PREVALENCE AND DETECTION OF VIRULENCE GENES OF SHIGATOXIGENIC *ESCHERICHIA COLI* SEROTYPES ISOLATED FROM CATTLE CARCASSES IN SLAUGHTER HOUSES IN NAIROBI, KENYA

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Prevalence and Detection of Virulence Genes of Shigatoxigenic *Escherichia coli* Serotypes Isolated From Cattle Carcasses in Slaughter Houses in Nairobi, Kenya

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DECLARATION

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

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DEDICATION

I dedicate this thesis to my first born daughter Amanda Kanja Gitonga and my Father A. N. Thirinja who encouraged me every step.

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LIST OF ABBREVIATIONS

A/E	Attaching and effacing
Bp	Base pairs
CLSI	Clinical Laboratory Standards Institute
CMR	Centre for Microbiology Research
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic*Escherichia coli*
HUS	Haemolytic Ureamic Syndrome
KEMRI	Kenya Medical Research Institute
LEE	locus of enterocyte effacement
MIC	Minimum Inhibition Concentration
mM	MilliMolar
µl	Microlitre
PAI	Pathogenicity Island

PCR	Polymerase Chain Reaction
rpm	Revolution per minute
STEC	Shiga Toxigenic *Escherichia coli*
STX	Shiga toxin
UV	Ultra violet light
WHO	World Health Organization

ABSTRACT

Food borne infections continue to exert a significant toll on human health globally and affect the ability to provide safe food. Shigatoxigenic *Esherichia coli* (STEC) typically causes an afebrile bloody colitis, and may be followed by Haemolytic Uraemic Syndrome. Most outbreaks of STEC infections have been linked to serotype *E. coli* O157:H7 strains. The pathogen excretes potent toxins called verotoxins or Shiga toxins so called because of their close resemblance and identical function to the Shiga toxin of *Shigella dysenteriae*. Human infection is documented to be associated with the consumption of contaminated meat (especially ground meat) or produce contaminated with animal manure that has not been cooked sufficiently to kill dysenteric *E. coli.* Contamination of carcasses and environment with *E. coli* from intestinal contents of cattle during slaughter is one of the most important roots of transmission to humans. The aim of this study was to determine contamination by shiga toxin producing *E. coli* of cattle carcass in licensed public abattoirs, and to assess the prevalence of virulence genes associated with these organisms. The abattoirs were selected from various regions of Nairobi which included Njiru area, Dagoretti market, Kiserian and private abattoirs including Hurlingham and Kenya Meat Commission. This was a cross-sectional laboratory based study, where a rectal swab was collected from every 10^{th} cow just before slaughter and a carcass swab sample collected immediately after removal of the hide from the hind quarters. Microbiological procedures included pre-enrichment and immunomagnectic separation, culture, biotyping and detection of virulence genes by PCR. From the 162 carcasses sampled, a total of twenty one (6.48%) Non Sorbitol Fermenting (NSF) colonies of *E. coli* O157 were isolated from cattle carcasses and a total of nineteen (5.86%) NSF colonies of *E. coli* O157 isolated from rectal content of the same cattle after Immunomagnetic separation, enrichment culturing, selective plating and biochemical tests. The majority (97.5%) of the forty *E. coli* O157 isolates were susceptible to Gentamycin, Nalidixic acid, Ceftriaxone, Ceftazidime, Ciprofloxacin and Cotrimoxazole. Resistance to Ampicilin at 47.5% was the highest prevalance while, resistance to co-amoxiclav, streptomycin and tetracycline was moderate at 25%, 12.5%

and 27.5% respectively.Using multiple PCR assays with specific primers for rfb_{O157}, $flic_{H7}$, *eae*, stx_1 and stx_2, thirty one out of 40 *E. coli* isolates studied were positive for various virulence genes. These genes were widely distributed among the isolates. Virulence genes were detected in 13 rectal swab isolates and remaining 18 were from carcass swab isolates. Only two isolatesfrom carcass swab samples contained all the five genes while only one rectal swab isolate had all five genes. Nine isolates contained the gene rfb_{O157} only and 3 contained the *eae* gene only. Two isolates contained stx_2 gene only. Virulence genes were detected in four carcass isolates, no virulence genes was detected in the rectal isolates of these same cattle suggesting- that STEC strains contaminate carcass yet they were not necessarily present in fecal matter of the same cattle. This confirms cross contamination of carcasses during the dressing of carcasses during slaughter.Though prevalence appears low; it's of significance as only very low doses are needed to cause infection. Finding of dispersed virulence genes in *E. coli* O157:H7 from cattle carcasses for slaughter strongly suggests their virulence potential as well as the fact cattle may act as reservoirs for transmission to humans. This finding is of major public health concern because when the environment and conditions are suitable, the virulence genes may link to form an entire complement capable of causing an outbreak. Necessary measures should be urgently put in place to minimize contamination of carcasses during slaughter in abattoirs and other measures including possible consideration for a vaccine development.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Escherichia coli is a Gram-negative, oxidase-negative, rod-shaped bacterium from the family *Enterobacteriaceae*. It is able to grow both aerobically and anaerobically, preferably at 37°C, and can either be nonmotile or motile, with peritrichous flagella (Croxen *et al.*, 2013). It is a non sporulating organism that is an important cause of both sporadic cases and outbreaks of diarrhea in the world posing a threat to public health globally (Araujo *et al.*, 2007). Diarrhoeagenic *E. coli* are divided into five distinct categories, or patho-types each with a distinct pathogenic scheme; enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and enterohemorrhagic E. coli (EHEC) (Ferens and Hovde, 2011). These strains are capable of eluding healthy host defenses due to several specific combinations of virulence factors. Discrimination of these serotypes during routine microbiological diagnostics is challenging and molecular methods targeting elements coding for virulence traits seem to be most reliable form of identification. (Usein *et al.*, 2009)

Most outbreaks of EHEC infections have been linked to *E. coli* O157:H7strains (Johnson *et al.*, 2006; Karmali *et al.*, 2010) which suggest that this serotype is more virulent than other serotypes (Hedican *et al.*, 2009). *Escherichia coli* O157:H7 was unknown until it was discovered in Oregon and Michigan State of USA, in 1983 (Riley *et al.*, 1983) as the

causative agent in two outbreaks of haemorrhagic colitis which were characterized by severe abdominal cramp pain with watery diarrhea, followed by grossly bloody diarrhea and little or no feces.

While STEC O157:H7 has been classified as a n adulterant in beef since 1994, the U.S. Department of Agriculture (USDA) has recently declared 6 more EHEC serogroups, i.e., O26, O45, O103, O111, O121, and O145 (also known as the "Big 6"), to be adulterants, as they are most commonly found than non-O157 STEC strains known to be associated with severe illness in humans (Johnson *et al.,* 2006).The presence of the Shiga toxin 1 or 2 gene (stx1 or stx2), typically acquired by a lambdoid bacteriophage, in an isolate of *E. coli* qualifies it as Shiga toxin-producing *E. coli* (STEC) or verocytotoxin-producing *E. coli* (VTEC) (Gyles, 2007; Serna and Boedeker, 2008; Croxen *et al.*,2013). Non-producing strains may become infected and produce shiga-like toxins after incubation with shiga toxin positive strains, just like resistant genes can be transferred among enteric pathogens in animals and humans (Mathew *et al.*, 2007).The elaboration of an oligomeric shiga toxin (Stx_1or Stx_2) contributes to the potential fatal haemorrhagic colitis and to the development of the systemic seqeulae of the disease leading to hemolytic uremic syndrome (HUS), characterized by hemolytic anemia and thrombocytopenia (Croxen *et al.*, 2013), by targeting globotriaosyl ceramide receptors in the kidney leading to eventual kidney failure in humans (Gyles, 2007; Spinale *et al.*, 2013). Despite there being over 400 STEC serotypes identified, only a subset of these have been correlated to illness in humans (Croxen *et al.*, 2013).

Escherichia coli O157:H7 is a human pathogen worldwide associated with meat products, dairy products, vegetables, and water (Magwira *et al.*, 2005).The shiga like toxin requires highly specificreceptors on the cells' surface in order to attach and enter the cell. Cattle, which do not carry these receptors, may harbor toxigenic bacteria without any ill effect andcolonize gastrointestinal tract such us rectoanal junction(Cobbold *etal.*, 2007; Chase-Topping *et al., 2007*). Ruminants such as cattle (both meat and dairy) are widely known to be major reservoirs for pathogenic STEC,

and exposure to their fecal matter represents an important source of human illness. Contaminated run off and irrigation water can also taint nearby water sources, affecting rivers, lakes, and private drinking water wells. Foods that have been involved in human illness include uncooked hamburger, sausage, raw milk and dairy products, apple cider, lettuce, spinach, and sprouts. These have been the most common source of transmitting verotoxigenic *E. coli* organisms in sporadic cases and in outbreaks of STEC infection (Chauret, 2011).Hence presence of pathogen in cattle feces is strong indication of possible disease outbreak in the area.

Prevalence in cattle has a seasonal distribution with studies in the Northern hemisphere indicating low prevalence during winter and high during summer (Vital *et al.*, 2008). Individually cattle can be transiently colonized with *E. coli* O157 for 30 to 60 days (Chase-Topping *et al.*, 2007). The source of pathogen that colonizes is not well established but studies suggest intake of feeds and water contaminated with coliforms. Others suggest insect contamination like flies (Sanderson *et al.*, 2005) attributed to high probability of infection with low doses, for instance, aninteresting study done by Besser *et al.*,2001 where they were able to induce shedding in three of four calves exposed to only 10^4 CFU of *E. coli* O157

.*E. coli* O157:H7 are present in the intestines of cattle as a component of the native microbiota and they can contaminate slaughterhouse environment and carcasses transmitting to humans during slaughter (Hale *et al.*, 2012). Studies have shown transfer of *E. coli* O157:H7 from food contacting or non-food contacting surfaces to meat products (Arthur *et al.*, 2009). In 2004 the pathogen was detected on conveyor belts in beef-processing plants (Rivera-Betancourt *et al.*, 2004). Hide removal operations also potentially constitute another very important source of cattle carcass contamination during slaughter (Arthur *et al.*, 2007). Contamination of carcass meat with *E. coli* O157:H7 can occur during dressing, primarily during the skinning, but also during the evisceration phase. Once the *E. coli* is transferred to the carcass surface, handling

and trimming operations can spread the pathogen to the beef trimmings (Chase & Topping *et al.*, 2007).

Treating *E.coli* O157 infections with antibiotics presents a challenge (Wong *et al.*, 2012).Wong *et al.* (2012) warn that treating *E. coli O157:H7* infections may result in the release of shigatoxins into the blood stream of the infected individuals resulting in hemolytic uremic syndrome. This therefore presents a great challenge in the treatment approach to be adopted in the case of *E. coli* O157:H7 infections. Antibiotic resistance may occur either spontaneously by selective pressure or because of antibiotic miss-use by humans or overuse by farmers on their beef cattle (Buvens *et al.*, 2010). Indirect intervention approaches have been used to reduce prevalence of *E. coli* O157 in feces of cattle presented for slaughter. These approaches include, feeding cattle with probiotics (intake of beneficial bacteria to compete with pathogen), vaccination and bacterophage treatment (Younts-Dahl *et al.*, 2005; Peterson *et al.*, 2007) and animal disinfecting and washing before slaughter (Arthur *et al.*, 2007).

Since there is no currently available specific treatment for hemolytic uremic syndrome, there is an urgent need for effectivepreventive measures based on a detailed understanding of the epidemiology of STEC infections. Such measures will also be dependent on the availability of rapid, sensitive, simple, and reproducible procedures for the detection of these pathogensand for the characterization of their toxins both in samples from human specimens and those of nonhuman origin such as food,water and animals (Fegan and Desmarchelier, 2002) as is the case of this study which aims to detect STEC in rectal and carcass swabs and investigate possible contamination of cattle carcasses by Shiga toxigenic *E. coli* serotypes in abattoirs in Nairobi, as potential cause of infection in humans.

1.2 Statement of the problem

Diarrheal disease is a leading cause of morbidity and mortality in developing countries. Case studies in these countries show that children below five years of age are mostly affected (Ethelberg *et al.*, 2009; Gould *et al.,* 2013). Prevalance and distribution of diarrhoeagenic *E. coli* categories and the disease burden they contribute is not well defined in Kenya and STEC is the least studied in food.

There have been many studies conducted to determine *E. coli* O157 and *E. coli* O157:H7 but in only carcass or in rectal samples at abattoirs worldwide, including Kenya. Kang'ethe *et al.* (2007) carried out a study in Dagoretti Division of Nairobi on farm cattle in the area and reported a prevalence rate of 5.2%. One of the isolated *E. coli* O157:H7 had genes encoding *VT1*. This was a significant find although he had a small sample size and only involved rectal swabs hence, further studies on a wider scope are necessary.

Identification of diarrhoeagenic *E. coli* requires that they should be differentiated from nonpathogenic strains of the normal flora. Serotypic markers are rarely sufficient by themselves reliably to identify a strain as diarrhogenic *E. coli.* Serotyping may generate false-negatives since H7 and O157 antigens may not be expressed, for instance, out of 100 meat isolates, 42 were H7-negative by latex agglutination but H7 positive by polymerase chain reaction (Narang *et al.*, 2009).

The burden of infection concerns the public health system worldwide and there is need to improve current evaluation of specific enteric pathogens in any region including developing Nations where there is paucity of epidemiological data on potential reservoirs of STEC infection and possible transmission dynamics.

Compared to serological methods, molecular methods are the sensitive and the most specific techniques for differentiating all types of the diarrhoeagenic *E. coli* from nonpathogenic stool flora, and for distinguishing one category from another depending

on virulent genes detected (Narang *et al.*, 2009). Hemolysin and protein gene *hlyA* and *eaeA*; the $rfbE_{O157}$, which encode the *E.coli* somatic antigen O157; $fliC_{H7}$, which encodes the *E. coli*structural flagella antigen H7; genes stx_1; stx_2 encoding shiga toxin production; *uidA*, which encodes ß-glucuronidase; the genes for the cytotoxic necrotizingfactors, heat-labile toxin; heat-stable toxin have all been used as markers for *E. coli* 0157:H7 (Marcon, 2011).

There is little data on prevalence of *E. coli* O157 and *E. coli* O157:H7 in both rectal and carcass samples from the same animal in Kenya. Therefore, this study was conducted to assess the presence of *E. coli* O157:H7 from both cattle carcasses and their rectal samples, detecting virulence genes in their isolates and investigating possible contamination of cattle carcasses by Shiga toxigenic *E. coli* serotypes in abattoirs in Nairobi area, as potential cause of infection in humans consuming the meat and meat products. This data is critical in risk assessment and magnitude of the reservoir status of cattle in the food chain, and informs public health policies on methods for prevention of slaughter-house contamination.

1.4 Hypothesis

Cattle for slaughter in Nairobi abattoirs do not carry pathogenic serotypes of shiga toxigenic *Escherichia coli* that contaminate carcasses.

1.5 Study objectives

1.5.1 General Objective

To determine the prevalence and virulence properties of shiga toxigenic *Escherichia coli* serotypes isolated from carcasses in Nairobi slaughterhouses.

1.5.2 Specific Objectives

1. To determine prevalence of shiga toxigenic *Escherichia coli* among rectal content and carcasses surfaces slaughtered in Nairobi abattoirs.
2. To determine antimicrobial susceptibility of shiga toxigenic *Escherichia coli* and other serotypes isolated from rectal content and carcasses swabs at different slaughter houses in Nairobi.
3. To detect occurrence of virulence genes among isolated shiga toxigenic *Escherichia coli* serotypes from carcasses in different Nairobi abattoirs.

CHAPTER TWO

LITERATURE REVIEW

2.1 Escherichia coli pathogenic strains.

After initial description of *Escherichia coli* by Escherich in 1886, it became clear that distinctive *E. coli* isolates were frequently found in clinical specimens of patients with specific clinical syndrome. Most *E. coli* isolates are intestinal commensals but some have highly adapted *E. coli* clones which have evolved and have the ability to cause a spectrum of diseases in robust humans (Cohen *et al.*, 2005; Araujo *et al.*, 2007).

Pathogenic strains of *E. coli* differ from commensals in that they express virulence factors, molecules directly involved in pathogenesis but ancillary to normal metabolic functions (Croxen & Finlay, 2010). These factors disrupt normal host physiology and thereby cause disease. Diarrhoeagenic *E. coli* includes emerging pathogens of worldwide public health importance. These pathogenic strains are categorized into at least six distinct groups, these are: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffuse-adhering *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), and enterohemorrhagic *E. coli* (EHEC) (Croxen & Finlay, 2010).

Enteropathogenic *Escherichia coli* (EPEC) was the first pathotype of *E. coli* to be described. Although large out- breaks of infant diarrhea due to EPEC have largely disappeared from industrialized countries, EPEC remains an important cause of potentially fatal infant diarrhea in developing countries (Scaletsky *et al.*, 2010). A

characteristic histopathology is associated with EPEC infections; known as ‘attaching and effacing’ (A/E), the bacteria intimately attach to intestinal epithelial cells and cause striking cytoskeleton changes, including the accumulation of polymerized actin directly

beneath the adherent bacteria. The microvillus of the intestine is effaced and pedestal-like structures on which the bacteria perch frequently rise up from the epithelial cell.

Enterotoxigenic *Escherichia coli* (ETEC) causes watery diarrhea, which can range from mild, self-limiting disease to severe purging disease. The organism is the main cause of diarrhea in travelers to developing countries (Gupta *et al.*, 2008). ETEC colonizes the surface of the small bowel mucosa and elaborates enterotoxins, which give rise to intestinal secretion. Colonization is mediated by one or more proteinaceous fimbrial or fibrillar colonization factors (CFs), which are designated by CFA (colonization factor antigen), CS (coli surface antigen) or PCF (putative colonization factor) followed by a number. ETEC enterotoxins belong to one of two groups: the heat-labile enterotoxins (LTs) and the heat-stable enterotoxins (STs). ETEC strains might express only an LT, only an ST, or both LTs and STs.

Enteroaggregative *Escherichia coli* (EAEC) are defined as *E. coli* that do not secrete LT or ST and that adhere to HEp-2 cells in a pattern known as auto- aggregative, in which bacteria adhere to each other in a 'stacked-brick' configuration (Okhuysen &Dupont, 2010).

Enteroinvasive *Escherichia coli* (EIEC) are biochemically, genetically and pathogenically closely related to *Shigella* spp. Numerous studies have shown that Shigella and *E. coli* are taxonomically indistinguishable at the species level (Yang *et al.*, 2009), but, owing to the clinical significance of Shigella, a nomenclature distinction is still maintained. EIEC might cause an invasive inflammatory colitis, and occasionally dysentery, but in most cases EIEC elicits watery diarrhea that is indistinguishable from that due to infection by other *E. coli* pathogens (Okeke, 2010).

Diffusely adherent *Escherichia coli* (DAEC) is defined by the presence of a characteristic, diffuse pattern of adherence to HEp-2 cell monolayers. DAEC have been implicated as a cause of diarrhea in several studies, particularly in children >12 months

of age (Snelling *et al.,* 2009). Approximately 75% of DAEC strains produce a fimbrial adhesin called F1845 or a related adhesin F1845 belongs to the Dr family of adhesins, which use DAF, a cell-surface gly- cosylphosphatidylinositol-anchored protein, which normally protects cells from damage by the complement system, as the receptor(Guignot *et al.,* 2009). DAEC strains induce a cytopathic effect that is characterized by the development of long cellular extensions, which wrap around the adherent bacteria (Guignot *et al.*,2009).

2.2 Enterohemorrhagic *Escherichia coli*

Enterohemorrhagic *Escherichia coli* (EHEC) cause hemorrhagic colitis and are often associated with devastating or life-threatening systemic manifestations. The most severe sequelae, the hemolytic uremic syndrome (HUS), results from shiga toxins (Stxs) produced by the bacteria in the intestine and act systemically on sensitive cells in the kidneys, brain, and other organs (Gyles, 2007). Although most EHEC strains produce Stxs, *E.coli* O157:H7 are especially virulent and are responsible for the majority of HUS

cases of bacterial etiology worldwide (Gyles, 2007; Serna and Boedeker, 2008). It is well documented that the *E. coli* strains of the O157:H7 serotype differ widely in their ability to cause human disease, colonize animal carriers, and survive in the environment (Croxen *et al*., 2013)

2.2.1 Enterohemorrhagic *Escherichia coli* in Humans

The principal illness caused by EHEC is hemorrhagic colitis, which is characterized by acute abdominal cramps and bloody diarrhea, and may progress to life-threatening clinical symptoms such as hemolytic uremic syndrome (HUS) (Mayer*et al.,* 2012). Haemolytic ureamic syndrome (HUS), is defined as the triad of acute renal failure, thrombocytopenia, and microangiopathic haemolytic anaemia (Johnson *et al*., 2006).

Ruminants are not sensitive to shiga toxins (Stxs) due to an absence of vascular Stx receptors (Scheutz*et al.*, 2012). The *Stx* genes are not necessarily associated with morbidity, and STEC may be carried asymptomatically by humans (Wong *et al.*, 2012).*E. coli* O157 infection and HUS are largely pediatric illnesses, although they can occur at any age. A recent 6-year review of active and passive surveillance data collected from multiple sites in the United States showed that the highest incidence of both *E. coli* O157 infection and HUS occurred among children of <5 years old (Gould *et al.*, 2009).

While the attention devoted to EHEC O157:H7 is justified by the pathogenicity, low infectious dose, and ability of the bacteria to survive in extra-intestinal environments, a number of non-O157:H7 EHEC cause severe human disease and are often implicated in HUS, and their animal reservoirs and modes of transmission are not well understood (Karch *et al.*, 2005). Almost half (47%) of 424 HUS isolates collected in Germany from 1996 to 2003 were of a canonical O157:H7 serotype, 17% were sorbitol-fermenting O157: NM, and the rest were O26:H11/NM, O145:H25/H28/NM, O111:H8/NM, O103:H2, and others (Karch *et al.*, 2005; Croxen *et al.*, 2013). Non-EHEC STEC can be pathogenic to humans with the disease severity highly dependent on serotype and a combination of virulence factors (reviewed in Gyles, 2007; Hussein, 2007), Non-O157 STEC were found in 5.6% (90/1602) of healthy workers at a slaughter company in Korea (Hong *et al.*, 2009) and O157 STEC in 1.1% (4/350) of farm workers in Italy (Silvestro *et al.*, 2004). EHEC lack the biochemical characteristics differentiating them from nonpathogenic *E. coli*, and thus present a special detection challenge and are perhaps insufficiently investigated. However, non-O157 STEC pathogens appear to be less virulent than the O157 serotype. Some studies however suggest that non-O157 STEC infection causes milder disease than O157 infection (Hedican *et al.*, 2009).

Incubation period before infection is symptomatic and takes three to eight days. HUS outbreaks report a distinctive gastrointestinal illness characterized by severe abdominal colic, initially watery diarrhea followed by grossly bloody diarrhea with little or no fever (Wong *et al.*, 2012). The diarrhea may range from mild and nonbloody to bloody stools with no leukocytes. Biopsies of the colon from many patients also show focal necrosis and neutrophil infiltration. The overall pattern resembles a combination of ischaemic and infection injuries similar to those described in toxin-mediated *Clostridium difficile*-associated colitis and pseudo membranous pathologies are seen in many patients (Johnson *et al.*, 2006).

2.2.2 Identification and detection of *Escherichia coli* O157:H7

Regional clinical laboratory routinelytests all stool cultures for *E. coli* O157, using its inabilityto ferment sorbitol on sorbitol-MacConkey (SMAC) agar, a medium where sorbitol is substituted (Croxen *et al.*, 2013) for the lactose in thestandard MacConkey formulation however, *E. coli*O157 infections may be missed on SMAC because of strains that fermentsorbitol and cannot be differentiated from normal intestinal flora (Hermos *et al.*, 2011). Almost all EHEC O157 display delayed (negative) fermentation of D-sorbitol with >99% (Croxen *et al.*, 2013).

The bacteria are commonly identified by plating on sorbitol MacConkey agar supplemented with cefixime and potassium tellurite (CT-SMAC) (Bielaszewska *et al.*, 2011) and confirmed serologically and biochemically as pale colonies positive for O157 and H7. Sorbitol MacConkey agar may be supplemented with 4-methylumbelliferyl-beta-d-glucuronide that is converted by most non-O157:H7 *E. coli* to UV-fluorescing 4-methylumbelliferone, or with light-visible 8-hydroxyquinoline-beta-d-glucuronide (Ferens and Hovde, 2011). On this medium, EHEC O157:H7 appear pale and nonfluorescent under UV light. Selective plating will occasionally generate false-positives and false-negatives because rare O157:H7 isolates may be sorbitol-fermenting and glucuronidase positive (Sanchez *et al.*, 2010).

Identification of diarrhoeagenic *E. coli* requires that they should be differentiated from nonpathogenic strains of the normal flora. Serotypic markers are rarely sufficient by themselves reliably to identify a strain as diarrhogenic *E. coli.* Serotyping may generate false-negatives since H7 and O157 antigens may not be expressed, for instance, out of 100 meat isolates, 42 were H7-negative by latex agglutination but H7 positive by polymerase chain reaction (Narang *et al.*, 2009). An O157-negative rough phenotype can be identified by phage testing (Rozand and Feng, 2009; Rump *et al.*, 2010). *Escherichia coli* serotypespossesses a typical lipopolysaccharide (LPS) outer membrane structure which features repeating O antigen polysaccharide polymers expressed on the surface of the bacterium (DebRoy *et al.*, 2011).

Strains of serotype O157: H^- (nonmotile) which do ferment sorbitol rapidly have emerged asimportant causes of human diseases duringthe past decade.Such strains are missed by diagnostic procedures recommended forthe detection of *E. coli* O157:H7 and their significance in otherparts of the world might thus be underestimated (Bielaszewska *et al.*, 2007); further studies on this should be encouraged. In some parts of the world, especially in Europe, sorbitol-fermenting nonmotile isolates of O157: H- emerged, characterized by a higher incidence of progression to HUS than canonical O157:H7 (Buvens *et al.*, 2010; Orth *et al.*, 2009) however, serotype O157:H7, which serves as a marker for EHEC is usually enough for its identification routinely.

Direct plating on CT-SMAC can enumerate viable EHEC O157 at densities >100 colony forming units (CFU)/g sample but may fail to detect highly stressed bacteria unable to survive in a selective medium .The detection limit can be lowered by enrichment, that is, incubation in broth before plating and/or concentration by immunomagnetic separation (IMS) with anti-O157 beads (Zhu *et al.*, 2011), but recovery is highly dependent on technique and still likely to underestimate a true prevalence of *E. coli* O157:H7 (Vidovic *et al.*, 2007). Low prevalence or EHEC O157 may reflect scarcity of the bacteria, but can also result from subjecting samples to exacting storage and culture conditions. For example, a year-long survey of two feedlots in Alberta yielded surprisingly few (namely,

2) isolates of EHEC O157:H7 by direct plating swabs transported in a chilled selective medium onto CT-SMAC (Bielaszewska *et al.*, 2007). A more recent prevalence survey of 21 Alberta feedlots employed nonselective overnight enrichment culture at 42°C followed by IMS, and revealed the presence of *E. coli* O157 in up to 15% of cattle and 57% of pens (Van Donkersgoed *et al.*, 2009).

Molecular methods are the sensitive and the most specific techniques for differentiating all of the diarrhoeagenic *E. coli* from nonpathogenic stool flora, and for distinguishing one category from another depending on virulent genes detected (Narang *et al.*, 2009). Hemolysin and protein gene *hlyA* and *eaeA*; the $rfbE_{\mathbf{O157}}$, which encode the *E.coli* somatic antigen O157; $fliC_{\mathrm{H7}}$, which encodes the *E. coli*structural flagella antigen H7; genes stx_1; stx_2 encoding shiga toxinproduction; *uidA*, which encodes ß-glucuronidase;the genes for the cytotoxic necrotizingfactors, heat-labile toxin; heat-stable toxin have all been used as markers for *E. coli* 0157:H7 (Torres *et al.*, 2009).

Isolates of EHEC are commonly matched by patterns generated by pulsed-field gel electrophoresis (PFGE) of genomic DNA digests. The resulting multitude of genetic patterns may be misleading, as shown by the results of oral challenge of cattle with a single strain of the EHEC O157 that yielded 12 different PFGE profiles resulting from five chromosomal deletions (Yoshii *et al.*, 2009). In general, genetic analysis of EHEC O157:H7 relatedness is hampered by a high degree of variation that may reflect widespread genetic drift of unclear practical significance. For example, highly related lineages and subpopulations of the pathogen exhibit varied expression of genes of the locus of enterocyte effacement due to genomic instability of a prophage-dense region adjacent to the *pch* sequences (Yang *et al.*, ***2009***). Also, nonparental PFGE patterns of <89% similarity to cognate strain were observed among randomly selected progeny of a single colony of *E. coli* O157: NM (Bielaszewska *et al.*, 2007).

2.2.3 Epidemiology of *Escherichia coli* O157:H7

Escherichia coli O157:H7 has been isolated from many sporadic cases of diarrhea, bloody diarrhea, and HUS, outbreaks in Africa (Arimi *et al.*, 2005). Available information indicates that the pathogen has wide geographic distribution. *Escherichia coli* O157-related diarrhea outbreaks that occurred before 2003 have been reported in South Africa, Swaziland, and Malawi, Kenya; Nigeria and Ivory Coast (Koyange *et al.*, 2004). In the Central African Republic in Zemio, a small village located on the Democratic Republic of Congo border, outbreaks of bloody diarrhea in 1996 were attributed to *E. coli* O157 from molecular test results. In 1996 in the Central African Republic and in 1998 in Cameroon, the major contributing factors of the *E. coli O157* outbreak were consumption of smoked *zebu*meat (Koyange *et al.*, 2004).

Results of sampling for the presence of EHEC O157:H7 are paradoxical: the bacteria occur worldwide, but in many prevalence studies are found infrequently. The reasons for this discrepancy are complex, and may reflect either scarcity of the bacteria in excreta and environment resulting from the sporadic nature of EHEC O157 carriage and low numbers of the bacteria residing in colonized animals, or insufficiently sensitive sampling and culturing approaches. For example, nonenrichment testing of 425 calves in Argentina yielded only two isolates of *E. coli* O157:H7 (Ferens and Hovde, 2011), whereas another study of pre-slaughter cattle using enrichment found EHEC O157:H7 in 4.1% of fecal samples, a result congruent with a high incidence of HUS in the country (Masana *et al.*, 2010). In Norway, only <0.1% of cattle, sheep, and pigs from >800 farms carried *E.coli* O157:H7 detected by selective enrichment (Ferens and Hovde, 2011), but 4.6% (23/504) of cattle imported to the country tested positive with nonselective enrichment (Ferens and Hovde, 2011). Another example is where a farm prevalence of EHEC O157 was 18% (88/481) in Scotland in a study employing IMS and a rigorous sampling pattern (Chase-Topping *et al.*, 2007). However, some studies yielded very low prevalence data despite using enrichment and IMS. A two-country study found *E.coli* O157 in 4/50 farms in Ohio (5/750 animals), but in 0/50 farms

($n = 680$) in Norway (LeJeune *et al.*, 2006a); in Greece, only one goat isolate of *E. coli* O157:H7 was found at 25 goat, sheep, and cattle farms (Ferens and Hovde, 2011); in Australia, 1/505 dairy cows from >200 farms harbored two EC O157 isolates, but the samples were analyzed after prolonged refrigeration (Hall *et al.*, 2008); in France, a representative survey of abattoir cattle did not find STEC O157, although 18.1% fecal samples were stx^+ by polymerase chain reaction, and STEC were found in 67/154 (Ferens and Hovde, 2011) and in 66% of patients (Espie *et al.*, 2008).

One of highest prevalence was noted in Japan, where up to a third of animals were positive for the pathogen on 3/4 farms; in this study, large fecal samples (10 g) were subjected to 18 h nonselective enrichment culture at 42°C (Sasaki *et al.*, 2011). Among Scandinavian countries, an exceptionally high STEC O157 prevalence was noted using nonselective enrichment and IMS in Denmark, in 10/60 farms, and 21% (88/412) of tested animals (Ferens and Hovde, 2011) as well as lettuce (Ethelberg *et al.*, 2010). Hence, low prevalence results usually reflect a reduced probability of detecting EHEC O157 due to one or more aspects of methodology, including single sampling visits, small numbers of tested animals/farm, absence of comprehensive farm surveys, and selective or no enrichment applied to stored samples.

2.2.4 Reservoir and carriage of *Escherichia coli* O157:H7

Presumably, *Escherichia coli* O157:H7 attachesand replicates at a site or sites in the gastrointestinal tracts of colonized animals and results in bacteria in the feces for a long duration. Experiments with individual Holstein calves and a crossover study found that the addition of chitosan microparticles to feed decreased *Escherichia coli* O157:H7 shedding (Jeong *et al.*, 2011). The major animal carriers are healthy domesticated ruminants, primarily cattle (Gyles, 2007) and, to a lesser extent, sheep and possibly goats (La Ragione *et al.*, 2009). A study by Naylor S. in 2003 provided compelling evidence that a primary siteof *E. coli*O157:H7 colonization in cattle is the recto anal junctionof the gastrointestinal tract (Cobbold *et al.*, 2007).

A number of studies performed in different countries compared PFGE profiles and virulence factors of bovine and human isolates and concluded that the majority of bovine isolates of EHEC O157 do not occur, or are underrepresented, in people (Ferrens & Hovde, 2011). For example, a comparison of 63 bovine isolates and 86 human isolates in Denmark revealed that human isolates associated with severe disease constituted a minor fraction of the bovine strains, and that little overlap existed between the two sets in a number of phage types (Soderlund *et al.*, 2012); also, analysis of phage insertion points revealed greater genetic diversity of bovine isolates compared to human ones (Besser *et al.*, 2007). Although bovine strains of EHEC O157 share numerous virulence factors with human strains, including *stxs* and *eae*, and may be considered potential human pathogens (Soderlund *et al.*, 2012), bovine isolates generally harbor fewer virulence factors while exhibiting greater tolerance for adverse conditions (Vanaja *et al.*, 2009). This contention is supported by the finding that the isolates from healthy cattle were less virulent in gnotobiotic pigs than human clinical isolates (Baker *et al.*, 2007). Non-O157 STEC isolated from healthy cattle also harbor relatively few virulence factors; 77% of 222 STEC isolates from slaughtered cattle in France presented only one of *stx*, *eae*, or *ehx* genes, and only 3/222 presented these 3 virulence genes together (Baker *et al.*, 2007).

Carriage of clinical *E.coli* O157:H7 isolates by cattle may simply reflect a high probability of pathogen transmission from cattle to people as a consequence of the predominance of beef and dairy cattle among domesticated animals, and the voluminous output of bovine manure. Enterohemorrhagic *Escherichia coli* is a special case of zoonotic STEC infections, and there is a clear association of cattle density and the occurrence of all STEC-related gastroenteritis in humans; in Ontario, there was a correlation with cattle density, but not with presence of sheep or goats (Oporto *et al.*, 2008), and in Germany the risk increased by 68% per additional 100 cattle/km^2 (Echtenkamp *et al.*, 2008). However, studies that measured intestinal prevalence of EHEC O157 in cattle and sheep at slaughter consistently show higher prevalence in cattle. In the United Kingdom, the bacteria were found in 4.7% of cattle and 1.7% of

sheep (Launders *et al.*, 2013), in 15.7% of cattle versus 2.2% of sheep, and in 4.7% of cattle and 0.7% of sheep (Milnes *et al.*, 2009); in Holland, abattoir survey revealed *E.coli* O157 in 10.6% cattle and 4.0% sheep (Friesema *et al.*, 2009). This relationship was also true when overall prevalence of the pathogen was very low; in Norway, *E.coli* O157:H7 was found in 0.19% of cattle (3/1541) and 0/665 sheep (Aspan & Eric, 2010).

Prevalence studies of *E. coli* O157:H7 are associated with two major caveats: first, the bacteria may be harbored extra-intestinally with little correlation to fecal shedding (Boqvist *et al.,* 2009), and second, intestinal carriage is usually intermittent or short-term (Ferens and Hovde, 2011). The bacteria were much more prevalent among all age groups on the ears than in fecal samples in a survey in Sweden (Boqvist *et al.*, 2009); hide prevalence of *E.coli* O157:H7 was as high as 92% on belly swipes of feedlot cattle (Kalchayanand *et al.*, 2009). *E.coli* O157 were isolated from the oral cavity or hides of 130/139 feedlot cattle, including 50 cattle that were fecal-negative (Ferens and Hovde, 2011). Possibly, extra-intestinal prevalence is reduced by high insolation; in a survey of 13 dairy herds in Louisiana, STEC O157:H7 were found in 0%, 0.7%, and 25.2% of samples from oral cavity, skin, and feces, respectively, whereas hide prevalence was 10 times higher than fecal prevalence (60.6% vs. 5.9%) in a study performed at three Midwestern beef plants found (Ferens and Hovde, 2011).

Age-dependent carriage has been studied also where low prevalence of *E.coli* O157:H7 in calves may be related to the presence of protective antibodies in colostrum and milk, as indicated by results of some studies with colostrum-deprived animals, or to EHEC-adverse microbiota and other factors of immature GIT. For instance, in Italy, 3.6% of adult cattle but not veal calves (0/437) carried intestinal EHEC O157 at slaughter (Ferens and Hovde, 2011); One hundred and fifty-four (7.3%) of 2133 samples were positive for STEC serogroups in Nigeria. The pathogens were detected in the feces of cattle (15.2%), sheep (10.7%), goats (7.5%) and pigs (5.6%) as well as in beef (3.8%), goat-meat (1.7%) and pork (4.0%). All seven investigated STEC serogroups were found in cattle, all except O145 were found in sheep, three serogroups (O157, O26 and O111)

were found in goats and three (O157, O111 and O128) in pigs.(Ojo *et al.*, 2010). EHEC O157 were found in fresh fecal samples collected in abattoirs in Holland over 2 successive years from 10% of the adult cows (27/270), but only once each year from veal calves (1/183, 1/214) (Oporto *et al.*, 2008); Similar to cattle, in 378 weaned and 265 suckling lambs tested for *E.coli* O157 all five isolates were found in weaned lambs (Battisti *et al.*, 2006), and colostrum-deprived lambs inoculated with *E.coli* O157:H7 shed more bacteria than conventionally reared animals (La Ragione *et al.*, 2009).

Typical *Escherichia coli* O157:H7 are prevalent in bovines other than *Bos taurus*. Fecal prevalence of the bacteria in farmed bison was greater than in cattle and ranged from 17% to 83% across days (Reinstein *et al.*, 2007); high prevalence was noted in water buffalo in Bangladesh (Islam *et al.*, 2008) and 11 *E.coli* O157:H7 strains were isolated from 300 healthy water buffalo in Turkey (Seker *et al.*, 2010). Small ruminants can be a significant source of human EHEC infection (La Ragione *et al.*, 2009), and EHEC O157:H7 were identified in sheep (Lenahan *et al.*, 2007) and matched to clinical strains. In Italy, most adult sheep tested in a slaughterhouse were positive for virulent O157 STEC (Franco *et al.*, 2009); 8/13 STEC strains isolated from ovine dairy products in Mexico were O157 (Caro *et al.*, 2007); *E.coli* O157:H7 were isolated from pelts and carcasses in a lamb-processing plants (Kalchayanand *et al.*, 2009). *Escherichia coli* O157:H7 was found in 8% of beef, and 2% of lamb and goat meat samples in Ethiopia (Hiko *et al.*, 2008), and was isolated from goats in Spain (Orden *et al.*, 2008). STEC O157 was more often found in goats (9%) than in cows (7%) in Bangladesh (Islam *et al.*, 2008). Some ruminants appear remarkably free of EHEC O157, for instance, no STEC or anti-Stx antibodies were detected in fecal and serum samples from 400 camels in five east African countries (El-Sayed *et al.*, 2008).

The seasonal differences are apparent, especially in countries featuring winters. In northern Italy, up to 17% of abattoir cattle carried STEC O157 in spring and summer, but only up to 2.9% in winter (Gyles *et al.*, 2007), and in a 15-month farm survey, peak prevalence in heifers was eightfold higher than the low and occurred in July and August

(Ferens and Hovde, 2011). In a 15-month post-outbreak U.K. survey of a cattle farm, *Escherichia coli* O157:H7 were found in 4.3% (153/3593) of rectal swabs, with the peak prevalence in May/July and briefly in November after transfer of cattle into a building, with no shedding from December to May (Friesema *et al.*, 2008); in another survey a decrease of CFU/g digesta and feces occurred in the winter (Sartz *et al.*, 2008). A year-long survey of 93 abattoirs in the United Kingdom showed peak carriage of *E.coli* O157:H7 from June to August (Grant *et al.*, 2008).

In warm climates, prevalence of EHEC in cattle may be less season dependent; in an October to December survey of a herd in Florida ($n = 296$), 3% of fecal samples were culture positive for EC O157:H7, and 9% of cows were culture positive at least once (Friesema *et al.*, 2008); Only a few exceptions to high summer prevalence were noted: in the United Kingdom, STEC O157 were isolated from ~1% of 1356 herds with no regard to season (Grant *et al.*, 2008), but the samples were from cases of bovine gastrointestinal disease and so not random or systematic. For instance, no seasonal preference was found during a 1-year survey on a finishing cattle farm where animals were housed indoors (Friesema *et al.*, 2011). A caveat that applies to longitudinal surveys is that most test only fecal samples, but peak seasonal prevalence in feces do not necessarily coincide with hide contamination (Callaway *et al.*, 2009). Since viable extra-intestinal bacteria may be present at the farm, even when fecal samples are negative, and the intestinal recolonization can occur at any time, the longitudinal studies of *E.coli* O157:H7 prevalence should include nonfecal samples.

2.2.5 Transmission and Human Infection of *Escherichia coli* O157:H7

Most cattle pass *E.coli* O157 at <100 CFU/g of feces, that is, close to the lower limit of detection by IMS, and it is likely that studies underestimate the true prevalence (LeJeune *et al.*, 2006b). Moreover, *Escherichia coli* O157:H7 present in the bovine GIT may not necessarily be excreted with every bowel evacuation and are unevenly distributed in feces; when 120 fresh fecal pats from two feedlots were sampled multiple times,

prevalence increased from 8.2% with one sample/pat to a plateau of 20% with five samples/pat (Echeverry *et al.*, 2005). Cattle transmit *Escherichia coli* O157:H7 to humans by shedding the pathogen in their feces. Fecal shedding may be brief or more extended (Cobbold *et al.*, 2007)

The concept of "supershedders" (animals shedding relatively high numbers of the bacteria for a given herd) posits that some cattle are predisposed to carry exceptionally high numbers of EHEC ($>10^3$–10^4 CFU/g) and are primarily responsible for spreading the pathogen at the farm (Cobbold *et al.*, 2007). Presence of supershedders secreting >200 CFU/g feces was associated with high pen hide prevalence (Arthur *et al.*, 2009). U.K. farms harboring high-shedding cattle exhibited high prevalence of low-level shedding, consistent with a possibility of higher-level transmission (Chase-Topping *et al.*, 2007); thus, 78% of 952 farms were negative for EC O157, but at 2% of farms 90%–100% of fecal pats were positive (Croxen *et al.*, 2013).

An experimental system used to study the spread of *Escherichia coli* O157:H7 by natural circumstance involves "Trojan" carriers, whereby dosed EHEC-positive animals are introduced to negative penmates. Typically, the bacteria are rapidly transmitted. Within 24 h of placement of individual *E. coli* O157:H7-shedding animals in six pens, the bacteria were present in environmental samples, within 48 h on hides of 20/30 animals, and from days 3–23 in fecal samples of 15/30 calves (Chauret *et al.*, 2011). Calves located in adjacent or across pens started shedding within 8 days of introduction of a positive calf to a room (Chase-Topping *et al.*, 2007).

Although the majority of bovine strains of EHEC O157 are not transmitted to humans, there is little doubt that cattle are the main source of human EHEC infections (Ferens and Hovde, 2011). Other important sources of human infection are, direct contact with cattle and other ruminants, contaminatedbathing water, beef products, unpasteurized milk, vegetables,fruits, and drinking water (Chauret, 2011).

Ground beef is a particularly efficient transmission vehicle of EHEC due to the ease of cross-contamination, dispersion of the bacteria throughout the substrate, and poor efficiency of dry heat as a sterilizing agent, whereas bacteria contaminating the surface of a meat slab are unlikely to survive heat exposure. In the first U.S. outbreak in which EHEC was recognized as a class of human pathogens, 17/19 nursing home residents with hemorrhagic colitis ate hamburgers, but only 28/67 healthy residents, and indirect evidence pointed to ground meat in a number of other outbreaks (Bosilevac *et al*., 2011).Outbreaks between 1982 and 2002, ground beef was the vehicle in 75/183 foodborne outbreaks (Bosilevac *et al*., 2011). Isolates from beef products and from cattle were matched to the outbreak isolates and to isolates from clinical cases (Hussein, 2007) and specifically to HUS.

One- third of the meat samples from randomly selected stores in Bologna (northern Italy) contained *E. coli,* and 3/149 were positive for STEC O157 (Stampi *et al*., 2004). In a cluster of *E.coli* O157:H7–associated HUS and gastroenteritis cases, PFGE-identical isolates were found in ground meat samples in processing plant, in grocery store, and patients (Vogt and Dippold, 2005). Contaminated equipment, such as meat grinders, was linked to food-borne *E. coli* O157:H7 infections (Bosilevac *et al*., 2011). Sausages, dry-cured salami, and other food items containing uncooked meat were shown to transmit *E.coli* O157:H7 (Sartz *et al*., 2008), and pork salami transmitted a severe *E.coli* O157 infection (Conedera *et al*., 2007).

Uncooked/unpasteurized cow and goat milk was a vehicle of infection in several clusters of HUS caused by *E.coli* O157:H7 and EHEC O157:H7 were found in sheep dairy products (Caro *et al*., 2007). PFGE-identical isolates were obtained from patients and the implicated dairy (Denny *et al*., 2008). *Escherichia coli* O157:H7 can survive and sometimes grow in different cheeses made from unpasteurized milk (Jordan and Maher, 2006; Schlesser *et al*., 2006; Wahi *et al*., 2006 STEC were detected in cheese after 12-month ripening (Caro and Garcia-Armesto, 2007). *Escherichia coli* O157:H7 were found in 2/268 bulk tank milk samples and on 8/30 dairy farms in Tennessee (Ferens &

Hovde, 2011), and 4.3% (36/859) of samples of bulk tank milk from U.S. dairies were positive for virulence factors associated with O157:H7 (*eaeA*, *tir*, and *stx*) (Karns *et al*., 2007).

Produce provides a variety of vehicles for transmission of EHEC as the bacteria can attach to intact or processed fruits and vegetables as well as survive in fruit juice. *Escherichia coli* O157:H7 survived for 20 days in apple cider (Chauret, 2011) and outbreaks were traced to this vehicle (Ferrens and Hovde, 2011). Major outbreaks have been linked to lettuce (Ethelberg *et al*., 2011) are implicated in numerous HUS cases (Grant *et al*., 2008). In some major EHEC outbreaks, pathogen was carried on spinach or other produce harvested in the proximity of cattle farms (Jay *et al*., 2007),

Manure is a good vehicle of EHEC and large outbreaks were associated with public events held on grazing areas presumably strewn with manure, especially in rainy weather. Isolates of EHEC O157 from music festival attendees were matched to a cattle herd (Ferens and Hovde, 2011). A scouting event held in Scotland on a muddy field grazed by sheep resulted in an 8% attack rate (18/226), with PFGE-identical isolates from patients, the field, and sheep feces (Strachan *et al*., 2001); Escherichia coli O157: H7 survived for 2 months in garden soil fertilized with manure (Ferens and Hovde, 2011), and four PFGE-identical isolates were obtained from an implicated cow and three people visiting a meadow strewn with manure (Le jeune *et al*., 2006)

Escherichia coli O157:H7 can grow in sterile fresh water at low carbon source concentration (Franz *et al*., 2008), and waterborne origin was implicated in a number of sporadic cases and outbreaks of STEC O157 infection (Castro *et al*., 2012) and STEC O157:H7 (Muniesa *et al*., 2006). EHEC outbreaks were associated with swimming in lakes and pools (Verma *et al*., 2007), and consumption of water from private water supply (Mannix *et al*., 2007). Interestingly, *E.coli* O157:H7 do not survive well in the Ganges river water due to heat-labile noncellular antimicrobials, presumably colicins or antimicrobial peptides (Nautiyal, 2009).

Direct contact with ruminants at petting zoos or through interactions with infected people within families, daycare centers, and healthcare institutes represent another source of EHEC transmission (Nguyen and Sperandio, 2012). Even brief physical human–animal contact can transmit EHEC; children are at most risk, as highlighted by an HUS case in a visitor to a petting zoo (Nguyen and Sperandio, 2012). In a study of STEC transmission in Germany, acquisition of isolates from 202 human cases analyzed risk factors by comparing age- and region-matched controls; in children <3 years, touching a ruminant was the greatest risk source (Werber *et al*., 2007).

Person-to-person transmission of EHEC may contribute to outbreaks from a primary source (Seto *et al*., 2007) and may reach 14% or higher; secondary cases occurred in 20/89 households with STEC O157 infection in the United Kingdom (Werber *et al*., 2008), and significant interpersonal spread was noted in 18-case O157 outbreak in Ireland, with identical/close PFGE patterns in isolates from patients and a rectal animal swab (Mannix *et al*., 2007). Asymptomatic infected people may be an unappreciated source of the pathogen (Gilbert *et al*., 2008).

2.2.6 Pathogenesis of *Escherichia coli* O157:H7

Karmali first identified VTEC as the infectious agent responsible for HUS after correlating *E. coli* infection in patients with diarrhea and HUS with the presence of a toxin that produced significant irreversible cytotoxic effects in Vero cells (Croxen *et al.*, 2013). O'Brien and LaVeck later purified the toxin from an enteropathogenic strain of *E. coli* and determined that the toxin was structurally and antigenically similar to the Shiga toxin produced by *Shigella dysenteriae* type 1 (Johannes *et al.*, 2010).

Shiga toxin is composed of two major subunits as shown in **Figure 1.1**, designated A and B (Croxen *et al.*, 2013). The B subunit forms a pentamer that binds to globotriaosylceramide-3 (Gb3) (Mayer *et al.*, 2012), and this specificity determines where Shiga toxin mediates its pathophysiology. The A subunit exhibits an RNA N-

glycosidase activity against the 28S rRNA (Johannes *et al.*, 2010) that inhibits host protein synthesis and induces apotosis (Karmali *et al.*, 2010). In humans, EHEC colonizes the large intestine (Steyert *et al.*, 2011). Shiga toxin released by EHEC binds to endothelial cells expressing Gb3, allowing absorption into the bloodstream and dissemination of the toxin to other organs (Nguyen & Sperandio, 2012). The tissues and cell types expressing Gb3 varies among hosts, and the distribution of Gb3 targets the pathology of toxin-mediated disease to cells expressing Gb3 (Obrig, 2010). For example, renal glomerular endothelium expresses high levels of Gb3 in humans, and Shiga toxin production results in acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, all typical characteristic of HUS (Mayer *et al.*, 2012).

After being ingested, *Escherichia coli* O157:H7 enters the rumen of cattle. In order to reach the RAJ for colonization, EHEC must first breach the acidic barrier of the stomachs. EHEC has an intricate acid resistance (AR) system that enables it to survive through the acidic environment of the stomach, as exemplified by its low infectious dose of 10–100 colony-forming units (Croxen *et al.*, 2013). Three important AR systems have been identified in *E. coli*: the AR system 1 (glucose-repressed or oxidative), AR system 2 (glutamate-dependent), and AR system 3 (arginine-dependent). The relative importance of each AR system *in vivo* is still being delineated; however, induction and function of these systems *in vitro* varies depending on the type of culture medium used and growth conditions (Hong *et al.*, 2009).

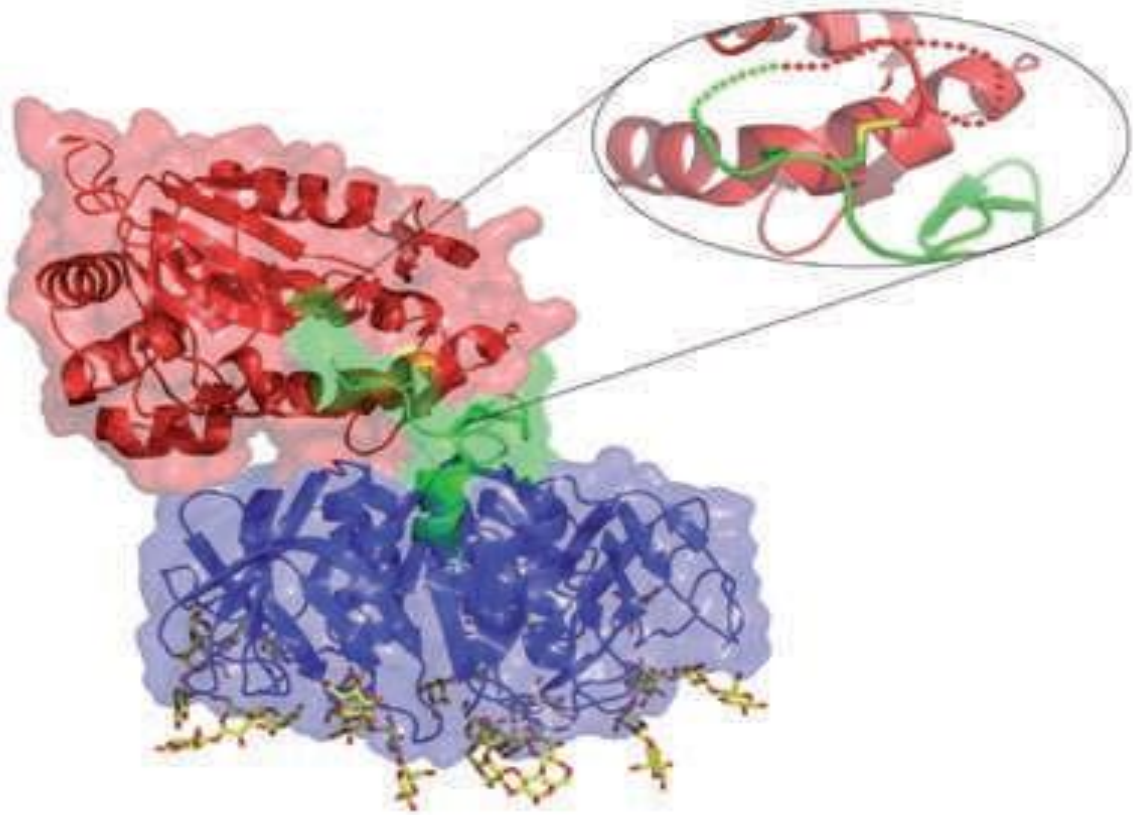


Figure 1.1: Shiga toxin structure

Shiga holotoxin, consisting of one A subunit (StxA), which is cleaved into pink and green fragments and five fragments that constitute the homopentameric B subunit (StxB). A ribbon diagram of Shiga toxin, highlighting globotriaosylceramide (Gb3)-binding sites on StxB. Gb3 is shown in a balland- stick representation. Also shown is an enlargement of StxA at the site of furin cleavage (Arg25-Met252), and showing the disulphide bond that links the two stxA fragments.

After passage through the acidic barrier, the pathogen forms attaching and effacing (A/E) lesions on the mucosal epithelium at the RAJ, allowing for its colonization at the RAJ. A/E lesions are characterized by destruction of microvilli, intimate attachment of the bacteria to the cell, and accumulation of polymerized actin beneath the site of

bacterial attachment to form a pedestal-like structure cupping individual bacteria (Cobbold *et al.*, 2007). The chromosomal virulence genes of EHEC are organised as cluster referred to as 'pathogenicity island' (Croxen and Finlay, 2010).Examples of this include the lysogenic lambdoid bacteriophage, locus for enterocyte effacement (LEE), PAI and the O genetic island. The genes required for formation of A/E lesions are encoded within the chromosomal pathogenicity island known as the locus for enterocyte effacement (LEE) (Croxen and Finlay, 2010).

2.2.6.1 Locus for Enterocyte Effacement

The LEE consists of approximately 41 genes, divided into five major operons (*LEE1-5*), that encode for a type 3 secretion system (T3SS), regulators, chaperones, and effector proteins. The LEE-encoded regulator (Ler), the first gene encoded in *LEE1*, acts as the master transcription factor of the pathogenicity island, regulating expression of the entire LEE (Croxen and Finlay, 2010). The structure of the T3SS resembles a "molecular syringe" where EHEC can inject effector proteins through the T3SS needle directly into the cytoplasm of the target cells. One important secreted protein that is injected into the host is the translocated intimin receptor (Tir). Once released into the host cytoplasm, Tir is directed to the host cytoplasmic membrane and is inserted as a hairpin-like structure, with its N- and C-terminus in the cytoplasm and central domain exposed to the surface. The central domain of Tir interacts with the LEE-encoded surface protein intimin to form a tight attachment of the bacteria to the eukaryotic cell (Campellone, 2010).

Another non-LEE encoded effector protein, *E. coli* secreted protein F-like protein from prophage U (EspFu), is secreted into the cell and works co-operatively with Tir to recruit host proteins to subvert host cytoskeleton and actin polymerization. EspFu recruits actin nucleation-promoting factor Wiskott-Aldrich syndrome protein (N-WASP) and insulin receptor tyrosine kinase substrate p53 (IRSp53), an important regulator for actin cytoskeleton reorganization. This results in accumulation of actin beneath attached

bacteria, forming the characteristic pedestal-like structure (Campellone *et al.*, 2004; Nguyen & Sperandio, 2012).

In vitro studies demonstrate the crucial role A/E lesion formation plays in pathogen attachment to cultured cells. Various groups have investigated whether the formation of A/E lesions is also required for pathogen to attach to bovine intestinal epithelial cells to promote colonization in cattle. Immunofluorescence staining of tissues reveals that *E.coli* O157:H7 tightly adheres predominately to the epithelial cells in the RAJ of cattle (Cobbold *et al.*, 2007). Dziva et al. used signature-tagged transposon mutagenesis (STM) to identify EHEC genes required for colonization and survival in cattle. Transposon insertions in the genes encoding for the T3SS machinery resulted in reduced fecal shedding of pathogen (Nguyen and Sperandio, 2012). Similarly, deletion of the *LEE4* operon, which encodes for essential structural components of the T3SS, resulted in reduced EHEC ability to colonize cattle (Sharma *et al.*2012). These data suggest that the secretion apparatus is important for colonization in cattle. Tir and intimin have also been shown to play an important role in intestinal colonization in neonatal calves and piglets (Croxen *et al.,* 2013) and in adult cattle and sheep. Together the data indicate that LEE-mediated adherence of EHEC to intestinal epithelia is important for promoting colonization in cattle.

In recent years, several non-LEE encoded effectors—EspJ, NleB, NleE, NleF, and NleH—also have been shown to influence EHEC survival and colonization. Although EspJ is not required for A/E lesion formation in HEp-2 cells or human intestinal explants, *in vivo* studies in mice show that EspJ aids in the passage of EHEC through the host's intestinal tract, suggesting a role for EspJ in host survival and pathogen transmission (Nguyen and Sperandio, 2012). The mouse pathogen *Citrobacter rodentium*, which shares homology of many virulence factors with EHEC, had reduced colonization of *nleB, nleH, nleF* mutants in mice compared to the wild-type strain (Kelly *et al.*, 2006; Echtenkamp *et al.*, 2008; Garcia-Angulo *et al.*, 2008). Wild-type EHEC also outcompeted the *nleF* mutant in gnotobiotic piglets for colonization of the piglet

colon and RAJ (Echtenkamp *et al*., 2008). Co-infection of lambs with wild-type EHEC and an *nleH* mutant demonstrated a competitive advantage of the wild-type strain over the mutant (Hemrajani *et al*., 2008). In contrast, Hemrajani *et al*. found that the *nleH* mutant colonized the bovine gut more efficiently than wild-type EHEC.

2.2.6.2 Lysogenic lambdoid bacteriophage

Shiga toxins (stx) have effects on both the intestinal mucosa and intestinal epithilium. Stx induced damage to microvasculature endothelium is the underlying mechanism of the syndrome (Johannes and Romer, 2010). Structure of shiga toxins family contains two major immunologically non cross reactive groups of toxins, *Stx*$_1$ and *Stx*$_2$. A single EHEC strains may express either or both of this toxins. Stx$_1$ from EHEC is identical to shiga toxin from *S. dysenteriae* 1 (Johannes and Romer, 2010). The structural genes for *Stx*$_1$ and *Stx*$_2$ are encoded on lysogenic lambdoid bacteriophage; the genes for Stx are not phage encoded but chromosomally encoded. Production of *Stx*$_1$ by *E. coli* and *S. dysenteriae* is repressed by iron and reduced temperature, but expression of *Stx*$_2$ is unaffected by these factors.

The prototypic Stx$_1$ and Stx$_2$ toxins respectively share 55% and 57% sequence identity in the A and B subunits (Obrig, 2010). The A_1 peptide contains the enzymatic activity and A_2 serves to bind the A subunit to a pentamer of five identical 7.7-kDa B subunits. The B pentamer binds to the toxin to a specific glycolipid receptor, globotriaosylceramide or Gb_{3c}. After binding, the holotoxin is endocytosed through coated pits and is transported to the Golgi apparatus and then to the endoplasmic reticulum. The A subunit is translocated to the cytoplasm; specifically, the A_1 peptide is an N-glycosidase that removes a single adenine residue from the 28S rRNA of eukaryotic ribosome's, there by inhibiting protein synthesis. The resulting disruption of protein synthesis leads to death of renal endothelial cells (Obrig, 2010).

Different results have been obtained in natural and model hosts, which raise the question of whether *Stx* is involved in inflammation and diarrhea in the xenograft models. In a piglet model, *Stx* was not essential for gut virulence (Scheutz *et al.*, 2012). Similarly, epithelial adhesion and colonization of the bovine terminal rectal mucosa, which is currently considered the prime site for carriage and shedding, was unaffected by the absence of Stx. By contrast, in an infant rabbit model, Stx increased the severity and duration of EHEC-induced diarrhea and purified Stx was able to induce inflammation and diarrhea (Raji *et al.*, 2009). Nevertheless, there is a clear need for model systems that will allow investigation of the virulence properties of EHEC in the context of the complete human intestinal mucosa

2.2.6.3 High-Pathogenicity Island (HPI) of *Escherichia coli* O157:H7

The high-pathogenicity island (HPI) was first described in pathogenic *Yersinia spp* as a chromosomal determinant essential for virulence in mice. The Yersinia HPI is a 35 to 48 Kb chromosomal region containing genes such as *irp-9*, *ybt A*, *fyu A* involved in regulation, biosynthesis and uptake of the siderophore yersiniabactin (Croxen *et al.*, 2013).

2.2.6.4 O pathogenic Island

One of these, O1#148 encodes LEE, and the other eight encode a macrophage toxin and ClpB-like chaperone (OI#7), an RTX-like exoprotein and a transport system(OI#28), two urease gene clusters (O1#43 and O1#48), an adhesion and polyketide or fatty acid biosynthesis system, (OI#47), a T3SS and secreted proteins similar to the *Salmonella-Shigella* host cell invasion genes of SPI-1 (O#115), two toxins and a PagC like virulence factor (O#122). There are also smaller islands that harbor virulence associated genes such as fimbrial biosynthesis genes, iron uptake and utilization structures, antibiotic efflux and glutamate fermentation (Ogura et *al.*, 2009).

2.2.7 Antimicrobial resistance of *Escherichia coli* O157:H7

Antimicrobial resistance in *Enterobacteriaceae* poses a critical public health threat, especially in the developing countries (Canton *et al.,* 2008). Much of the problem has been shown to be due to the presence of transferable plasmids encoding multidrug resistance and their dissemination among different enterobacterial species (Carattoli, 2009). The usefulness of antimicrobial therapy for STEC infections is unresolved because the use of conventional antibiotics exacerbates Shiga toxin-mediated cytotoxicity. Currently no treatment is available for *E.coli* O157 infections (Goldwater & Bettelheim, 2012).

In an epidemiology study conducted by the Centers for Disease Control and Prevention, patients treated with antibiotics for EHEC enteritis had a higher risk of developing HUS (Tserenpuntsag *et al.,* 2005). Additional studies support the contraindication of antibiotics in EHEC infection; children on antibiotic therapy for hemorrhagic colitis associated with EHEC had an increased chance of developing HUS (Nguyen and Sperandio, 2012). Certain antibiotics, such as fluoroquinolones, induce Shiga toxin-encoding bacteriophages in vivo leading to increased expression of Shiga toxin genes (Croxen *et al.,* 2013). Antibiotics also may cause bacterial lysis, which could increase free Shiga toxin in the intestinal tract. Antibiotics promote Shiga toxin production by enhancing the replication and expression of *stx* genes that are encoded within a chromosomally integrated lambdoid prophage genome. *Stx* induction also promotes phage-mediated lysis of the EHEC cell envelope, allowing for the release and dissemination of Shiga toxin into the environment (McGannon *et al.,* 2010). In addition to their therapeutic use in human and veterinary medicine, antimicrobials are routinely used for disease prevention and growth promotion in animal production. This practice leads to the inevitable selection of antimicrobial resistance among commensals in the intestinal tracts of food animals, which poses a public health threat (Canton *et al.,* 2008) For instance, antimicrobial-resistant bacteria from food animals may colonize the human population via the food chain, contact through occupational exposure, or waste runoff

from animal production facilities (Chauret, 2011). Food animals, in particular mature cattle, which may be asymptomatic carriers of *E. coli* O157, including STEC (Gyles, 2007), when exposed to antimicrobial agents in the animal production environment, may serve as a reservoir of antimicrobial-resistant bacteria.

However, recent studies suggest that some antimicrobials, if administered early in the course of infection, may prevent disease progression to HUS (McGannon *et al.*, 2010). Because STEC infections are not aggressively treated with antimicrobial therapy, many isolates may yet be susceptible to numerous antimicrobials. Recent studies have revealed a trend towards increased antibiotic resistance of *E. coli* O157:H7 (Carratoli, 2008). For instance, in 2005, about 35% of *E. coli O157:H7* strains isolated from meat and meat products in Gaborone, Botswana, were resistant to cephalothin, sulfatriad, colistin sulfate and tetracycline (Magwira *et al.*, 2005). In a study carried out in 2009 (Abong'o *et al.*, 2009), it was shown that *E. coli O157:H7* isolates were resistant to 5 out of 8 antibiotics tested namely: gentamicin, ampicillin, nalidixic acid, erythromycin, and tetracycline. There was double and multiple resistances of four isolates. However, some isolates were either intermediately susceptible and/or fully susceptible to some antibiotics. (Abong'o *et al.*, 2009)

Strains of Enterobacteriaceae producing an extended spectrum beta-lactamase have become a concern in medical bacteriology as regards both antimicrobial treatment and infection control in hospitals (Drieux *et al.*, 2008). In the past few years, E. coli strains isolated from diarrhoeic calves have shown resistance to multiple, structurally unrelated antibiotics. Chromosomal and plasmid-borne integrons have been identified as one of the crucial factors for the development of multidrug resistance in Enterobacteriaceae as well as many other bacterial species by harboring and lateral gene transfer of gene cassettes (Arya *et al.*, 2008). Mutations are implicated in the emergence of resistance (Kumarasamy *et al.*, 2010).

Resistance to b-lactam antibiotics in Gram-negative pathogens is mostly mediated by b-lactamases. These antimicrobial agents by hydrolyzing their b-lactam ring. EBSL producing Enterobacteriaceae have caused multiple nosocomial outbreaks with a high impact on patients' morbidity (Drieux *et al.*, 2008). Other mechanisms contributing to β-lactam resistance involve AmpC-type cephalosporinases, alteration of porin channels with possible antibiotic efflux, hyperproduction of specific β-lactamases and inhibitor-resistant mutants (Aarestrup *et al.*, 2010). Resistance to fluoroquinolones in Gram-negative bacteria is based on two distinct mechanisms: Inhibition of bacterial DNA topoisomerases and, Decreased drug uptake by changes in the outer membrane and increased efflux (Tserenpuntsag *et al.*, 2005).

2.2.8 Management and control strategy of *Escherichia coli* O157:H7 infection

Genetic fingerprinting is a means by which epidemiologists have traced back infections to their probable sources. It also has been used to understand the ecology of *E. coli* O157:H7 and might be used as part of hazard analysis and critical control point (HACCP) programs for producers to reduce on-farm pathogens (Chauret, 2011). The number of cases of disease might be reduced by other various mitigation strategies for ground beef, for example, screening the animals pre-slaughter to reduce the introduction of large numbers of pathogens in the slaughtering environment. Good hygienic slaughtering practices reduce contamination of carcasses by faeces, but do not guarantee the absence of EHEC from products.Treatment of dairy wastewater in an artificial wetland resulted in a 2-log reduction of *E.coli* O157:H7 numbers (Chauret, 2011).

According to Codex Recommended International Code of Practice-General Principles of Food Hygiene, 2009, education in hygienic handling of foods for workers at farms, abattoirs and those involved in the food production is essential to keep microbiological contamination to a minimum. Education is essential to reduce risks associated with animal contact in public settings. Animal owners, exhibit operators, and their staff should be educated to make appropriate management decisions. In addition, the public

should be educated so that they can weigh the benefits and risks of animal contact and take appropriate measures to reduce risks (LeJeune & Davis, 2004).

Preventive measures for *E. coli* O157:H7 infection is similar to those recommended for other food borne diseases. Basic good food hygiene practice, can prevent the transmission of pathogens responsible for many foodborne diseases, and also protect against food borne diseases caused by EHEC. The only effective method of eliminating EHEC from foods is to introduce a bactericidal treatment, such as heating (e.g. cooking, pasteurization) or irradiation. Cook food thoroughly so that the centre of the food reaches at least 70°C. Vulnerable populations should avoid the consumption of raw or undercooked meat products, raw milk and products made from raw milk.Consumption of unpasteurized products should be avoided. Unpasteurized dairy products (e.g., milk, cheese, and yogurt) as well as unpasteurized apple cider or juices should not be consumed (Johnson & Nolan, 2009).

Hand-washing is the single most important prevention step to reduce transmission resulting from human–animal contact (LeJeune and Davis, 2004).Regular hand washing, particularly before food preparation or consumption and after toilet contact, is highly recommended, especially for people who take care of small children, the elderly or immunocompromised individuals, as the bacterium can be passed from person-to-person, as well as through food, water and direct contact with animals. Make sure to wash fruits and vegetables carefully, especially if they are eaten raw. If possible, vegetables and fruits should be peeled.At venues where human-animal contact occurs, signs regarding proper hand-washing practices are critical to reduce disease transmission (Vanaja *et al.*, 2009).

A number of EHEC infections have been caused by contact with recreational water. Therefore, it is also important to protect such water areas, as well as drinking-water sources, from animal feces and waste. Local public health authorities should inspect drinking water systems before use. Only potable water should be used for human

consumption. Back-flow prevention devices should be installed between outlets in livestock areas and water lines supplying other uses on the grounds. If the water supply is from a well, adequate distance should be maintained from possible sources of contamination (e.g., animal-holding areas and manure piles) (LeJeune & Wetzel, 2007).

Animals should be monitored daily by their owners or caretakers for signs of illness, and they should receive appropriate veterinary care. Animals should be housed to minimize stress and overcrowding, which can increase shedding of microorganisms. Options to reduce the burden of enteric pathogens need to be evaluated, particularly for animals that are at higher risk and that will be used in venues where animal contact is encouraged. Vaccination, preventive care, and pathogen control appropriate for the cattle should be provided. Manure and soiled animal bedding should be removed promptly. Animal waste and specific tools for waste removal (e.g., shovels and pitchforks) should be confined to designated areas restricted from public access. No food or beverages should be allowed in animal areas. In addition, smoking, carrying toys, and use of pacifiers, spill-proof cups ("sippy cups"), and baby bottles should not be permitted in animal areas (LeJeune & Davis, 2004).

The prevention of infection requires control measures at all stages of the food chain, from agricultural production on the farm to processing, manufacturing and preparation of foods in both commercial establishments and household kitchens.

CHAPTER THREE

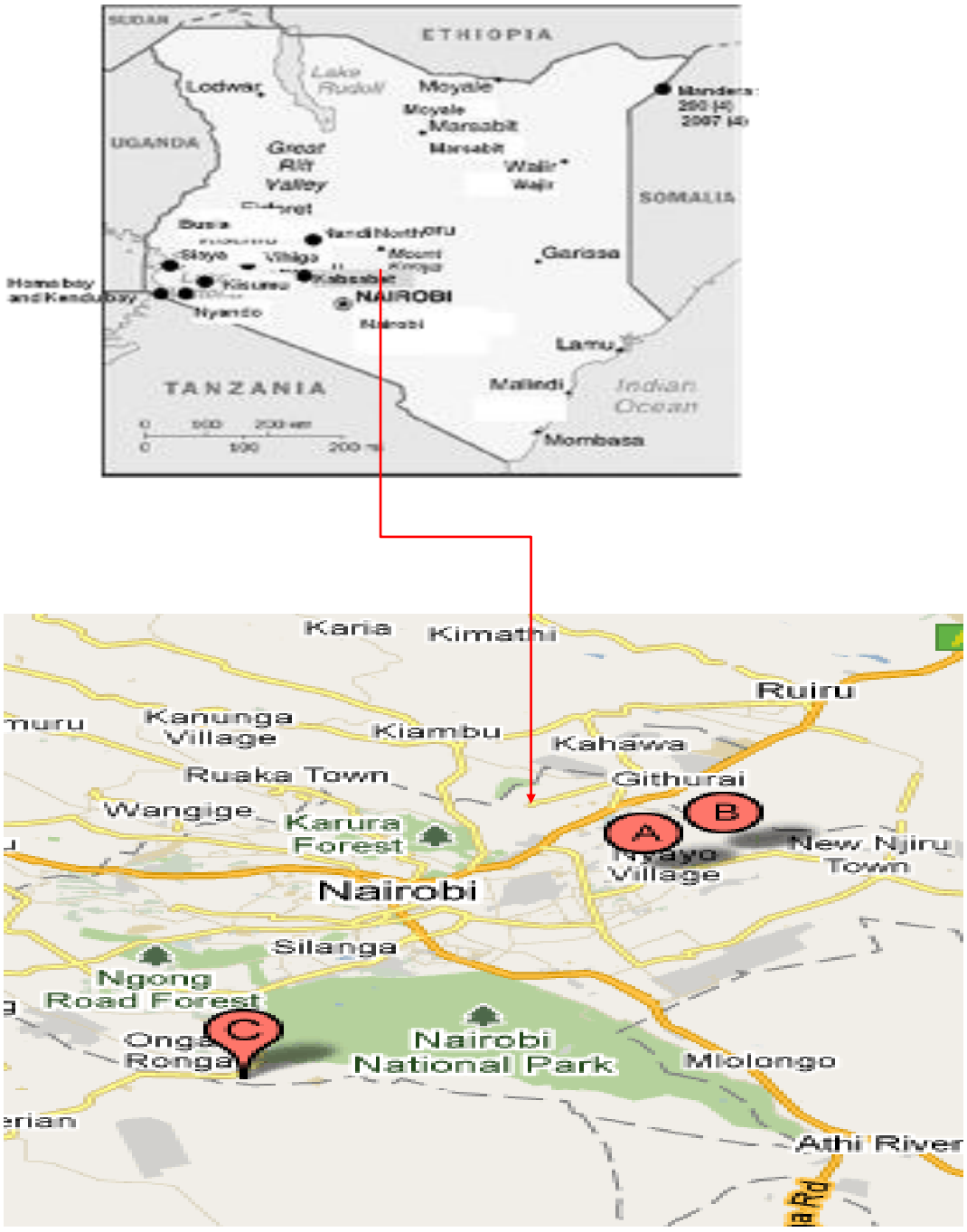
MATERIALS AND METHODS

3.1 Study and sampling Design

This was a cross-sectional laboratory based study design and a systematic random sampling design was used where the cattle selected depended on the average number of cattle slaughtered per day in a particular abattoir.

3.2 Study sites

Surveillance was done in the city of Nairobi which is the capital city of Kenya. Nairobi is the most populous city in East Africa, with an estimated urban population of about 3.5 million. The growth rate of Nairobi is currently 6.9% (Kenya Central Bureau of Statistics, 2009). Abbatoirs were selected from various regions and outskirts of Nairobi (Fig.3.1). From the Eastern part of Nairobi in the Njiru area, two abattoirs specializing in beef only for local market were chosen. Still in the Eastern part, The Kenya Meat Commision abbatoir at Athi river region was included which provides beef products for local and also for export market. From the Northern part of Nairobi in the Waithaka area in Dagorreti Market, two abattoirs supplying beef for local market were chosen. From the Southern Part of Nairobi in the Kiserian area, three abattoirs supplying beef for local market were included in the study.



KEY: **A- Abattoirs in Southern Part B&C-Abottoirs in Eastern Part (Image generated with google maps)**

Figure 3.1The map of Kenya showing sampling regions of slaughter houses.

3.3 Study population

The population of study included healthy animals delivered to all the slaughter houses meant for slaughter and eventual human consumption. Inclusion criteria included all cattle for slaughter ment for human cunsumption. Exclusion criteria were cattle slaughterd not for human consumption. Over half of the beef animals are mainly supplied by the Maasai community who herd their animals in free range in Kajiado District neighboring Nairobi to the South. Each beef abattoir handled over one hundred animals per week.

3.4 Sample Size determination

The rate of *E. coli* isolation in cattle carcasses has been given as approximately 28% (Gansheroff and O'brien, 2000). Assuming prevalence rate of 28% at 95% confidence level, the minimum estimated sample size was given as 100 using the Fisher *et al.*, (1992) formula, as shown below.

$$n = \frac{Z^2_{1-\alpha/2}\, P\,(1-P)}{d^2}$$

Where:

- N = Minimum sample size required
- $Z_{1-\alpha/2}$ =1.96
- α = the level of significance
- d = 0.05 (5% Absolute precision)
- P = expected prevalence of condition of interest

Therefore ;

$$\frac{(1.96)^2\ 0.28\ (1-0.28)}{0.05^2}$$

N = 309 (minimum sample size required)

3.5 Sample collection and management

Minimum sample size (n) required as calculated was 309 samples however, 162 samples of both rectal and carcass swabs were collected and processed **(Appendix 5),** making a total of 324 swab samples obtained from 162 slaughtered cattle from commercial abattoirs between July 2010 and December 2010. Swabs were from Styroplast® Company. Sampling of each group of abattoirs was done separately during this study. The number of cattles swabbed in each abbatoir was based on the number of cattles slaughtered in each abbatoir. The private abbatoirs such as Hurlingham recorded the lowest number of cattle slaughtered unlike the Njiru abbatoir. This was done to randomize the sampling and obtain as a wide a range of carcasses as possible. During visits, sampling procedure for most beef abattoir involved rectal swabbing done for every tenth carcass. Simple random sampling was used after drafting a list of all cattles and picking every 10^{th} cow lined up for slaughter.

Rectal swabs were collected from each of the 162 cows just before slaughter and placed in transport media, supplied by swab manufacturer. Carcass samples were collected immediately after removal of the hide (dressing step) from the carcass. Each carcass surface sample consisted of two pooled neck and rump subsamples. An area of 200 cm^2 of the neck and rump regions of the carcass was swabbed (100 cm^2 - 10 × 10 cm - of each region) using two different sterile cotton swabs. These two tubes from one carcass were labeled with the same laboratory number as the rectal swab but designated 'C' at the end instead of 'R'.All swabs collected were then placed into cool boxes at 4ºC

and transported to the laboratory within 2 hours on the same day of collection.Upon arrival at the laboratory, samples were analyzed immediately. All samples were processed at the Kenya Medical Research Institute-Centre for Microbiology Research laboratory located in Nairobi.

3.6 Identification of Shiga Toxigenic *Escherichia coli* isolates

To enhance isolation and identification of *E. coli* O157, necessary steps including immunomagnectic separation and polymerase chain reaction were done.

3.6.1 Pre-enrichment and immunomagnectic separation

For isolation, the swabs were placed in a modified tryptone soy broth (mTSB-Oxoid-CM 989; Basingstoke, England) supplemented with novobiocin (20 mg/L, N1628; Sigma, USA) in 10 mL tubes and then incubated at 41.5℃ for 24 h.A 1-ml aliquot of the broth and 20 ml of Dynabeads were added to an Eppendorf tube, and incubated with continuous mixing for 10 minutes at room temperature. A magnetic field was then applied to the side of the tube, and the beads with any adherence to STEC or *E. coli* O157 were drawn to the side of the tube. The culture supernatant was removed by aspiration, and the beads re-suspended in 1 ml of phosphate-buffered saline. This washing process was repeated twice. After the final wash step, the beads were then re-suspended in 100 ml of phosphate buffered saline solution.

3.6.2 Culture

The concentrated washed beads were inoculated onto sorbitol MacConkey agar supplemented with cefixime 0.05 mg ly1 and tellurite 2.5 mg. Cefixime Tellurite-Sorbital MacConkey (CT-SMAC) plates were then incubated at 37ºCfor 18 hours. The following day the plates were examined for non-sorbitol fermenting colonies.

3.6.3 Confirmation of Shiga Toxigenic Escherichia coli isolates by agglutination and biochemical tests

The suspected colonies were purified on plate count agar at 37ºC for 20-22 hours and subjected to the confirmatory tests.The isolates were examined by latex agglutination with *E. coli* O157 (#210753; Denka-Sheiken, Japan) and O157:H7 antisera (#211057; Denka-Sheiken, Japan). Colonies exhibiting a positive precipitation reaction with the O157 antiserum were identified as *E. coli*O157, while colonies showing a positive precipitation reaction with the H7 antiserum were identified as *E. coli* O157:H7.

Two to four sorbitol non-fermenting colonies from primary culture were selected for sub culture and isolates tested for their biochemical characteristics on triple sugar iron agar (TSI). Triple sugar iron agar is used to test for the ability of an organism to ferment the sugars glucose and/or lactose and produce hydrogen sulfide (H_2S). The process of sugar fermentation allows bacteria to produce ATP under oxygen limiting conditions, producing acids and/or gas (CO_2) as a byproduct. A 24-48 hour culture was picked by sterile wire needle and aseptically inoculated to a sterile TSI slant tube. Inoculation of the TSI slant was done by first stabbing the butt down to the bottom, withdrawing the needle, and then streaking the surface of the slant. A loosely fitting closure was used to permit access of air. Flaming of the neck of the TSI tube was done in addition to capping it and incubation was done at 37^{o}C for 18 to 24 hours.

Other biochemical tests included sulphur indole motility agar and Simmon's citrate agar.This was done by inoculating a SIM tube with the organism to be tested. Incubation followed for 48 hours. After incubation, Kovac's reagent was added to the top of the tube and pink or red color that formed indicated indole formation. Black precipitate in the medium indicated hydrogen sulfide formation. Growth away from the stab line indicated motility.

For Simmon's citrate, the procedure was carried out by inoculating slants with growth from an *E. coli* pure culture using a light inoculum. Incubation of all tubes was done at 35 ± 2°C for 24-28 hours in an aerobic atmosphere. A positive reaction was indicated by growth with an intense blue color in the slant. A negative reaction was evidenced by nogrowth to trace growth with no change in color (medium remains dark green).

Gram stain testwas also done to confirm morphological characteristics of the isolates. Organisms once identified were stocked in 1ml capacity vials containing Mueller Hinton broth and 15% (v/v) glycerol and stored at -70°C until analyzed. All the media reagents used were from Oxoid Ltd., Basingstoke, United Kingdom

3.7 Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using the Kirby Bauer disk diffusion technique (Bauer *et al.,* 1966) for 12 antimicrobials (Oxoid, Basingstoke, United Kingdom) (**Table 3.1**). The 12 antimicrobials represented all the major groups of drugs used for treatment of *E. coli* as grouped by Clinical Laboratory Standards Institute (CLSI, 2010). The pure isolates were emulsified in sterile normal saline water to conform to 0.5 McFarland turbidity standards. The isolates were spread out with the aid of sterile swabs onto Mueller Hinton agar (Oxoid, Basingstoke, United Kingdom) and the antibiotic disks applied. *E. coli* ATCC© 25922 was included in each run as a quality control. The plates were incubated at 37^{o}C overnight. A zone reader was used to record the zones of the inhibition. The results were interpreted according to the guidelines provided by the CLSI (2010).

Table 3.1 List of the various Antibiotics tested on isolates

Symbol	Antibiotic	Potency
K	Kanamycin	30µg
C	Chloramphenicol	30µg
S	Streptomycin	10µg
CRO	Ceftriaxone	30µg
SXT	Cotrimoxazole	25µg
TE	Tetracycline	30µg
NA	Nalidixic acid	10µg
Cip	Ciprofloxacin	3µg
Amp	Ampicillin	10µg
Amc	Augmentin	20:10µg
Caz	Ceftazidime	30µg
CN	Gentamicin	10µg

3.8 Identification of Shiga Toxigenic Escherichia coli virulence genes using molecular characterization techniques

Isolates were assayed for the presence of virulence genes by multiplex PCR using oligonucleotide primers indicated in **Table 3.2** (Promega, Madison). The virulence genes selected for screening in the isolateswere *rfbE*, responsible for the serotype O157, stx_1 responsible for verotoxin 1 production, stx_2 responsible for verotoxin 2productions, *eae* the determinant of attaching effacing properties and *flicH7*responsible for flagella.

Table 3.2 Primers used in the study

Gene	Primer	Oligonucleotide sequence (5'-3')	Fragment size (bp)	PCR conditions	Reference
stx$_1$	VT1-A	CGCTGAATGTCATTCGCTCTGC	302	90 ng, 55°C	Blanco *et al* 2003
	VT1-B	CGTGGTATAGCTACTGTCACC			
stx$_2$	VT2-A	CTTCGGTATCCTATTCCCGG	516	60 ng, 55°C	Blanco *et al* 2003
	VT2-B	CTGCTGTGACAGTGACAAAACGC			
Eae	EAE-1	GAGAATGAAATAGAAGTCGT	775	150 ng, 55°C	Blanco *et al* 2003
	EAE-2	GCGGTATCTTTCGCGTAATCGCC			
rfbE-	O157L	AACGGTTGCTTCATTTAG	470	-	Jin *et al*,2005
	O157R	GAGACCATCCAATAAGTGTG			
fliC	H7L	TACCACCAAATCTACTGCTG	560	-	Jin *et al*,2005
	H7R	TACCACCTTTATCATCCACA			

3.8.1 *E. coli* DNA extraction

E. coli pure colonies were grown on sorbitol MacConkey at 37ºC for 24 hours. Approximately three loop full colonies were re-suspended in 750 µl sterile lysed buffer in ZR BashinBead™ lysis tube, and vortexed for 5 minutes then centrifuged at 10,000 X g for 1 minute. 400µl of supernatant in a Zymon-spin™ spin filtered tube was then centrifuged at 7,000 rpm for 1 minute. To the filtrate, 1,200µl of DNA binding buffer was added then 800µl of this mixture centrifuged in a Zymon-Spin™IIC column in a collection tube at 10,000 X g for 1minute. Flow in collection tube was discarded and step repeated after which 200µl of DNA wash buffer was added to IIC column in a new collection tube and centrifuged at 10,000 X g for 1 minute. This column was then transferred to a sterile 1.5ml micro centrifuge tube where 100µl of DNA elution buffer was added directly to column matrix and centrifuged at 10,000 X g for 30 seconds to elute the DNA. The eluted DNA was transferred to a Zymo-spin™ IV-HRC spin filtered in a clean micro centrifuge tube and centrifuged for 1 minute at 8,000 X g. This filtered DNA was now suitable for PCR application.

3.8.2 Amplification of genes

Sets of oligonucleotide primer mixtures were prepared according to manufacturer instructions. The PCR assay for genes was carried out in a 28 µl reaction volume containing buffer with each of the four deoxynucleoside triphosphates (dNTPs), each of $fliC_{H7}$, $rfbE_{O157}$, stx_1 ,stx_2, *ehxA and eaeA* specific primers, Taq DNA polymerase, Magnesium chloride and stabilizers. A total volume of 3 µl of extracted sample nucleic acid was used in each PCR reaction. The reaction was carried out in a DNA engine known as Dynax® thermo cycler. It included 40 cycles of denaturation at 94ºC for 30 seconds, primer annealing at 60ºC for 1 minute 30 seconds, and primer extension at 72ºC for 1 minute 30 seconds. Known EHEC strains, *E. coli* O157:H7 ATCC 43895and sterile distilled water were added as positive and negative controls respectively.

3.8.3 Agarose gel electrophoresis for identification of virulence genes

A 100 ml of 1% agarose gel was prepared. This was done by dissolving 1.0 g of agarose powder in 100 ml of X1 TBE buffer in a flask and then heating it for 1-2 minutes in a microwave oven till the mixture was clear. The solution was then cooled to 55^{o}C. Ethidium bromide was added at this point to a final concentration of 0.05 ug/ml: 2 µl of stock before pouring into plastic tray. A plastic comb was placed in the slots on the side

of the gel tray and care was taken for the comb teeth not to touch the bottom of tray. The molten agarose was allowed to cool until it polymerized. The ethidium bromide added per 100 ml of buffer was to enhance visibility of the bands by UV light when taking pictures. After the gel had polymerized, the comb was removed from the gell by pulling straight up with care not to tear the wells. The gel tray was then placed in the gel box with the wells closest to the negative black electrode. Enough 1X TBE buffer, was added to fill the buffer tank and submerged in gel. Samples were loaded including a standard marker (BiolineHyperladder 1®). Current was switched on at 125amp and PCR products were electrophoresed for 40 min, viewed and photographed under UV light.

3.9 Ethical consideration and study approval

The study was approved by Board of Post graduate Studies (BPS) of Jomo Kenyatta University of Agriculture and Technology (JKUAT), Scientific Steering Committee (SSC) of Kenya Medical Research Institute (**Appendix 6),** and the National Ethical Review Committee and assigned number 1900 **(Appendix 7).** This was a sub-study under a larger project titled "An integrated surveillance study of antimicrobial resistance in *Salmonella* spp,*Campylobacter* spp *Escherichia coli* and *Enterococcus* spp from gut of healthy food animals and retail meat outlets in selected regions in Kenya" based at Centre for Microbiology Research, KEMRI.

3.10 Data Management

Data was stored in Ms Excel ® worksheet and also in a hard copy in a book. Results of data were presented in form of tables and graphs.

CHAPTER FOUR

RESULTS

4.1 Prevalence of shiga toxigenic *Escherichia coli* in cattle slaughtered in Nairobi abattoirs.

From a total of 162 cattle sampled, 40 non sorbitol fermenting *E. coli* were isolated (**Appendix 1**) after IMS, enrichment culturing, selective plating and biochemical tests. Ninteen carcass swabs and 21 rectal swabs tested positive for *E. coli* O157 strain. Twenty six samples that contained *E. coli* O157 were from the rectal swabs and carcass swabs fetched from the same cattle, while 14 isolates were six rectal swabs and eight carcass swabs fetched from different cattle. The results of samples positive from abattoir origin and how they were distributed are shown in **Table 4.1.** Nyongara abbatoir in northern Nairobi produced highest prevalence at 4.62% followed by Keekonyoke abbattoir in southern Nairobi at 2.16%. Kiserian abbatoir in southern Nairobi had the least prevalence at 0.31% followed by Thiani abbatoir in northern Nairobi at 0.62% (**Table 4.1**).

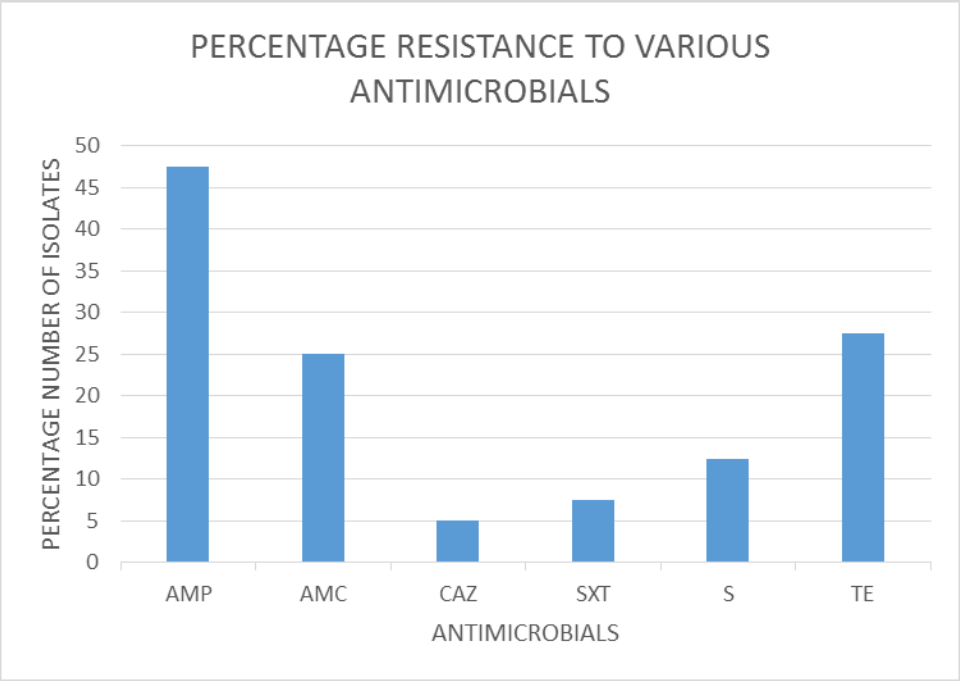
All the 40 STEC isolates were subjected to biochemical tesing including MIO, Simmon's citrate, TSI method and Gram stain test. Four isolates grew away from the stab line indicative of motility. Ten isolates exhibited ornithne decarboxylation by a purple coloration in the media that was spread throughout. All forty isolates tested Indole positive by producing a red color after addition of Kovac's reagent and also fermented sugars inthe triple sugar iron media in order to produce a yellow colored butt and slant.These general bio-chemical characteristics of the isolates were as shown in **Appendix 1**.

Table 4.1 Distribution of positive STEC samples amongst Nairobi slaughter houses

Slaughter houses	Number of cattle sampled	Number of positive Rectal samples based on IMS and biochemical methods	Number of positive carcass samples based on IMS and biochemical methods	Total Number of STEC positive samples&abbatoir prevalance
Thiani slaughter house	14	1	1	2(0.62%)
Kiserian slaughter house	16	0	1	1(0.31%)
Mumo slaughter house	20	2	3	5(1.54%)
Nyongara slaughter house	37	9	6	15(4.62%)
KMC slaughter house	13	1	1	2(0.62%)
Keekonyoke slaughter house	23	3	4	7(2.16%)
Njiiru slaughter house	31	4	2	6(1.85%)
Cooperative slaughter house	8	1	1	2(0.62%)
Total	**162**	**21**	**19**	**40(12.34%)**

4.2 Antimicrobial susceptibility patterns of STEC and other serotypes isolated from different slaughter houses in Nairobi

In this study, all the 40 NSF *E. coli* isolates, were subjected to antimicrobial susceptibility testing. They showed resistance to 6 out of 12 antibiotics namely: Streptomycin, Ampicillin, Augmentin, Cotrimoxazole and Tetracycline **(Figure 4.1).** Ten isolates were resistant to co-amoxiclav while 19 isolates were resistant to ampicillin. Eleven isolates were resistant to Tetracycline, 2 to Ceftazidime, 3 to Trimethoprim and 5 to Streptomycin as shown below **(Figure 4.1).**

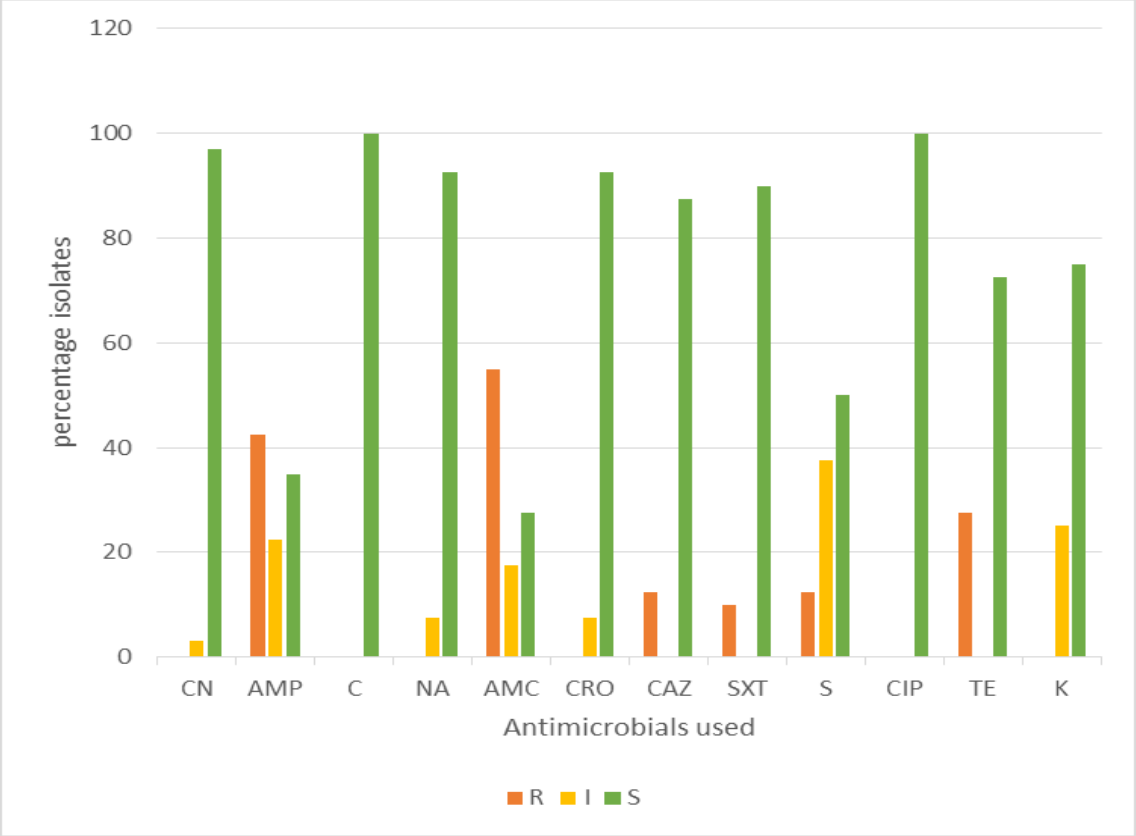


Key:

AMP-Ampicillin
AMC-Amoxicillin&clavulanic acid
SXT-Sulphamethoxazole
TE-Tetracycline
S-Streptomycin
CAZ-ceftazidime

Figure 4.1 Percentage isolates resistant to various antimicrobials

All isolates were susceptible to Ciprofloxacin and Chloramphenicol. Two (5%) isolates were resistant to Ceftazidime and only three isolates (7.5%) to Cotrimoxazole, while Gentamycin, Nalidixic acid and Ceftriaxone recorded zero resitance. Resistance to Ampicilin at 47.5% was the highest prevalance while, resistance to co-amoxiclav, streptomycin and tetracycline was moderate at 25%, 12.5% and 27.5% respectively.(**Figure 4.1 and 4.2**).



Key: CN-Gentamycin AMC-Amoxicillin &clavulanic acid C-Ceftriaxone
CAZ-Ceftazidime AMP-Ampicillin K-Kanamycin
NA-Nalidixic acid SXT-Sulphamethoxazole S-Streptomycin
CIP-Ciprofloxacin CRO-Chloramphenicol TE-Tetracycline

Figure 4.2 Percentage number of isolates resistant to various antimicrobials

All isolates (100%) showed sensitivity to Ceftriaxone and Ciprofloxacin antibiotics Streptomycin showed highest number (37.5%) of isolates at intermediate resistance. A large proportion of isolates from northern part (Dagoretti slaughter houses) of Nairobi showed resistance to more than one antibiotic as compared to a large proportion of isolates got from cattle from eastern Nairobi abattoirs. Different phenotypes of antibiotic resistance for different slaughter houses were observed for all *E. coli* isolates **Table 4.2 .**

Table 4.2 Antibiotic resistance phenotypes observed

Isolate number	Sample origin	Abbatoir	Resistance phenotype observed
212R	Northern	Nyongara slaughterhouse	S
212C	Northern	Nyongara slaughterhouse	AMC
223C	Northern	Mumo slaughterhouse	S, Amp, AMC, TE
225C	Northern	Mumo slaughterhouse	Amp, Te, SXT
233R	Northern	Nyongara slaughterhouse	Aug
234R	Northern	Nyongara slaughterhouse	S,Amp, AMC,Te,SXT
410C	Northern	Nyongara slaughterhouse	Amp, AMC, Te ,SXT
240R	Northern	Nyongara slaughterhouse	Amp
260C	Southern	Mumo slaughterhouse	Te
261C	Sourthern	Keekonyokie slaughter house	Caz, Amp ,Te
262R	Southern	Keekonyokie slaughter house	Amp, AMC
265C	Southern	Keekonyokie slaughter house	S
270R	Northern	Nyongara slaughterhouse	Amp, AMC
278R	Northern	Thiani slaughterhouse	Te
303R	Northern	Nyongara slaughterhouse	Amp
410R	Northern	Nyongara slaughterhouse	Te
418R	Eastern	Njiiru slaughterhouse	Amp
414C	Eastern	Njiiru slaughterhouse	Caz, S,Te
415C	Eastern	Njiiru slaughterhouse	Amp
414R	Eastern	Njiiru slaughterhouse	Amp
430R	Northern	Co-operative slaughterhouse	Amp
486R	Southern	Keekonyokie slaughter house	Te
450C	Southern	Kiserian slaughterhouse	Amp, AMC
486C	Southern	Keekonyokie slaughter house	Amp, AMC
500C	Southern	Keekonyokie slaughter house	Amp, AMC
500R	Southern	Keekonyokie slaughter house	Te
514C	Southern	Keekonyokie slaughter house	Amp,AMC
509R	Southern	Kenya Meat Commission	Amp

Twelve out of forty isolates were multidrug resistant. Thirteen isolates were fully susceptible to all antibiotics. Fourteen isolates were resistant to one antibiotic, 6 isolates were resistant to two antibiotics, 3 isolates were resistant to three antibiotics, 2 isolates were resistant to four antibiotics and one isolate was resistant to five antibiotics **Figure 4.3**.

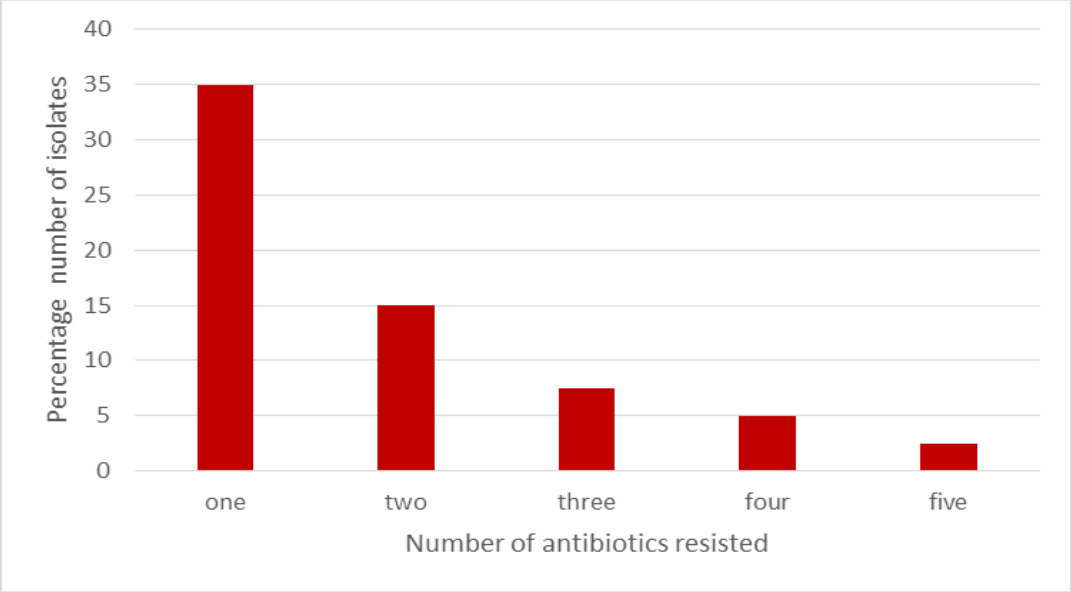
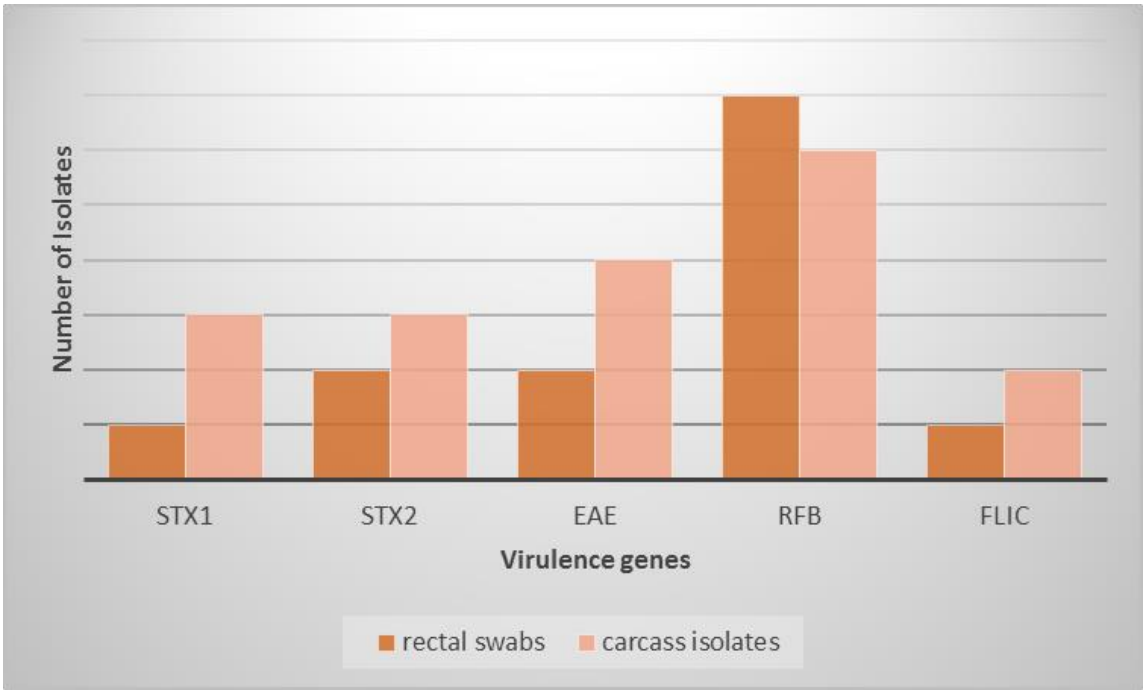


Figure 4.3 Number of antibiotics to which *E.coli* isolates showed resistance.

4.3 Occurrence and distribution of virulence genes among isolated STEC serotypes by use of PCR

Thirty one out of 40 *E. coli* isolates studied were positive for various virulence genes based on multiplex PCR assays using specific primers for rfb_{O157}, $flic_{H7}$, *eae*, stx_1 and stx_2. These genes were widely distributed amongst the isolates. Virulence genes were detected in 13 rectal swab isolates and remaining 18 were from carcass swab isolates **Figure 4.4**).The gene encoding for O somatic antigen $rfbE_{O157}$ was detected in 13 isolates, this was the most predominant gene in the study. The flagella encoding gene

fliC$_{H7}$ was detected in only three isolates, making it the least prevalent. Four and five isolates expressed *stx*$_1$gene and *stx2* respectively **Figure 4.4**.



Key;

Rectal Isolates **Carcass Isolates**

Figure 4.4 *E. coli* STEC pathotypes numbers positive for various virulence genes.

Only two isolates shown in **plate 4.1, plate 4.2** and **plate 4.3** contained all the five genes from carcass swab samples while only one rectal swab isolate had all five genes shown in **plate 4.3**. Nine isolates contained the gene *rfb*$_{O157}$ only, for instance as shown in **plate 4.5**, and 3 contained the *eae* gene only (**plate 4.4)**. Two isolates contained *stx2* gene only, (**Appendix 4).**

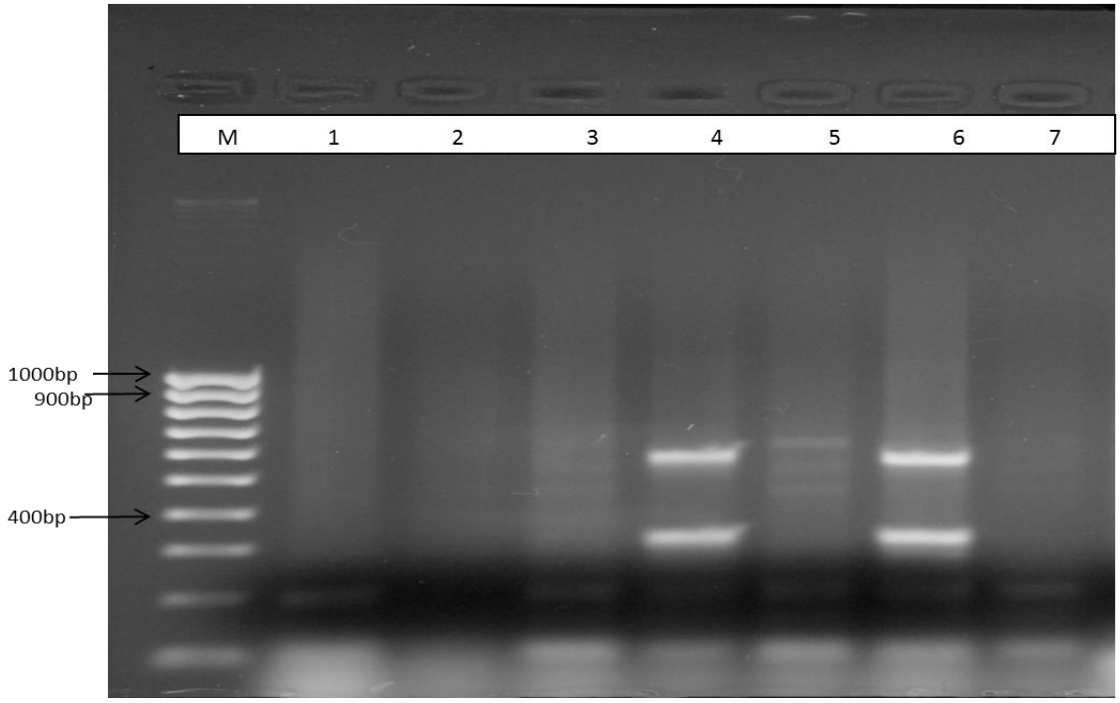


Plate 4.1. Electrophoresis of the multiplex-PCR products of STEC pathotype containing stx1 and *stx2* virulence genes

Lane M represented 100bp 5µl Hyperladder IV (Bioline H4K5) of upto 100bp. In lanes 1 to 3,5 and 6 no genes were amplified representing isolates no.415C, 303R, 262R, 270R and 500 R respectively lacked verotoxins. Lane 4 and 6,loaded with sample isolate no.234C and 410C respectively,both amplified *stx1*and stx_2 genes at 516 bp and 302 bp respectively.

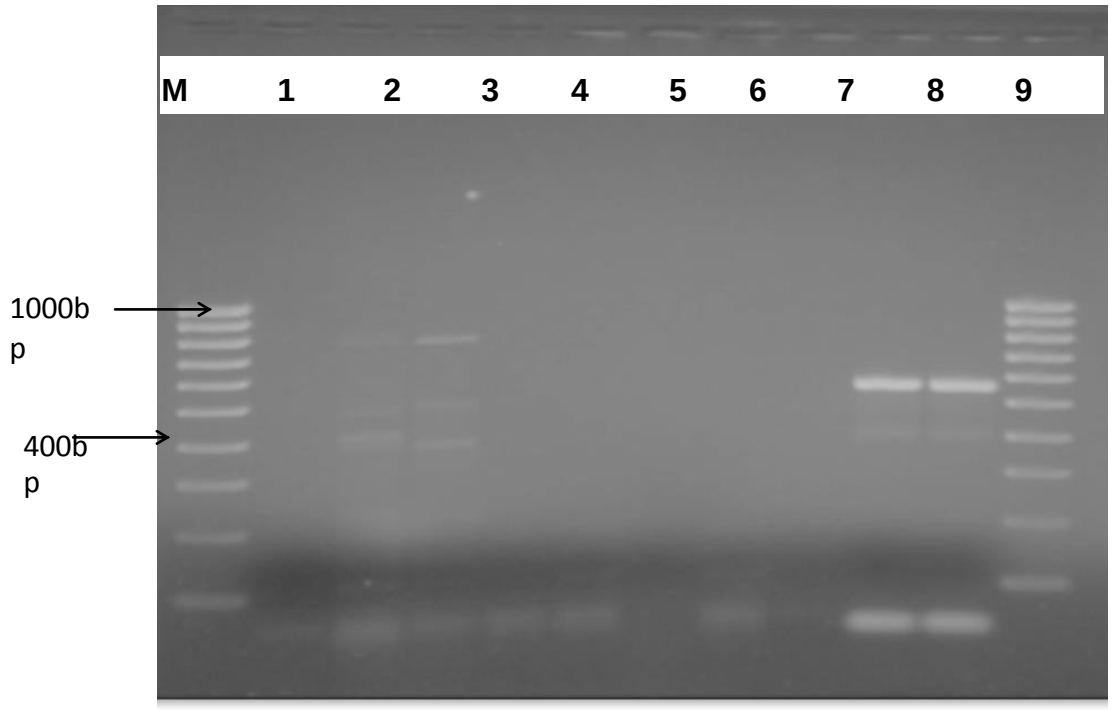


Plate 4.2: Electrophoresis of the monoplex-PCR products of STEC pathotype containig *flicH7* virulence genes.

Lane M represented 100 bp 5µl Hyperladder IV (Bioline H4K5) of upto 1000bp. Lane 1 was loaded with sterile distilled water and used as a negative control. In lanes 2 to 5 no genes were amplified representing isolates no.486C, 500R , 270R, and 262R respectively lacked verotoxins. Lane 9 and 10,loaded with sample isolate no 234R and 410C respectively, both amplified *flicH7* genes at 560 bp.

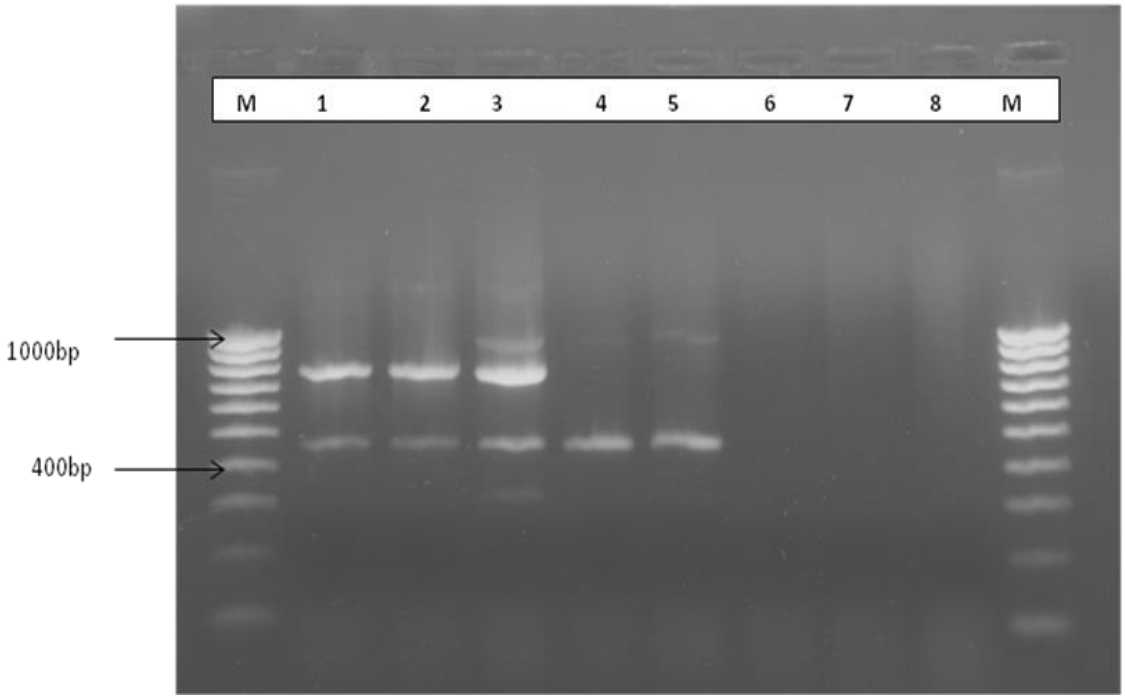


Plate 4.3: Electrophoresis of the multiplex-PCR products of STEC pathotype containing *rfb*E*O157* and *eae* virulence genes

Lane M represented100bp 5µl Hyperladder IV (Bioline H4K5) of upto 1000 bp.Lane 1 was loaded with ATTC003, used as a positive control and amplified *eae* and *rfbEO157* genes at 775 bp and 470 bp respectively. Lanes 2 and 3, loaded with sample isolate no.234R and 410C respectively, both amplified *eae* and *rfbEO157* genes at 775 bp and 470 bp respectively. Lanes 4 and 5, loaded with sample isolate no.262R and 270R respectively,both amplified *rfbEO157* genes at 470 bp. In lanes 6 and 7 no genes were

amplified representing that isolates number 415C and 486C respectively lacked verotoxins. Lane 8 was loaded with sterile distilled water and used as a negative control.

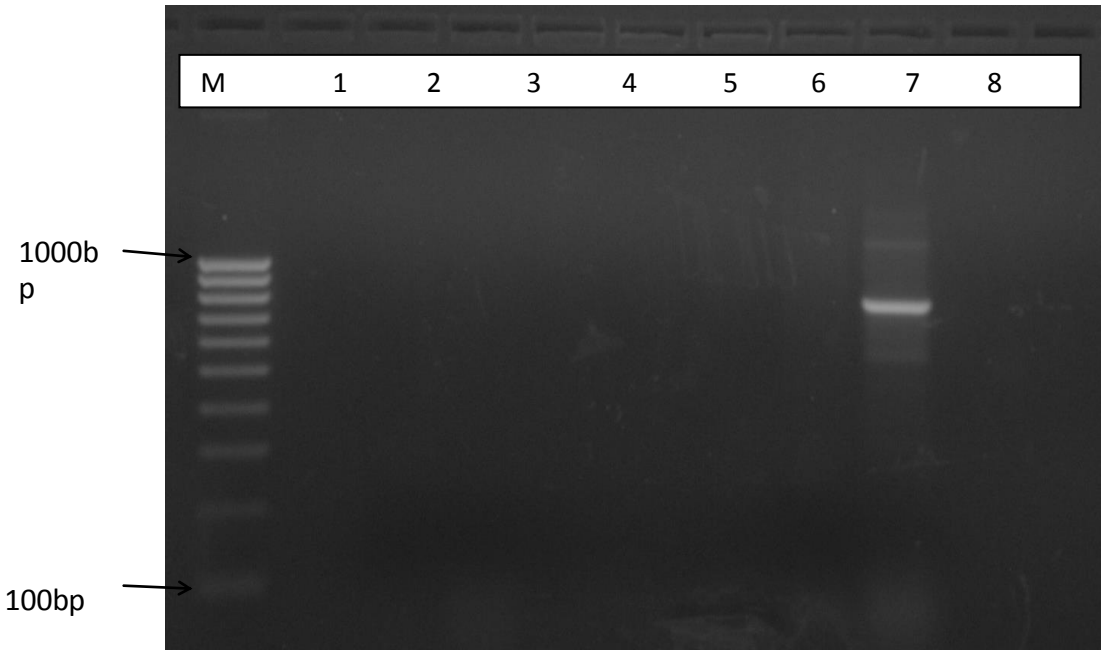


Plate 4.4: Gel electrophoresis of the multiplex-PCR products of STEC pathotype containing *eae* virulence genes

Lane M represented100 bp 5µl Hyperladder IV (Bioline H4K5) of upto 1000bp. In lanes 1 to 7 no genes were amplified representing that isolates no. 436R, 230C, 432R, 212C, 225C, 233R and 514C respectively lacked verotoxins. Lane 8, loaded with sample isolate no.261C, amplified *eae* at 775 bp. Lane 8 was loaded with sterile distilled water and used as a negative control.

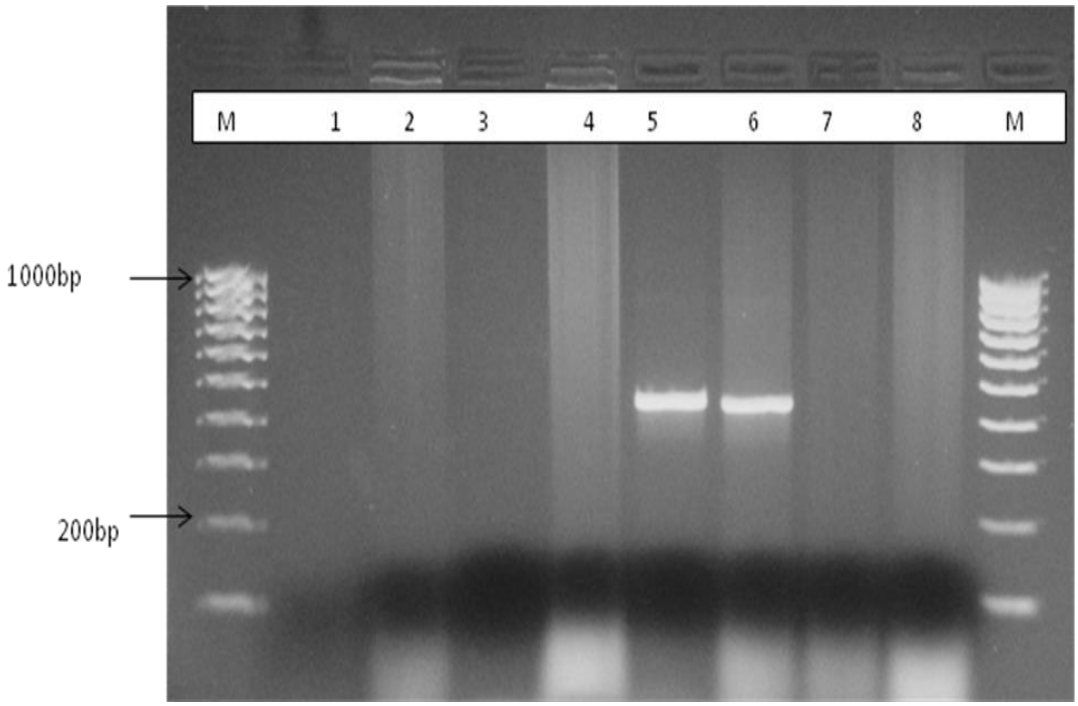


Plate 4.5: Gel electrophoresis of the multiplex-PCR products of STEC pathotype containing virulence genes *rfbEO157*

Lane M represented100bp 5µl Hyperladder IV (Bioline H4K5) of upto 1000bp. In lanes 1 to 4 and lane 7 no genes were amplified representing that isolates no.509R, 260R, 450C, 214R and 265C respectively lacked verotoxins. Lane 5 and 6,loaded with sample isolate no.486C and 500R respectively, both amplified *rfbE O157* genes at 470 bp. Lane 8 was loaded with sterile distilled water and used as a negative control.

CHAPTER FIVE

DISCUSSION

5.1 Prevalence of shiga toxigenic Escherichia coliO157 strains in cattle for slaughter in Nairobi abattoirs

At a prevalence of 6.78%, Northern region abattoirs of Nairobi had the highest prevalance of positive STEC isolates as compared to the Southern Region with 3.09% and the Eastern Region which had the lowest prevalence of 2.47%. Nyongara abbatoir in northern Nairobi had highest prevalence of STEC positive samples at 4.62%. This slaughter house in Dagoreti area proved to be the busiest public abbatoir during the field study with over two hundred cattle slaughtered per day hence contained the highest number of samples. Majority of the cattle are sourced from Northern Kenya Counties of Isiolo, Samburu and Marsabit. Other sources include the Rift valley counties of Kajiado and Narok. However six (1.85%) carcass samples as compared to nine (2.78%) rectal samples were positive for STEC, showing a moderate carcass contamination rate. Kiserian abbatoir in southern Nairobi had the least prevalence at 0.31% but also had a high carcass contamination rate where one (0.31%) carcass sample as compared to zero (0%) rectal samples was positive for STEC. Its immediate neighbor, Keekonyoke abattoir also showed high ratio of carcass contamination where four (2.16%) carcass samples as compared to three (1.85%) rectal samples were positive for STEC. Almost all cattle for slaughter in the two abattoirs are sourced from Maasai community of the Kajiado and Narok counties.

The Eastern region has the lowest prevalence due to the fact that it has two private abattoirs Cooperative slaughter house and Kenya Meat Commision. This caters for the country's export market and very strict international standards and policies have been implemented to minimize carcass contamination hence both showed low ratio of carcass contamination where only one carcass samples as compared to one rectal sample were

positive for STEC. Majority of cattle slaughtered originate from ranches in the vast Athi-river, and Kitengela area of Machakos, Kiambu and Kajiado counties and also from Northern regions of Kenya. The distribution of this pathogen during the study clearly differed by abattoirs and this can be explained by the fact that different abattoirs source their cattle for slaughter from different regions of the country. This eventually leads to different carcass contamination degree in each one of them for instance, the low contamination rate in private firms which source cattle from ranches.

In this study, a total of twenty one (6.48%) Non Sorbitol Fermenting (NSF) colonies of *E. coli* O157 were isolated from cattle carcasses and a total of nineteen (5.86%) NSF colonies of *E. coli* O157 isolated from rectal content of the same cattle. The reported prevalence in this study of 12.34 % is higher when compared to reports from other countries such as 5% from Philippines (Flores *et al.,* 2013) and 3.0% (4/132) of carcass samples in Ireland (Carney *et al.,* 2006). These previous studies show a wide range of isolation rates for *E. coli* O157 and *E. coli* O157:H7. Several factors that contribute to this include, diverse geographical origins of cattle, numbers of cattle, study design, number of herds and cattle, sex and age of cattle, abattoir conditions and treatment with antimicrobial substances during the process (Carney *et al.,* 2006). For instance, it has been reported that the prevalence of *E. coli* O157 varies with the seasons, generally increasing in the warm months of March-September in the northern hemisphere (Inat and Siriken, 2013; Varela-Hernandez *et al*., 2007). The variations may also be due, at least in part, to the sensitivity of the method used for detection. Indeed, the detection of *E. coli* O157:H7 from cattle fecal samples is known to be very difficult due to their low concentration. Therefore, direct inoculation of samples onto plates is not sensitive enough. Several enrichment culturing methods and isolation methods have been developed to counter this problem (Inat and Siriken, 2013).One of the more sensitive methods is IMS technique (Vidovic *et al.,* 2007), which has been employed in this present study that was not used in the past studies.

Ingestion of *E. coli* O157:H7 from an environmental source without establishment of a colonization site within the animal results in transient shedding of thebacteria in feces for a few days (Chase-Topping *et al.*, 2007). Hence, this present study; swab samples were collected from the rectum of the cattle instead of direct feces samples, because the recto-anal junction mucosa has been identified as the primary site of *E. coli* O157:H7 colonization in cattle (Sheng *et al.*, 2006; Varela-Hernandez *et al.*, 2007). In attempting to manage *E. coli* O157 and *E. coli* O157:H7 contamination in abattoirs, it is crucial to consider cross contamination during slaughter. *Escherichia coli* O157 have been reported to spread easily onto carcass surfaces from the hide or during evisceration (Inat and Siriken, 2013; Arthur *et al.*, 2007). The results of the present study support that hypothesis, with thirteen rectal and carcass samples of the same animal being positive. This is further supported from the fact that six carcass samples were positive with *E.coli* O157 strains whereas the rectal swabs of the same cattle did not show any pathogen. The finding that *E. coli* O157 strains are present in 12.96% in cattle carcasses from Nairobi slaughterhouses has important implications on public health sector of Kenya. Cattle are the main reservoir for Shiga toxin producing *E. coli* strains causing the hemolytic syndrome in humans (Ferens and Hovde, 2011) and frequently excrete these bacteria in their feces (Chase-Topping *et al.*, 2007).The major route of STEC transmission is indirect, by contaminated food but also, by direct transmission from animals to humans by contact (Hale *et al.*, 2012). The origins and subsequent rate at which carcass contamination with *E. coli O157* occurs have not been well established, but Hazard Analysis-Critical Control Point system is being used to decrease the risk of food borne illnesses. Hence, strict policies put in place to all abattoirs on minimal contamination of carcass with the gastric contents are essential.

5.2 Antimicrobial susceptibility of *E. coli O157* and other serotypes

One of the major problems that accompany *E. coli* O157:H7 infection is the danger of treating such patients with antibiotics (Panos *et al.*, 2006).Treating *E. coli O157:H7* infections may result in the release of shigatoxins into the blood stream of the infected individuals. It is believed that the release of such toxins affects the kidneys resulting in hemolytic uremic syndrome (Bielaszewska *et al.,* 2011). This therefore presents a great challenge in the treatment approach to be adopted in the case of *E. coli* O157:H7 infections.

The majority (97.5%) of *E. coli* isolateswere susceptible to Gentamycin, Nalidixic acid, Ceftriaxone, Ceftazidime, Ciprofloxacin and Cotrimoxazole. Fewer (5%) isolates were resistant to Cephalosporins and none to Gentamycin. These findings concur with data from several previous studies which have found that resistance to tetracycline derivatives, sulfa drugs, cephalosporins, and penicillin is common among *E. coli* isolated from food animals and meats (Tadesse *et al.*, 2011). These findings were in consistence with a study whereby resistance to tetracycline was prevalent (Dierikx *et al.*, 2012).

The genetic basis of resistance has been shown to be due to the presence of class 1 integrons conferring multi-drug resistance (Gaze *et al.*, 2005). A high level of resistance was observed against co-amoxiclav with only 55% being susceptible. The beta lactam antibiotics are the most frequently prescribed antimicrobial agents in clinical practice. It's worrying that most strains analyzed were resistant to the drugs used as 'first line' antibiotics in Kenya. In most developing countries, including Kenya, Ampicilin, Streptomycin and Sulphamethoxazole are used as 'first line' antibiotics for treating most bacterial infections (Kariuki *et al.*, 2006).

During this study, some rectal and carcass isolates from same cattle show different resistance to different antibiotics, whereas, some rectal and carcass isolates from different cattle show same resistance. This suggests cross contamination during

slaughter and spread of particular resistant isolate onto carcass surfaces from the hide or during evisceration (Arthur *et al.*, 2007). Resistance in *E.coli* may be transferrable to other gut flora, raising serious public health concern. The frequent occurrence of β lactamase genes in readily transmissible plasmids and their possible integration into bacterial chromosomes is of concern in the management of antimicrobial therapy in the community and in hospital centers.

In this study, the prevalence of resistant isolates from cattle may be attributed to antibiotic use in animal husbandry. This was not established by collecting data from farmers selling cattle for slaughter in Nairobi but it has been known to be a contributing factor to resistance in animal food bacterial isolates (Moneoang and Bezuidenhout, 2009). In 2000, WHO addressed the issue of antimicrobial overuse in food animals by publishing ‘‘global principles for the containment of antimicrobial resistance in animals intended for food’’ (WHO, 2000). One major requirement of the program is the surveillance of antimicrobial resistance in ‘‘indicator bacteria’’. *E. coli* is well suited for this purpose as it easily acquires resistance and is common in many different species (Bengtsson *et al.*, 2006). Increasing resistance in *E. coli* as an ‘‘early warning system’’ for the potential development of resistance in human pathogens. Thus it is wise to abolish the use of antimicrobial drugs as feed additives and promote alternative strategies like mass vaccination of husbandry, new feeding systems and improved management practices (Oguttu *et al.*, 2008) in order to minimize the need for use of antimicrobials in animal husbandry (Moneoang and Bezuidenhout, 2009).

5.3 Prevalence and distribution of virulence genes among isolated STEC serotypes from carcasses in different Nairobi abattoirs by use of PCR

Routine biochemical and serological tests for pathogen identification can only type the species or serogroup level, but cannot directly characterize virulence genes neither do they provide any information about potential pathogenicity of STEC serotypes. Serotyping may generate false-negative since H7 and O157 antigens may not be

expressed. In 2009, Narang found that, out of 100 meat isolates, 42 were H7 negative by latex agglutination but H7 positive by PCR detection (Narang *et al.*, 2009). Several genetic makers such as *rfbE, fliC* and certain virulent as attachment and effacing gene *eaeA, stx*$_1$ and *stx*$_2$ are useful targets for multiplex PCR amplification and differentiation of pathogen (Carney *et al.*, 2006).

The present study was able to establish various virulence factors in STEC serotypes isolated rectal swabs and carcass swabs from various slaughterhouses in Nairobi.Thirty one isolates from 40 tested amplified various virulence genes. Two isolates out of 40 were confirmed to be STEC strains O157:H7 with all four genes detected *rfbE, fliC, eaeA, stx*$_1$ and *stx*$_2$. This signifies that these strains produce Shiga toxins and harbor large plasmids which code for production of intimin encoding gene (*eaeA*).

Shiga toxin production alone does not seem to confer human pathogenicity on STEC. For instance, in this study isolate lab no. 223C, only stx$_2$ genes was detected, signifying its unlikely for this strain of *E.coli* to cause disease in humans. However, if combined with presence of other virulence genes like *eaeA* the serotype is often associated with disease in humans. However, there have been reports of an HUS-causing *E. coli* strain that lacks *eaeA* (Croxen *et al.*, 2013).

VT1 is a relatively uniform family of toxins (Melton-Celsa *et al.*, 1998), whereas the VT2 family is more diverse, comprising the variants VT2, VT2b, VT2c, VT2d, VT2e, VT2f and VT2g (Perrson *et al.*, 2007). The VT2 genotype of a strain apparently influences its pathogenic ability and the variant vtx2 has been found to be significantly more common in strains isolated from patients who had developed haemolytic uraemic syndrome and haemorrhagic colitis than did the other vtx gene variants (Aspan and Eriksson, 2010).

Figure 4.4 in chapter 4 show gene encoding somatic O (*rfbE)* as the predominant signifying success in our immunomagnetic separation which is a sensitive technique for targeting target O157 strains. In this study *rfbE* gene was detected as the only gene in eight isolates indicating the strains lacked gene encoding flagella H7. While the main EHEC serotype is *E. coli* O157:H7 other serotypes such as O111:H8 and O104:H21 are also diarrheogenic in humans (Ojo *et al.*, 2010). Some of these strains may carry virulence genes which produce shigatoxins as evident in our study where two strains had only *stx2* virulence genes but were not O157 strains.

In this study, virulence genes were detected in four carcass isolates **(Appendix 4),** no virulence genes was detected in the rectal isolates of these same cattle suggesting- that STEC strains contaminate carcass yet they were not necessarily present in fecal matter of the same cattle. This confirms cross contamination of carcassesduring the dressing of carcasses during slaughter. This relates to public health issues in our abattoirs as various health issues were observed during the field study leading to this. For instance, it was noted that personnel shared dirty protective clothing from one individual to another without washing. Sanitation facilities were inadequate and drainage poorly constructed forming dirty pools of stagnant effluent. Water supply was scarce hence same pool of water been used to clean different carcasses. There were no clear hygienic guidelines for personnel to follow and strict enforcement of these standards were lacking. Taking the sample total number of each as 162, prevalence of STEC virulence genes in rectal isolates was 8.02% while in carcass isolates was 11.11%. Out of the carcass isolates, two (1.2%) were confirmed virulent strains of *E.coli* O157:H7.Though prevalence appears low, it is of significance as only very low doses are needed to cause infection. This finding is a major public health concern because when the environment and conditions are suitable, the virulence genes may link to form an entire complement capable of causing an outbreak. Necessary measures should be taken urgently in effect to minimize contamination of carcass during slaughter in abattoirs.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

Cattles slaughtered in Nairobi abattoirs harbor various STEC serotypes at a prevalence of 12.34% as seen from this study. In particular, cattle for slaughter harbor *E.coli* O157:H7 at a prevalence of 1 %.

E. coli serotypes from Nairobi slaughter houses exhibited antimicrobial resistance to various commonly used antibiotics. Differences in antibiotic resistance frequencies were detected in *E. coli* isolates, from different sources. Previous studies suggest that they may transfer this resistance to other intestinal bacteria. This raises a possible limitation of therapeutic options in treating enteric infections in humans in future.

Finding of dispersed virulence genes in *E. coli O157:H7* from cattle carcasses for slaughter strongly suggests their virulence potential as well as the fact they may act as reservoirs. When the environment and conditions are suitable, the virulence genes may link to form an entire complement capable of causing an outbreak.

6.2 Recommendations

1. Strict guidelines on food handling and safety should be enforced in abattoirs and personnel well trained in contamination risk.
2. Refurbishment of abattoirs is necessary ensuring modern infrastructure with good sanitation put in place, running clean water and working drainages.

3. Hazard Analysis-Critical Control Point system should be used as well as implementing, evaluating and validating antimicrobial interventions to reduce the presence of potential pathogenic microorganisms.
4. More restrictive policies on the use of antibiotics in animals may result in an improvement of the current resistant patterns. Enforcement of legislation on prudent use of antimicrobials in animals should be paramount.
5. Presence of other serotypes of *E.coli* O157 other than with Flagella H7 creates a necessity for further characterization of virulence genes that could lead to better understanding of epidemiology of outbreaks associated with *E.coli*. Further genetic analysis of bacteria isolates to understand the evolution of these infections over years is important in prevention and control of infectious diseases.

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APPENDICES

APPENDIX 1: General biochemical tests results for isolates obtained.

Isolate number	**Gram stain**	**Appearance on Sorbitol McConkey**	**TSI**			**Simmon's Citrate**	**MIO**			**Agglutination with *E. coli O157* kit latex**
			Slant	Butt	H_2S		M	I	O	
212R	-	NSF	Acid	Acid	-	-	+	+	+	+
212C	-	NSF	Acid	Acid	-	-	_	+	+	+
214R	-	NSF	Acid	Acid	-	-	+	+	_	+
223R	-	NSF	Acid	Acid	-	-	_	+	-	+
223C	-	NSF	Acid	Acid	-	-	-	-	+	+
225C	-	NSF	Acid	Acid	-	-	-	+	+	+
229R	-	NSF	Acid	Acid	-	-	-	+	+	+
230C	-	NSF	Acid	Acid	-	-	-	+	+	+
233R	-	NSF	Acid	Acid	-	-	-	+	+	+
234R	-	NSF	Acid	Acid	-	-	-	+	+	+
234C	-	NSF	Acid	Acid	-	-	-	+	+	+
240C	-	NSF	Acid	Acid	-	-	-	+	+	+
240R	-	NSF	Acid	Acid	-	-	-	+	+	+
260R	-	NSF	Acid	Acid	-	-	-	+	+	+
260C	-	NSF	Acid	Acid	-	-	-	+	+	+
261C	-	NSF	Acid	Acid	-	-	-	+	+	+
262R	-	NSF	Acid	Acid	-	-	-	+	+	+
265C	-	NSF	Acid	Acid	-	-	-	+	+	+
270R	-	NSF	Acid	Acid	-	-	-	+	+	+
270C	-	NSF	Acid	Acid	-	-	-	+	-	+

			Slant	Butt	H_2S		M	I	O	
278R	-	NSF	Acid	Acid	-	-	+	+	+	
278R	-	NSF	Acid	Acid	-	-	_	+	+	
303R	-	NSF	Acid	Acid	-	-	+	+	_	
410R	-	NSF	Acid	Acid	-	-	_	+	-	+
418R	-	NSF	Acid	Acid	-	-	-	-	-	-
410C	-	NSF	Acid	Acid	-	-	-	+	-	+
414C	-	NSF	Acid	Acid	-	-	-	+	-	+
415C	-	NSF	Acid	Acid	-	-	-	+	-	+
447R	-	NSF	Acid	Acid	-	-	-	+	-	+
432R	-	NSF	Acid	Acid	-	-	-	+	-	+
430R	-	NSF	Acid	Acid	-	-	-	+	-	+
430C	-	NSF	Acid	Acid	-	-	-	+	-	+
486R	-	NSF	Acid	Acid	-	-	-	+	-	+
436R	-	NSF	Acid	Acid	-	-	-	+	-	+
450C	-	NSF	Acid	Acid	-	-	-	+	-	+
486C	-	NSF	Acid	Acid	-	-	-	+	-	+
500C	-	NSF	Acid	Acid	-	-	-	+	-	+
509R	-	NSF	Acid	Acid	-	-	-	+	-	+
500R	-	NSF	Acid	Acid	-	-	-	+	-	+
514C	-	NSF	Acid	Acid	-	-	-	+	-	+

M-Motility **+ positive** **O-ornithine**

I-Indole **- negative** **NSF-non-sorbitol**

APPENDIX 2: Antimicrobial susceptibility testing standard zones of inhibition CLSI, (2008).

Antibiotics	Resistant	Intermediate	Sensitive
Ciprofloxacin (5µg)	≤15	16-20	≥21
Gentamicin (10µg)	≤12	13-14	≥15
Ampicillin (10µg)	≤13	14-16	≥17
Chloramphenicol (30ug)	≤12	13-17	≥18
Augmentin (30µg)	≤13	14-17	≥18
Kanamycin (30µg)	≤14	15-17	≥18
Tetracycline (30ug)	≤11	12-14	≥15
Nalidixic Acid (30µg)	≤13	14-18	≥19
Ceftazidime (30ug)	≤14	15-17	≥18
Cotrimoxazole (30µg)	≤10	11-15	≥16
Ceftriaxone (30µg)	≤13	14-20	≥21
Streptomycin (10µg)	≤10	12-14	≥15

APPENDIX 3: Resistance patterns (disk inhibition zone diameters in mm) for *E. coli* isolates.

LAB NO.	CAZ	S	CN	AMC	AMP	C	NA	TE	CRO	SXT	CIP	K
ATCC 25922	(S)24	(S)25	(S)27	(S)29	(S)22	(S)25	(S)26	(S)24	(S)24	(S)25	(S)22	(S)21
212R	(S)19	(I)13	(S)17	(S)24	(I)15	(S)24	(S)24	(S)16	(S)20	(S)19	(S)24	(S)19
212C	(S)24	(R)6	(S)15	(S)24	(I)15	(S)24	(S)24	(S)16	(S)21	(S)24	(S)24	(S)24
214R	(S)20	(I)13	(S)17	(R)6	(R)6	(S)22	(I)14	(S)17	(S)24	(S)20	(S)22	(S)24
223R	(S)22	(S)16	(S)16	(S)24	(S)22	(S)24	(S)19	(S)16	(S)24	(S)24	(S)24	(S)24
223C	(S)20	(R)6	(S)17	(R)6	(R)6	(S)23	(S)20	(R)6	(S)19	(S)22	(S)23	(I)16
225C	(S)20	(S)19	(S)18	(S)24	(R)6	(S)24	(S)19	(R)6	(S)19	(R)6	(S)24	(S)24
230R	(S)19	(I)12	(S)17	(S)24	(S)17	(S)20	(S)19	(S)15	(S)19	(S)24	(S)21	(I)17
230C	(S)19	(I)12	(S)17	(I)24	(I)14	(S)20	(S)22	(S)20	(S)19	(S)22	(S)24	(I)24
233R	(S)20	(I)12	(S)16	(I)24	(R)6	(S)19	(I)14	(S)21	(S)18	(S)23	(S)24	(S)24
234R	(S)19	(R)6	(S)17	(R)6	(R)6	(S)24	(S)24	(R)11	(S)24	(R)6	(S)26	(I)17
234C	(S)21	(S)20	(S)17	(S)18	(I)15	(S)24	(S)24	(S)17	(S)24	(S)19	(S)24	(S)24
240C	(S)22	(S)16	(S)19	(S)24	(I)16	(S)24	(S)24	(S)18	(S)19	(S)24	(S)26	(S)24
240R	(S)20	(S)16	(S)17	(I)24	(R)6	(S)24	(I)15	(S)16	(S)22	(S)21	(S)24	(S)24
260R	(S)21	(S)21	(S)18	(S)24	(S)24	(S)19	(S)24	(S)16	(S)24	(S)24	(S)26	(S)24
260C	(S)24	(I)13	(S)20	(S)20	(S)19	(S)19	(S)19	(R)6	(S)19	(S)24	(S)24	(I)17
261C	(R)6	(I)12	(S)21	(S)20	(R)6	(S)19	(S)19	(R)6	(I)24	(S)24	(S)24	(S)24
262R	(S)20	(S)20	(S)15	(R)6	(R)6	(S)18	(S)20	(S)17	(S)18	(S)18	(S)25	(S)24
265C	(S)23	(R)6	(S)17	(I)15	(I)16	(S)20	(S)20	(S)15	(S)18	(S)18	(S)25	(I)17
270R	(S)24	(I)14	(S)15	(R)6	(R)6	(S)19	(S)20	(S)19	(S)18	(S)18	(S)25	(S)24
270C	(S)23	(I)13	(S)18	(S)18	(I)16	(S)19	(S)20	(S)20	(S)18	(S)16	(S)25	(S)21
278R	(S)22	(I)13	(S)16	(S)19	(S)19	(S)20	(S)20	(R)6	(S)19	(S)18	(S)25	(S)24
278C	(S)24	(I)13	(S)18	(S)19	(S)18	(S)18	(S)20	(S)19	(S)17	(S)17	(S)25	(I)17
303R	(S)24	(S)24	(S)15	(I)15	(R)6	(S)19	(S)19	(S)20	(S)18	(S)18	(S)21	(S)24
410R	(S)23	(S)20	(S)18	(S)20	(S)19	(S)19	(S)20	(R)6	(S)19	(S)17	(S)24	(S)24

418R	(S)24	(S)24	(S)17	(S)17	(R)6	(S)19	(S)18	(S)17	(S)19	(S)18	(S)24	(S)24
410C	(S)20	(I)12	(S)17	(R)6	(R)6	(S)18	(S)20	(R)6	(S)20	(R)(6)	(S)24	(S)24
414C	(R)6	(R)6	(S)17	(S)19	(S)20	(S)20	(S)20	(R)6	(I)24	(S)17	(S)24	(S)22
415C	(S)24	(S)18	(S)24	(I)15	(R)6	(S)20	(S)19	(S)17	(S)18	(S)18	(S)27	(S)24
414R	(S)24	(I)13	(I)13	(I)15	(R)6	(S)19	(S)20	(S)15	(S)18	(S)17	(S)24	(I)17
432R	(S)21	(S)24	(S)24	(S)24	(S)18	(S)19	(S)20	(S)17	(S)18	(S)18	(S)24	(I)17
430R	(S)19	(S)17	(S)16	(I)15	(R)6	(S)24	(S)19	(S)16	(S)24	(S)19	(S)22	(S)24
430C	(S)20	(S)19	(S)19	(S)20	(S)24	(S)20	(S)20	(R)6	(S)24	(S)17	(S)25	(S)22
486R	(S)21	(S)19	(S)16	(S)19	(I)16	(S)24	(S)20	(S)20	(S)24	(S)19	(S)22	(S)24
436R	(S)19	(S)20	(S)16	(S)19	(S)20	(S)20	(S)21	(S)20	(S)24	(S)17	(S)24	(S)22
450C	(S)19	(S)19	(S)15	(R)6	(R)6	(S)24	(S)20	(S)16	(S)24	(S)19	(S)24	(S)24
486C	(S)20	(S)20	(S)16	(R)6	(R)6	(S)24	(S)20	(S)20	(S)24	(S)18	(S)24	(S)23
500C	(S)24	(I)14	(S)17	(R)6	(R)6	(S)24	(S)20	(S)19	(S)24	(S)19	(S)27	(I)16
509R	(S)23	(S)19	(S)16	(S)19	(S)24	(S)20	(S)20	(S)20	(S)24	(S)17	(S)22	(S)19
500R	(S)19	(I)13	(S)19	(S)19	(I)15	(S)24	(S)19	(R)6	(S)24	(S)18	(S)24	(S)24
514C	(S)19	(S)22	(S)16	(R)6	(R)6	(S)22	(S)20	(S)20	(S)24	(S)19	(S)23	(S)20

KEY:

CAZ-Ceftazidime, **S**-Streptomycin, **CN**-gentamycin,
AMP-Ampicillin, **AMC**-Amoxycillin/ Clavulanic Acid,
C-Chloramphenicol, **NA**-Nalidixic acid, **TE**-Tetracycline,
CRO-Ceftriaxone, **SXT**-Sulphamethoxazole, **CIP**-Ciprofloxacin,
K-Kanamycin

APPENDIX 4: Virulence gene distribution among *E. coli* isolates.

Isolate No.	Virulence genes				
	stx2	***stx1***	***eae***	***rfb* E_{O157}**	***flic*** $_{H7}$
212R	-	-	-	-	-
212C	-	-	-	-	-
214R	-	-	-	-	-
223R	-	-	-	+	-
223C	+	-	-	-	-
225C	-	-	-	-	-
230R	+	-	-	-	-
230C	-	-	-	-	-
233R	-	-	-	-	-
234R	+	+	+	+	+
234C	-	-	-	-	-
240R	-	-	-	-	-
240C	+	+	+	+	+
260R	-	-	-	-	-
260C	-	-	-	+	-
261C	-	-	+	-	-
262R	-	-	-	+	-
265C	-	-	-	-	-
270R	-	-	-	+	-
270C	-	-	-	+	-
278C	-	-	-	-	-
278R	-	+	-	+	-
303R	-	-	-	-	-
410R	-	-	-	-	-

410C	+	+	+	+	+
418R	-	-	-	-	-
414R	-	-	-	-	-
414C	-	-	+	-	-
415C	-	-	-	-	-
432R	-	-	-	-	-
430R	-	-	+	-	-
430C	-	-	-	-	-
436R	-	-	-	-	-
451C	-	-	-	-	-
486R	-	-	-	+	-
486C	-	-	-	+	-
500R	-	-	-	+	-
500C	-	-	-	+	-

APPENDIX 5: Description of samples collected in different slaughter houses in Nairobi.

LAB NO	DATE	SOURCE	SAMPLE TYPE	Location	Study site
210	22/7/2010	Nyongara S. H.	Rectal swab & carcass swab	Dagoretti	Northern
211	22/7/2010	Nyongara S. H	Rectal swab & carcass swab	Dagoretti	Northern
212	22/7/2010	Nyongara S. H	Rectal swab & carcass swab	Dagoretti	Northern
213	22/7/2010	Nyongara S. H	Rectal swab & carcass swab	Dagoretti	Northern
214	22/7/2010	Nyongara S. H	Rectal swab & carcass swab	Dagoretti	Northern
215	22/7/2010	Nyongara S. H	Rectal swab & carcass swab	Dagoretti	Northern
216	22/7/2010	Nyongara S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
217	22/7/2010	Nyongara S. H	Rectal swab & carcass swab	Dagoretti	Northern
218	22/7/2010	Nyongara S. H	Rectal swab & carcass swab	Dagoretti	Northern
219	22/7/2010	Nyongara S. H	Rectal swab & carcass swab	Dagoretti	Northern
220	2/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
221	2/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
222	2/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
223	2/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
224	2/8/2010	Mumo S. Hse.	Rectal swab &	Dagoretti	Northern

			carcass swab		
225	2/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
226	2/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
229	2/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
230	9/8/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
231	9/8/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
232	9/8/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
233	9/8/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
234	9/8/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
235	9/8/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
236	6/7/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
237	6/7/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
238	6/7/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
239	6/7/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
240	6/7/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
241	6/7/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
242	6/7/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
243	23/8/2010	Mumo S. Hse.	Rectal swab &	Dagoretti	Northern

			carcass swab		
244	23/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
245	23/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
246	23/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
247	23/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
250	23/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
251	23/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
252	23/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
253	23/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
258	23/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
259	23/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
260	23/8/2010	Mumo S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
261	23/8/2010	Keekonyoke S. Hse	Rectal swab & carcass swab	Kiserian	Southern
262	23/8/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
263	23/8/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
264	23/8/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
265	23/8/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
266	23/8/2010	Keekonyoke S.	Rectal swab &	Kiserian	Southern

		Hse.	carcass swab		
267	23/8/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
268	23/8/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
269	23/8/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
270	13/9/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
271	13/9/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
272	13/9/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
273	13/9/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
274	13/9/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
275	13/9/2010	Thiani S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
276	13/9/2010	Thiani S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
277	13/9/2010	Thiani S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
278	13/9/2010	Thiani S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
279	13/9/2010	Thiani S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
292	13/9/2010	Thiani S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
293	13/9/2010	Thiani S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
294	13/9/2010	Thiani S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
295	13/9/2010	Thiani S. Hse.	Rectal swab &	Dagoretti	Northern

			carcass swab		
296	13/9/2010	Thiani S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
297	13/9/2010	Thiani S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
298	13/9/2010	Thiani S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
299	13/9/2010	Thiani S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
300	13/9/2010	Thiani S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
301	20/9/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
302	20/9/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
303	20/9/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
308	20/9/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
309	20/9/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
310	20/9/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
311	4/10/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
312	4/10/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
313	4/10/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
314	4/10/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
315	4/10/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
316	4/10/2010	NjiiruS.H	Rectal swab &	Kayole	Eastern

			carcass swab		
317	4/10/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
318	4/10/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
319	4/10/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
320	4/10/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
321	12/10/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
322	12/10/2010	Nyongara S.H.	Rectal swab & carcass swab	Dagoretti	Northern
410	12/10/2010	Nyongara S. Hse.	Rectal swab & carcass swab	Dagoretti	Northern
411	18/10/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
412	18/10/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
413	18/10/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
414	18/10/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
415	18/10/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
418	18/10/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
420	18/10/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
423	2/11/2010	cooperative S.H	Rectal swab & carcass swab	Dagoretti	Northern
424	2/11/2010	cooperative S.H	Rectal swab & carcass swab	Dagoretti	Northern
425	2/11/2010	cooperative S.H	Rectal swab &	Dagoretti	Northern

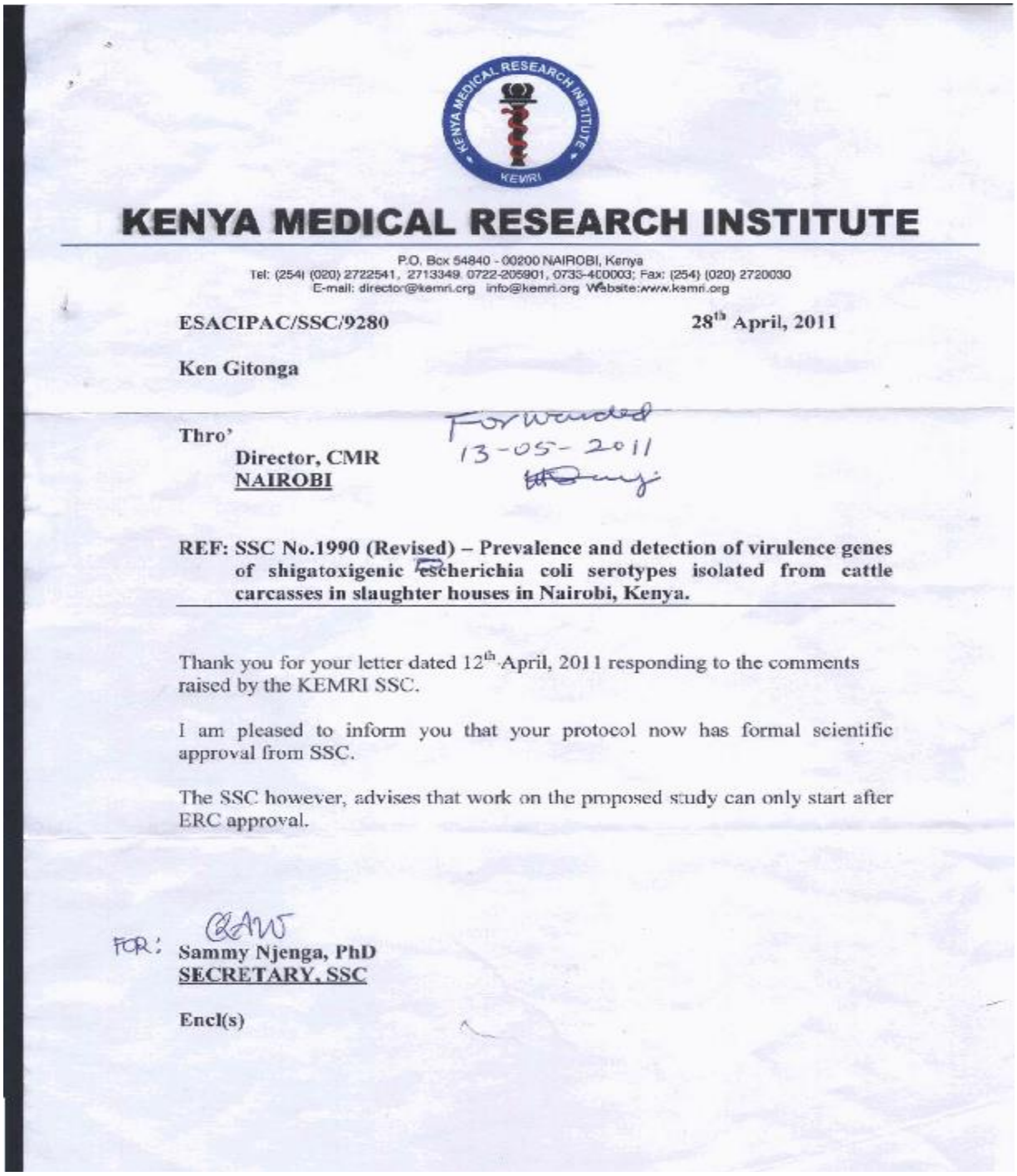
			carcass swab		
426	2/11/2010	cooperative S.H	Rectal swab & carcass swab	Dagoretti	Northern
427	2/11/2010	cooperative S.H	Rectal swab & carcass swab	Dagoretti	Northern
428	2/11/2010	cooperative S.H	Rectal swab & carcass swab	Dagoretti	Northern
429	2/11/2010	cooperative S.H	Rectal swab & carcass swab	Dagoretti	Northern
430	2/11/2010	cooperative S.H	Rectal swab & carcass swab	Dagoretti	Northern
431	8/11/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
432	8/11/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
433	8/11/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
434	8/11/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
435	8/11/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
436	8/11/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
439	8/11/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
440	8/11/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
441	8/11/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
442	8/11/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
444	8/11/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
446	8/11/2010	NjiiruS.H	Rectal swab &	Kayole	Eastern

			carcass swab		
447	8/11/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
449	8/11/2010	NjiiruS.H	Rectal swab & carcass swab	Kayole	Eastern
451	15/11/2010	Kiserian S.H	Rectal swab & carcass swab	Kiserian	Southern
453	15/11/2010	Kiserian S.H	Rectal swab & carcass swab	Kiserian	Southern
454	15/11/2010	Kiserian S.H	Rectal swab & carcass swab	Kiserian	Southern
455	15/11/2010	Kiserian S.H	Rectal swab & carcass swab	Kiserian	Southern
456	15/11/2010	Kiserian S.H	Rectal swab & carcass swab	Kiserian	Southern
457	15/11/2010	Kiserian S.H	Rectal swab & carcass swab	Kiserian	Southern
458	15/11/2010	Kiserian S.H	Rectal swab & carcass swab	Kiserian	Southern
459	15/11/2010	Kiserian S.H	Rectal swab & carcass swab	Kiserian	Southern
460	15/11/2010	Kiserian S.H	Rectal swab & carcass swab	Kiserian	Southern
468	15/11/2010	Kiserian S.H	Rectal swab & carcass swab	Kiserian	Southern
470	15/11/2010	Kiserian S.H	Rectal swab & carcass swab	Kiserian	Southern
471	15/11/2010	Kiserian S.H	Rectal swab & carcass swab	Kiserian	Southern
477	15/11/2010	Kiserian S.H	Rectal swab & carcass swab	Kiserian	Southern
478	15/11/2010	Kiserian S.H	Rectal swab & carcass swab	Kiserian	Southern
479	15/11/2010	Kiserian S.H	Rectal swab &	Kiserian	Southern

			carcass swab		
480	15/11/2010	Kiserian S.H	Rectal swab & carcass swab	Kiserian	Southern
481	17/11/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
482	17/11/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
483	17/11/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
484	17/11/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
485	17/11/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
486	17/11/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
487	17/11/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
494	17/11/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
495	17/11/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
496	17/11/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
497	17/11/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
498	17/11/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
499	17/11/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
500	17/11/2010	Keekonyoke S. Hse.	Rectal swab & carcass swab	Kiserian	Southern
506	22/11/2010	KMC	Rectal swab & carcass swab	Athi River	Eastern
507	22/11/2010	KMC	Rectal swab &	Athi River	Eastern

			carcass swab		
508	22/11/2010	KMC	Rectal swab & carcass swab	Athi River	Eastern
509	22/11/2010	KMC	Rectal swab & carcass swab	Athi River	Eastern
510	22/11/2010	KMC	Rectal swab & carcass swab	Athi River	Eastern
511	22/11/2010	KMC	Rectal swab & carcass swab	Athi River	Eastern
512	22/11/2010	KMC	Rectal swab & carcass swab	Athi River	Eastern
513	22/11/2010	KMC	Rectal swab & carcass swab	Athi River	Eastern
514	22/11/2010	KMC	Rectal swab & carcass swab	Athi River	Eastern
515	22/11/2010	KMC	Rectal swab & carcass swab	Athi River	Eastern
516	22/11/2010	KMC	Rectal swab & carcass swab	Athi River	Eastern
517	22/11/2010	KMC	Rectal swab & carcass swab	Athi River	Eastern
518	22/11/2010	KMC	Rectal swab & carcass swab	Athi River	Eastern

APPENDIX 6: KEMRI SCIENTIFIC STEERING COMMITTEE APPROVAL



KENYA MEDICAL RESEARCH INSTITUTE

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E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

ESACIPAC/SSC/9280 **28th April, 2011**

Ken Gitonga

Forwarded
13-05-2011

Thro'
Director, CMR
NAIROBI

REF: SSC No.1990 (Revised) – Prevalence and detection of virulence genes of shigatoxigenic escherichia coli serotypes isolated from cattle carcasses in slaughter houses in Nairobi, Kenya.

Thank you for your letter dated 12th April, 2011 responding to the comments raised by the KEMRI SSC.

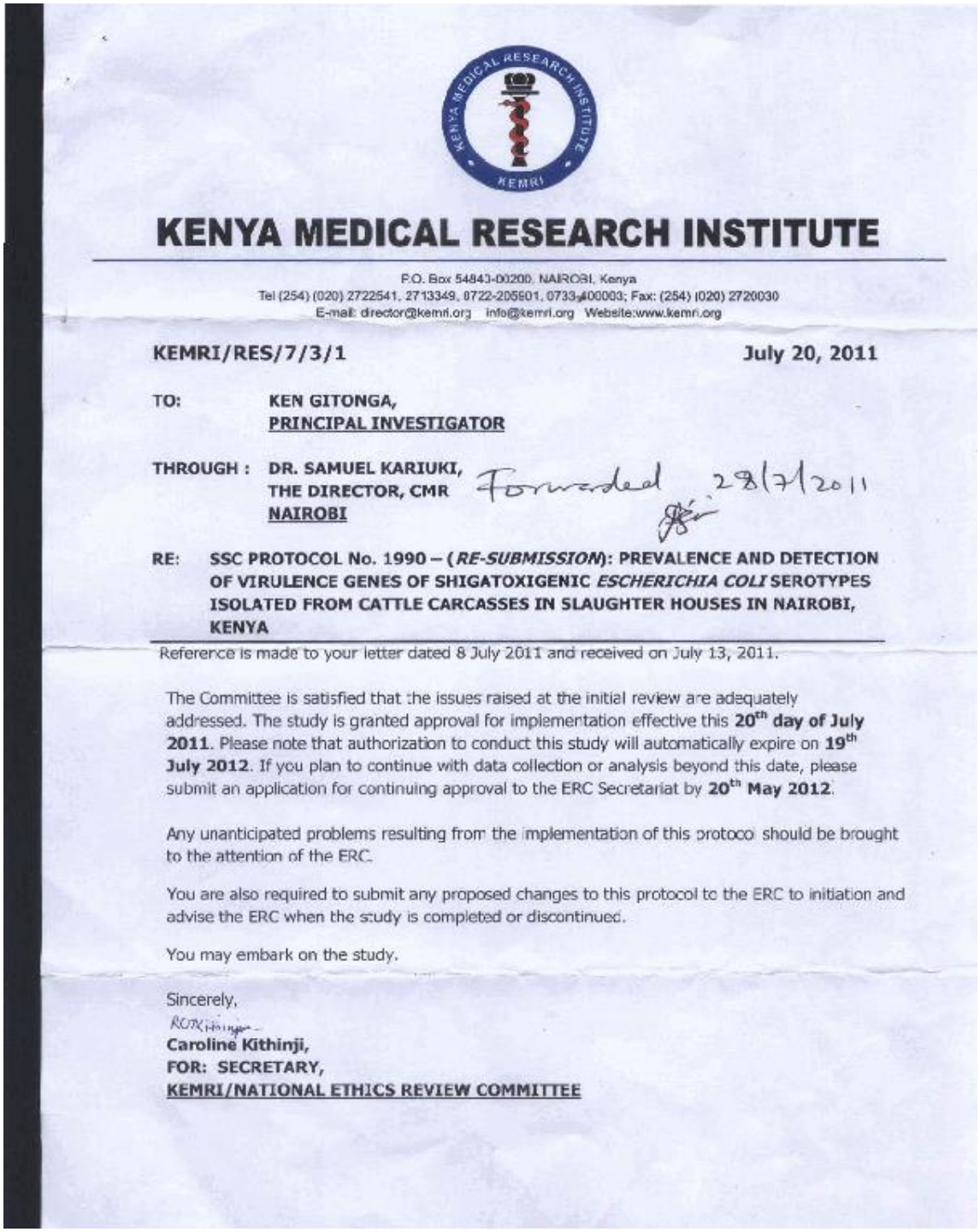
I am pleased to inform you that your protocol now has formal scientific approval from SSC.

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

FOR: **Sammy Njenga, PhD**
SECRETARY, SSC

Encl(s)

APPENDIX 7:NATIONAL ETHICS REVIEW COMMITTEE APROVAL



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KEMRI/RES/7/3/1 **July 20, 2011**

TO: **KEN GITONGA,**
PRINCIPAL INVESTIGATOR

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

Forwarded 28/7/2011

RE: **SSC PROTOCOL No. 1990 – (*RE-SUBMISSION*): PREVALENCE AND DETECTION OF VIRULENCE GENES OF SHIGATOXIGENIC *ESCHERICHIA COLI* SEROTYPES ISOLATED FROM CATTLE CARCASSES IN SLAUGHTER HOUSES IN NAIROBI, KENYA**

Reference is made to your letter dated 8 July 2011 and received on July 13, 2011.

The Committee is satisfied that the issues raised at the initial review are adequately addressed. The study is granted approval for implementation effective this **20th day of July 2011**. Please note that authorization to conduct this study will automatically expire on **19th July 2012**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **20th May 2012**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC.

You are also required to submit any proposed changes to this protocol to the ERC to initiation and advise the ERC when the study is completed or discontinued.

You may embark on the study.

Sincerely,

Caroline Kithinji,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE