. coli* isolates were multidrug resistant with potential to produce ESBLs.

6.2. Recommendations

1. Large scale study is recommended to establish the specific non O157 STEC serotypes in milk in the study area with more samples.
2. Furthermore, there is need to scale up awareness on risks of consuming raw milk in order for consumers to make informed decisions when buying milk.
3. More elaborate studies are required to confirm the role of environmental contamination as a source of antimicrobial resistant-*E. coli* found in raw milk.

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APPENDICES

Appendix I: *Escherichia coli* strain MEZEC42 8, complete coding sequence downloaded from the NCBI-database.

>VCYJ01000008.1: *Escherichia coli* strain MEZEC42, complete coding sequence

Appendix II: *Escherichia coli* O157:H7 shiga toxin 1 and 2 genes complete coding sequences downloaded from the NCBI-database.

>AB071845.1:2871-3830 *Escherichia coli* O157:H7 stx2 gene, complete cds

>AB035142. *Escherichia coli* genes for Shiga toxin1, complete cds

Appendix III: *Escherichia coli eae* gene downloaded from the NCBI-database.

>Z11541.1: *Escherichia coli eae* gene protein

Appendix IV: *Escherichia coli* bla$_{TEM}$, bla$_{CTX\text{-}M}$ and bla$_{SHV}$ genes complete coding sequences downloaded from the NCBI-database.

>AB201242.1:215-1075 *Escherichia coli* bla$_{TEM\text{-}1}$ gene, complete cds

>KM211691.1 *Escherichia coli* bla$_{CTX\text{-}M}$ gene, complete cds

>NG_049989.1: *Escherichia coli* bla$_{SHV}$ gene, complete cds

Appendix V: *Escherichia coli tet*B and *tet*C, genomic sequences downloaded from the NCBI-database.

>HQ018801.1: *Escherichia coli* O157:H7 *tet*B gene, genomic sequence

>HQ018801.1: *Escherichia coli* O157:H7 *tet*C, genomic sequence

Appendix VI: CLSI Zone diameter interpretative standards for antimicrobial resistance for Enterobacteriaceae

Antibiotics	Resistance(mm)	Intermediate(mm)	Susceptible
Tetracycline (30 μg)	≤ 11	(12–14)	≥ 15
Gentamycin (10 μg)	≤ 12	(13–14)	≥ 15
Kanamycin (30 μg)	≤ 13	(14–17)	≥ 18
Sulphamethazole/trimethoprim (25	≤ 10	(11–15)	≥ 16
Chloramphenicol (30 μg)	≤ 12	(13–17)	≥ 18
Streptomycin (10 μg)	≤ 11	(12–14)	≥ 15
Amoxicillin-clavulanate (20/10	≤ 13	(14–17)	≥ 18
Ampicillin (10 μg)	≤ 13	(14–16)	≥ 17
Cefotaxime (30 μg)	≤ 22	(23–25)	≥ 26
Ceftazidime (30 μg)	≤ 17	(18–20)	≥ 21
Cephalexin (30 μg)	≤ 14	-	≥ 15
Ciprofloxacin (5 μg)	≤ 15	(16–20)	≥ 21
Erythromycin (15 μg)	≤ 13	(14–17)	≥ 18
Nalidixic acid (30 μg)	≤ 13	(14–18)	≥ 19

Appendix VII: Accession numbers of nucleotide sequences of milk-borne *E. coli* submitted to NCBI Gen-Bank

>MH818568-MH818570 and MH818570 for ltg

> MH744737 and MH818217 for SHV

Appendix VIII: A Questionnaire on animal diseases encountered, type and frequency of treatment and antibiotics used in disease control in Isiolo County, Kenya.

How do you handle milk in the farms in terms of:-

Collection…………………………………..……………………………………………….

Transportation……………………………..……………………………………………..

Marketing………………………….……………………………………………………..

How is the milk consumed………………………………………………………………..

Raw……………………………………….…..Boiled…………….………………………..

What are the common livestock diseases encountered in your farm……………………………...

What are the common drugs used to treat animals in your farm ………………………………….

Do you consult any field veterinarians when your animals are unwell? YES/NO………………………………………………………………………………….....

If yes, what is the level of training of field veterinarians?

Veterinary surgeon……………………………...Artificial insemination experts………………..

Paraprofessionals………………………………..Quacks……………………….…………...…….

What dosage of the drugs do you use in various species and ages of animals?

Cattle………………………………………Goats………………………………………………

Sheep…………………………….…………Camels………...………………..…………………

What is the average duration of treatment………………………………………………………..

How many animals are you milking?

Cattle………………………………..……………………….………………………………...

Goats…………………………………………………………...…………………………...

Sheep……………………………………………………………………………..………………

Camels…………………………………………………………………………………………

Have you had any cases of mastitis………………………………………..…………………...

How do you manage mastitis……………………………………………….……………………

What is the efficiency of reporting cases of mastitis and other notifiable diseases contributing to incidences of mastitis to the Livestock Department offices?..

Have the milking animals been treated in the last three months? YES/NO If yes, what medicines were they given? ....

Do you observe any withdrawal period after administering drugs to the milking animals? YES/NO………………………………………………………………………………….

If yes how long is the withdrawal period…………………………………………………………

Do you boil your milk before consumption? YES/NO If yes, How do you do it…………………

Have you ever had a health problem related to milk consumption? YES/NO If yes, what where the symptoms/clinical signs?...

Did you seek medical attention……………………………………………………………...