MAKERERE  UNIVERSITY

PATHOTYPE AND ANTIBIOTIC RESISTANCE PROFILES OF *ESCHERICHIA COLI* ISOLATED FROM FAECES OF CHICKENS OF CENTRAL AND EASTERN REGIONS AND CATTLE FROM KAMPALA CITY ABATTOIR

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DECLARATION

I **Robert Egessa**, declare that this is my original work, and has not been published or presented for the award of any other degree in any institution.

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LIST OF ABBREVIATIONS

A/E	Attaching and Effacing
AAF	Aggregative adherence antigen
BFP	Bundle forming protein
cAMP	Cyclic adenosine monophosphate
CFA	Colonisation factor antigen
cGMP	Cyclic guanosine monophosphate
DAEC	Diffuse adhering *Escherichia coli*
DAF	Decay accelerating factor.
DEC	Diarrhoeagenic *Escherichia coli*
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EAF	Enteropathogenic adherence factor
EAEC	Enteroaggregative *Escherichia coli*
East	Enteroaggregative heat stable toxin
Efa1	EHEC factor for adherence
e.g.	For example
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
EsP(A-G)	EPEC secreted proteins A-G
et al	(*et alii*) and others
etc	(*et cetera*) and so forth
ETEC	Enterotoxigenic *Escherichia coli*
g	Gram

GB3	Globotriaosyl ceramide
HC	Haemorrhagic colitis
Hly	Hemolysin
HUS	Haemolytic uremic syndrome
Ipa	Invasion plasmid antigen
LEE	Locus of enterocyte effacement
LIFA	Lymphocyte inhibitory factor A
LT	Heat labile toxin
mg	Milligram
ml	Millilitre
MAP	Mitogen activated protein
ORF	Open reading frame
PAI	Pathogenicity island
ST	Heat stable toxin
STEC	Shiga toxin-producing *Escherichia coli*
Tir	Translocated intimin receptor
UK	United Kingdom
USA	United States of America
VT	Verocytotoxin
VTEC	Verocytotoxin-producing *Escherichia coli*
w/v	Weight per volume

ABSTRACT

Escherichia coli are zoonotic bacteria important to both public health and veterinary medicine. This study was carried out to identify the different diarrhoeal *E. coli* pathotypes and determine their resistance to selected antibiotic drugs in comparison to the non-pathotypes. A total of 288 faecal samples were collected from cattle and chickens. 24 STEC and four EPEC were detected in cattle and chicken faeces respectively. The 24 STEC either carried only stx_1 (8 %), stx_2 (37.5 %), or both stx_1 and stx_2 (42 %), stx_2 and *eaeA* (12.5 %) genes. Ten (41.7%) STEC were antibiotic resistant as follows: ciprofloxacin (12.5%), chloramphenicol (8.3%), nalidixic acid (8.3%) and tetracycline (33.3%). Of the four EPEC, only two were resistant, one to ciprofloxacin and the other to tetracycline. Comparison of antimicrobial resistance (AMR) between *E. coli* pathotypes and non-pathotypes showed that non-pathotypes were more resistant (79.8%) than the pathotypes (42.9%).

In conclusion, the fact that only EPEC was identified in healthy chickens and STEC in healthy cattle suggests presence of host preference factors for these pathotypes. Presence of such virulent pathotypes indicates a potential threat in the human food chain. Antibiotic resistant pathotypes were observed suggesting that healthy cattle & chicken faeces present a high risk of spreading antimicrobial resistant pathogenic *E. coli* to man, as well as indicating improper use of antimicrobial drugs.

I therefore recommend that; monitoring of hygiene in slaughter places and dairy processing plants, and continued surveillance of emerging AMR among the pathogenic strains especially in the healthy animals be carried out. Studies should be carried out to establish the relationship between pathotype carriage and host preference, and the distribution of antibiotic resistance genes among *E. coli* pathotypes of chickens, cattle and humans.

CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

In Africa, especially in the rural areas, people traditionally keep livestock such as poultry, cattle, goats and pigs in close proximity to the homestead or even inside the domicile especially for the case of poultry. In such situations, humans and livestock come into close contact both directly and through cross contamination of the shared environment. It was estimated that 75% of emerging human infectious diseases are zoonotic, with livestock serving as reservoirs of infection (Rwego *et al.*, 2008).

Escherichia coli (*E. coli*) infection is not an exception to this observation because many of the pathogenic strains have been isolated from animals, with most studies showing that cattle are a major reservoir of highly virulent STEC serotypes (Kaddu-Mulindwa *et al.*, 2001; Majalija *et al.*, 2007). Pathogenic *E. coli* strains isolated from humans and animals within the same locality have been shown to be genetically similar (Rwego *et al.*, 2008, Majalija *et al.* 2007), clearly indicating that these pathotypes in animals infect humans as well. *Escherichia coli* is therefore of special concern as a livestock-associated bacterial zoonosis and contact between humans and livestock may increase the risks of *E. coli* transmission to humans.

Pathogenic *E. coli* are associated with intestinal and extra-intestinal infections. The diarrhoea causing *E. coli* (DEC) have been categorized into six pathotypes which include; enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E.*

coli (EIEC) and diffusely adherent *E. coli* (DAEC) (Kaper *et al.*, 2004; Bando *et al.*, 2009).

Antimicrobial drugs have been since the mid-20th century used to treat and control bacterial infections. Overuse of these antimicrobial agents has resulted in the emergence of drug resistant strains as a result of selection in favour of those able to survive. It has been suggested that the evolution of resistance genes in the presence of antimicrobial agents do not depend on phylogenetic, ecological or geographical boundaries. Hence antimicrobial use and the resulting resistance in one ecological niche may have consequences for resistance in another niche (FAO /WHO/OIE, 2003).

However, much of these significant advances in our understanding of *E. coli* transmission and antimicrobial resistance come from developed nations, and little is known in the developing countries like Uganda. Studies on pathogenic agents carried in animals reared in close contact with humans are therefore important in widening our understanding of zoonotic transmission in developing countries.

1.2 Statement of the problem

Much as *E. coli* is a commensal organism, pathogenic strains have emerged and cause serious diarrhoeal disease in humans and animals. Animals such as cattle in close contact with humans have been implicated in shedding of pathogenic *E. coli* for human infections. Chicken and cattle are reared in close proximity and are in close contact with humans; yet few studies have been performed to show the *E. coli* pathotypes in faecal samples of such animal species in Uganda. Available information appears to show that animal species can harbour any pathotype depending on the ecology and ability of such animal species to favour such virulence genes of particular pathotypes. The relationship between *E. coli* pathotypes in selected animal species (cattle and chicken) and their response to selected antibiotics in Uganda is lacking. Since resistance is spreading between humans, animals and environment, there is need to explore the distribution of resistant bacteria in animals that are in close contact with humans and more so studies on prevalence of resistance in animal pathogens.

1.3 Objectives of the study

1.3.1 General objective

This study aimed at identifying the different diarrhoeal *E. coli* pathotypes isolated from faecal samples of cattle and chicken, and determining the extent to which these pathotypes are resistant to commonly used antibiotic drugs in comparison to the non-pathogenic ones.

1.3.1.1 Specific objectives

- To investigate the distribution of different diarrhoeal *E. coli* pathotypes and virulence genes carried in faecal samples of cattle and chickens.

- To determine the extent to which *E. coli* pathotypes isolated from cattle and chickens are resistant to selected antibiotic drugs.

1.4 Hypotheses

- The distribution of different *E. coli* pathotypes in the different animals is similar.
- Resistance of pathogenic *E. coli* isolates to selected antibiotic drugs is low.
- Animal species as a source of pathogenic *E. coli* isolates affects the response of *E. coli* isolates to antibiotic drugs.
- There is an association between isolates with particular virulence genes or their subtypes and antibiotic resistance.

1.5 Significance of the study

This study was carried out to avail information on *E. coli* pathotype distribution in selected animal species of Uganda and their response on selected antibiotics. The findings therefore warrant a more critical appraisal of zoonotic pathogens in animal species under study. Such knowledge is relevant to the pharmaceutical industry, policy makers, public health and veterinary medicine for the control of such infections in future. Findings of this study are also relevant in providing insights into other studies on *E. coli* pathotype carriage in the healthy animals.

CHAPTER TWO

LITERATURE REVIEW

2.1 Classification of *E. coli*

The bacteria *E. coli* are facultative anaerobic intestinal bacteria of the genus *Escherichia* within the family *enterobacteriaceae* (Nataro & Kaper, 1998). A majority of strains of *E. coli* are nonpathogenic or commensal while the pathogenic strains appear to be increasing. The nonpathogenic strains maintain and support digestion in the host as well as defend them against enteric pathogens (Schierack *et al.*, 2006), but can cause infection in a situation where the host is immunosuppressed or when gastrointestinal barriers are violated (Nataro & Kaper, 1998).

The various pathotypes of *E. coli* are characterized by shared O (lipopolysaccharide, LPS) and H (flagella) antigens. The O and H antigens define serogroups or serotypes. Serogroups are defined by O antigen only while serotypes are defined by O and H antigens and sometimes K (capsular) antigens (Kaper *et al.*, 2004).

2.2 Biochemical properties of *E. coli*

E. coli are easily recovered from faecal samples on general or selective media by incubating at 37°C under aerobic conditions. Such media used may include: blood agar, a non-selective medium; MacConkey or eosin methylene-blue agars, which selectively grow members of the *Enterobacteriaceae* allowing differentiation of enteric organisms on the basis of morphology (Nataro & Kaper 1998; Murray *et al.*, 2003). Identification of *Enterobacteriaceae* is then carried out using biochemical tests such as indole, methyl red, citrate and Voges proskauer. For epidemiologic or clinical

purposes, *E. coli* strains are often selected from agar plates after presumptive visual identification. The indole test is positive in 99% of *E. coli* strains and is the single best test for differentiation of *E. coli* from other members of the *Enterobacteriaceae* (Nataro & Kaper 1998).

2.3 Transmission routes for Diarrhoeagenic *E. coli* (DEC)

Transmission is via the oral route and is food or water borne but person-to-person transmission contributes to the severity of the outbreaks. In humans, faecal contamination of food and drinking water serves as the major route of human *E. coli* infection. Infection of animals is a result of living in a contaminated environment (Kuhnert *et al.*, 1999).

2.4 Major Pathotypes of *E. coli*

The ability of DEC strains to cause disease is based on possession of specific virulence attributes (Kaper *et al.*, 2004; Stenutz *et al.*, 2005; Schierack *et al.*, 2006). These virulence attributes enable *E. coli* to colonise mucosal site, evade host defenses, multiply and damage the host (Nataro & Kaper, 1998). Pathogenic *E. coli* are divided into particular groups known as pathotypes based on their pathogenicity mechanisms, virulence markers, adhesion patterns to cultured epithelial cells and clinical symptoms provoked (Bando *et al.*, 2009). A pathotype is a group of strains of a single species that cause a common disease using a common set of virulence factors (Kaper *et al.*, 2004; Wiles *et al.*, 2008). The various *E. coli* pathotypes causing diarrhoeal disease have been described in detail in the following sections.

2.4.1 Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* was the first pathotype to be described as a cause of diarrhoea in children (Kaper *et al.*, 2004). It causes profuse watery diarrhoea in infants and individuals in developing countries (Higgins *et al.*, 1997; Kuhnert *et al.*, 1999; Clarke, 2001; Kaper *et al.*, 2004). Enteropathogenic *E. coli* also causes vomiting and fever besides watery diarrhea (Kuhnert *et al.*, 1999; Stenutz *et al.*, 2005). This pathotype is of two types; Typical or type I and atypical or type II, differing in the carriage of *bfp* gene (Albert *et al.*, 2009).

Typical EPEC have both *eae* and *bfp* genes (Albert *et al.*, 2009) and have been established to be rarely isolated from animals (Farooq *et al.*, 2009) while atypical EPEC have only *eae* gene (Albert *et al.*, 2009) and have been demonstrated in both humans and animals including chicken, pigeons and ducks (Farooq *et al.*, 2009). The most prevalent serogroups within this group of *E. coli* associated with human and food borne outbreaks are: O14, O18ac, O20, O25, O26, O44, O55, O86, O91, O111, O114, O119, O125ac, O126, O127, O128, O142 and O158 (Kuhnert *et al.*, 1999; Clarke, 2001)

2.4.2 Enterohaemorrhagic *E. coli* (EHEC)

Enterohaemorrhagic *E. coli* produce a toxin active on vero cells hence also called Verocytotoxigenic *E. coli* (VTEC). They also produce cytotoxic effect on HeLa cells in culture and because this effect could be neutralized by antiserum produced against Stx protein of *S. dysenteriae*, they were named Shiga-like toxin producing *E. coli*, STEC (Clarke, 2001; Mainil & Daube, 2005). This pathotype causes bloody diarrhoea (haemorrhagic colitis), non-bloody diarrhea and haemolytic uremic syndrome (HUS) in

adults and children infected (**Kuhnert** *et al.*, 1999; Clarke, 2001; Kaper *et al.*, 2004; Mellies *et al.*, 2007). The bloody diarrhea is due to damage of the colon it infects (Mellies *et al.*, 2007). The pathotype also causes diarrhoea in calves of two weeks to two months of age but are asymptomatically carried by adult cattle, the principal reservoir of EHEC. Enterohaemorrhagic *E. coli* have also been isolated from sheep, goats, other wild ruminants and, piglets where it causes oedema (Mainil & Daube, 2005). Studies on faecal samples of chicken showed absence of STEC. However, presence of STEC has been demonstrated in pigeons (Farooq *et al.*, 2009).

Enterohaemorrhagic *E. coli* strains of the O157:H7 serotype, are the most important EHEC pathogens in human disease (Clarke, 2001; Kaper *et al.*, 2004; Mainil & Daube, 2005). Other serotypes particularly those of the O26 and O111 serogroups can also cause disease and are more prominent than O157:H7 in many countries (Kaper *et al.*, 2004).

2.4.3 Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* causes watery diarrhoea ranging from mild, self-limiting disease to severe purging disease. The organism is an important cause of childhood diarrhoea in the developing world and the main cause of diarrhoea in travelers to developing countries (Kaper *et al.*, 2004; Stenutz *et al.*, 2005). It also causes diarrhoea in newborn animals mainly piglets (Kuhnert *et al.*, 1999). The most common ETEC serogroups are; O6, O8, O11, O15, O20, O25, O27, O78, O128, O148, O149, O159 and O173 (Stenutz *et al.*, 2005).

2.4.4 Enteroaggregative *E.coli* (EAEC)

Enteroaggregative *E. coli* are recognized as a cause of often persistent diarrhoea in children and adults in both developing and developed countries (Clarke, 2001; Kaper *et al.*, 2004). The pathotype adheres to HEp-2 cells in an auto-aggregative pattern (Kaper *et al.*, 2004; Stenutz *et al.*, 2005) in which bacteria adhere to each other in a stacked-brick configuration (Kaper *et al.*, 2004). The serogroups identified within the EAEC group are O3, O7, O15, O44, O77, O86, O111, O126 and O127 (Stenutz *et al.*, 2005).

2.4.5 Enteroinvasive *E. coli* (EIEC).

This pathotype causes dysentery and it is biochemically, genetically and pathogenically closely related to *Shigella* species (Kuhnert *et al.*, 1999; Clarke, 2001; Kaper *et al.*, 2004). In most cases, EIEC elicits watery diarrhoea indistinguishable from that due to infection by other *E. coli* pathogens (Stenutz *et al.*, 2005; Kaper *et al.*, 2004). The most common EIEC serogroups are; O28ac, O29, O112ac, O124, O136, O143, O144, O152, O159, O164 and O167 (Stenutz *et al.*, 2005).

2.4.6 Diffusely adherent *E. coli* (DAEC)

This pathotype produces a diffuse pattern of adherence (DA) to epithelial cells in a classical laboratory assay of adherence to HEp-2 or Hela cells (Clarke, 2001; Kaper *et al.*, 2004; Bouguenec & Servin, 2006). The pathotype has been classified on the basis of its adherence pattern due to presence of some types of adhesins and absence of virulence determinants of other *E. coli* pathotypes (Kyaw *et al.*, 2002). Several studies have implicated DAEC as a cause of diarrhoea in children of more than 12 months of age (Kaper *et al.*, 2004).

2.5 Virulence determinants of different pathotypes of *E. coli*

Several virulence factors of *E. coli* occur and these include; attachment factors, host cell surface modifying factors, toxins and secretion systems and these vary according to the pathotype (Kuhnert *et al.*, 1999). The major and other virulence determinants for the different pathotypes of *E. coli* are described in the next sections.

2.5.1 Virulence determinants in EPEC

Enteropathogenic *E. coli* are mainly characterized by the presence of a 35-kb pathogenicity island (PAI) known as locus of Enterocyte Effacement, LEE (Higgins *et al.*, 1997; Badea *et al.*, 2002; Kaper *et al.*, 2004; Mellies *et al.*, 2007) with a 41 core Open Reading Frame (ORF) organized into several polycistronic operons (Badea *et al.*, 2002).

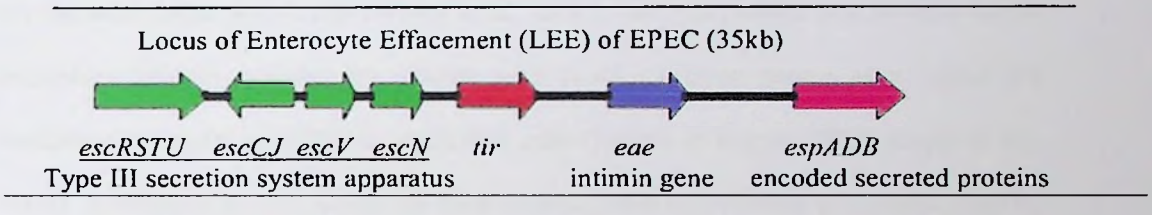

Figure 1 Different genes involved in EPEC pathogenesis

The LEE genes encode determinants that elicit the Attaching and Effacing (A/E) lesions. Most of the 41 ORF of the core LEE PAI encode a type III secretion system, associated chaperones and effector proteins (Higgins *et al.*, 1997; Badea *et al.*, 2002; Kaper *et al.*, 2004). The role of type III secretion system is to direct the secretion of several LEE encoded proteins known as EPEC secreted proteins, EsPs (Badea *et al.*, 2002), of which EspA, B and D are essential for normal A/E lesion formation (Wales *et al.*, 2005). The mechanism of A/E lesion formation begins with non-intimate adhesion and protein translocation (Figure 2a). During this process, Esp-A, -B and -D are

exported via a type III secretion system and form a bi-functional organelle with EspA forming filaments on bacterial surface in an EspD-dependent process. The filaments adhere to host cells and permit adhesion of the bacterium to the host cell. Secreted proteins; EspB and D may create a pore in eukaryotic cell membrane and together form a molecular syringe for introduction of macromolecules into host cell cytosol (Wales *et al.*, 2005).

Translocated intimin receptor (Tir) is then translocated and inserted in the host cell membrane. Signal transduction then occurs (Figure 2b) and results in host cell cytoskeletal reorganisation leading to effacement of microvilli. Translocated intimin receptor (Tir) focuses filamentous (F) actin, forming a pedestal, and binds intimin in the bacterial outer membrane (Wales *et al.*, 2005). Intimin protein is a 94-kDa outer-membrane protein encoded by intimin gene (*eae*). Intimin serves as a ligand for intimate attachment of EPEC to epithelial cells (Nataro & Kaper, 1998; Kaper *et al.*, 2004). Formation of A/E lesion has been demonstrated in a number of animals such as cattle, goats, sheep, pigs, ducks and pigeons (Wales *et al.*, 2005).

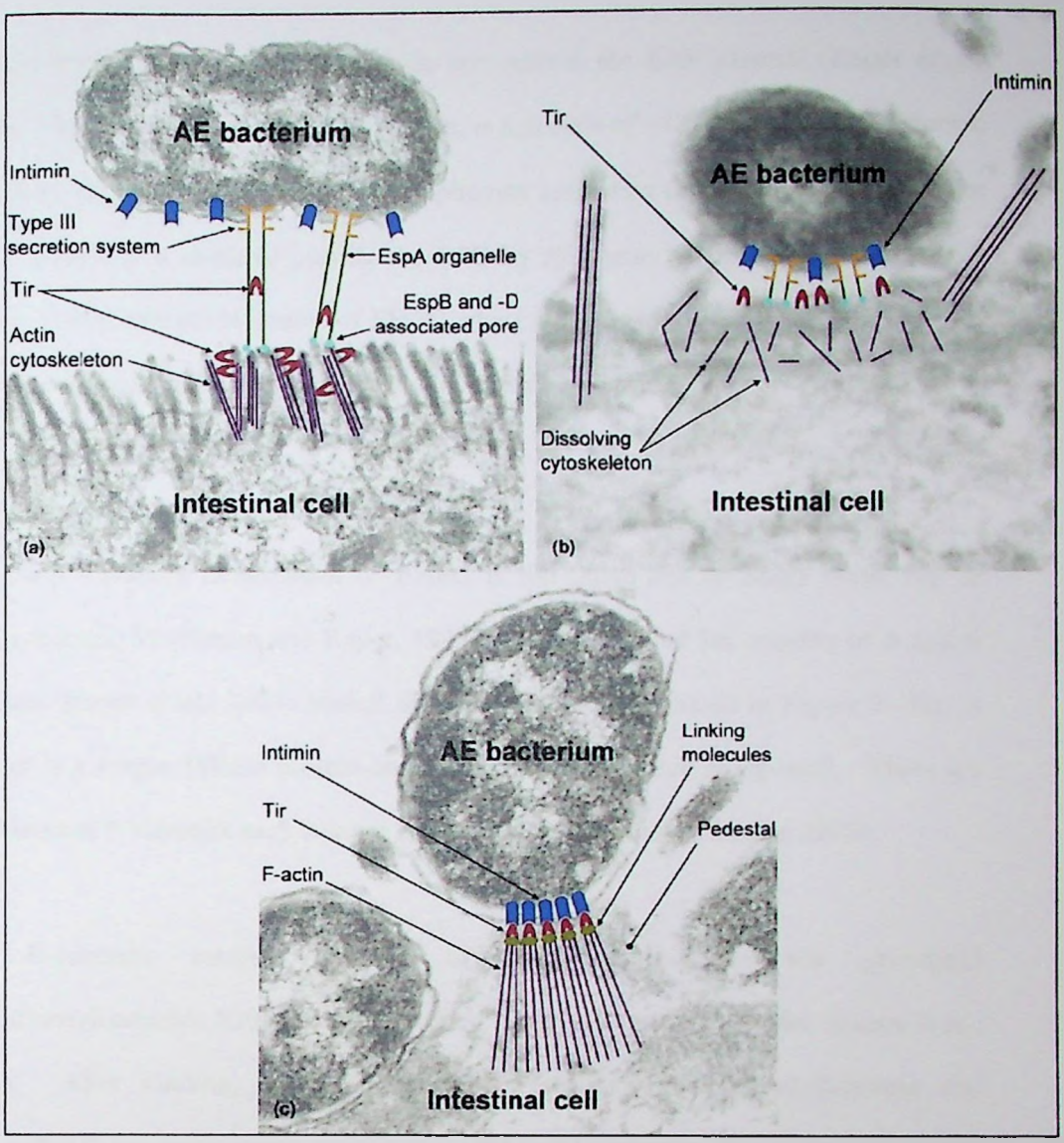

Figure 2 Formation of attaching and effacing lesions (Wales *et al.*, 2005)

Typical EPEC strains possess EPEC adherence factor (EAF) plasmid (70-100 kb) that encodes a type IV pilus called bundle-forming pilus, BFP (Badea *et al.*, 2002; Kaper *et al.*, 2004). This plasmid mediates inter-bacterial adherence and possibly adherence to epithelial cells. In fact EPEC strains null for *bfpA*, the gene encoding the major subunit of BFP, have attenuated virulence in human volunteers (Mellies *et al.*, 2007). So-called

atypical EPEC contain the LEE but do not contain the EAF plasmid (Kaper *et al.*, 2004). Besides the above virulence factors, is a protein of ~385 kDa called lymphocyte inhibitory factor (LifA) that inhibits lymphocyte activation (Badea *et al.*, 2002; Kaper *et al.*, 2004). It is encoded outside the LEE by *lifA* gene (Badea *et al.*, 2002). This protein is also present in strains of EHEC where it is known as Efa1 (EHEC factor for adherence) and an adhesive property has been attributed to it (Kaper *et al.*, 2004).

2.5.2 Virulence determinants in EHEC

The main virulence determinant of EHEC is the Shiga-like or Shiga toxin, Stx or Verocytotoxin, Vt (Nataro and Kaper, 1998). The structure of Stx consists of A and B subunits (Kaper *et al.*, 2004; Mainil & Daube, 2005) as depicted in Figure 3. The A subunit is a single 33KDa protein and the biologically active component. There are five identical B subunits each being a 7.5KDa protein (Mainil & Daube, 2005).

The B-subunits mediate binding of the holotoxin to the glycolipid globotriaosylceramide (Gb_3) on the target cell surface (Kaper *et al.*, 2004; Stamm *et al.*, 2002). After binding, it is internalized by receptor-mediated endocytosis and transported to the endoplasmic reticulum via the golgi apparatus. The A sub-unit is then translocated into the cytoplasm using sec61 transmembrane protein complex. The 4.5KDa C-terminal A2 peptide is then cleaved off leaving the active A1 peptide (27.5KDa). The A1 peptide with its N-glycosidase activity cleaves a purine residue from 28S rRNA altering the function of ribosomes. This makes ribosomes to no longer bind elongation factors one and two (EF1 and EF2 respectively) inhibiting protein synthesis causing cell death (Mainil & Daube, 2005).

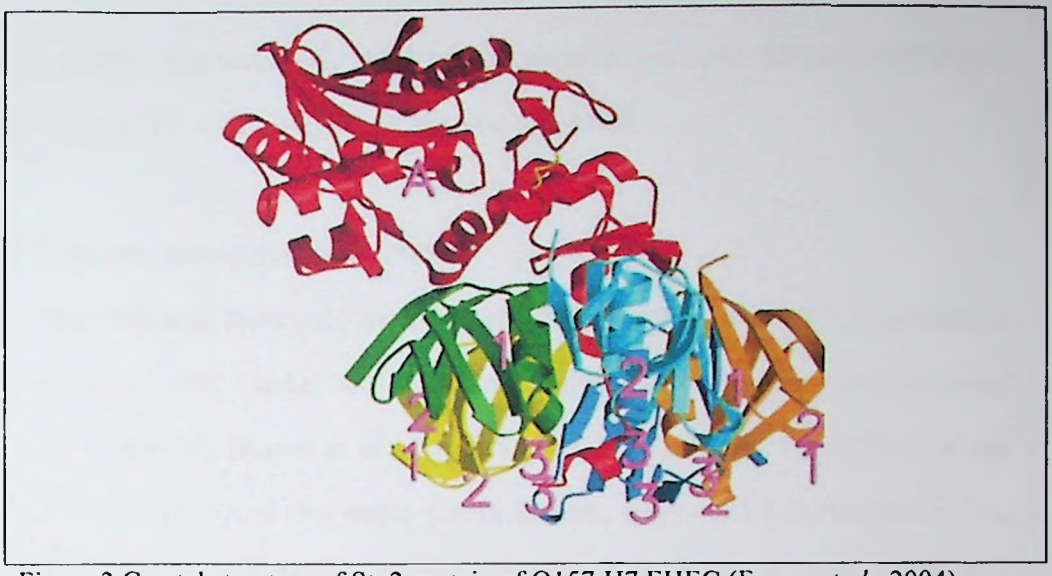

Figure 3 Crystal structure of Stx2 protein of O157:H7 EHEC (Fraser *et al.*, 2004)

The A-subunit is indicated red, whereas the five B-subunits are indicated as; orange, cyan, green, yellow, and blue. The magenta letter A marks the active site in the A-subunit. The side chains of the cysteine residues that link A1 and A2 are depicted in yellow. The sites equivalent to Gb3-binding sites on the B-pentamer of Stx1 are shown by magenta numbers that distinguish the type of binding site.

The Stx family contains two subgroups; Stx1 and Stx2 proteins encoded by stx_1 and stx_2 genes respectively (Kuhnert *et al.*, 1999; Murray *et al.*, 2003; Kaper *et al.*, 2004) sharing approximately 55% amino acid homology (Kaper *et al.*, 2004). The Stx1 toxins are antigenically close to Stx from *S. dysenteriae* type 1 while Stx2 is distantly related (Mainil & Daube, 2005). Several variant forms of stx_2 genes: stx_{2c}, stx_{2d} and stx_{2f} have been described (Murray *et al.*, 2003). These differ much more from each other in their antigenicity, toxicity and gene sequences. The variants stx_{2c}, stx_{2d} and stx_{2f} have been described in human strains and stx_{2e} in porcine strains (Mainil & Daube, 2005).

Enterohaemorrhagic *E. coli* also possesses adhesins (Clarke, 2001) such as intimin protein, a large 362-kDa protein encoded on the 93-kb plasmid present in O157:H7 and other EHEC strains (Kaper *et al.*, 2004; Mellies *et al.*, 2007); other toxins include alpha

hemolysin, *hly* (Kuhnert *et al.*, 1999) besides shiga toxins. Like EPEC, EHEC strains often possess LEE with *eae* genes (Kuhnert *et al.*, 1999).

2.5.3 Virulence determinants in ETEC

The major virulence factors are the heat stable (ST) and heat labile (LT) enterotoxins (Kuhnert *et al.*, 1999; Clarke, 2001; Kaper *et al.*, 2004). The genes for these toxins are located on plasmids (Kaper *et al.*, 2004; Stenutz *et al.*, 2005). The LT toxins are oligomeric and consist of two major groups namely, LT-I and LT-II (Kuhnert *et al.*, 1999; Clarke, 2001) encoded by *eltI* and *eltII* respectively (Kuhnert *et al.*, 1999). The LTII is primarily found in animal isolates (Kuhnert *et al.*, 1999).

Heat stable toxins are small, monomeric and plasmid mediated comprising of two unrelated classes namely STa (STI) and STb (STII) (Kuhnert *et al.*, 1999; Clarke, 2001) encoded by *stIA* and *stIB* respectively (Kuhnert *et al.*, 1999). The STb is also primarily found in animal isolates (Kuhnert *et al.*, 1999). A single plasmid normally carries a toxin and colonization factor antigen, CFA (Kuhnert *et al.*, 1999; Stenutz *et al.*, 2005). Strains of ETEC might express only an LT, only an ST, or both LTs and STs (Kaper *et al.*, 2004). The two proteins (STa and STb) differ in structure and mechanism of action (Kuhnert *et al.*, 1999; Kaper *et al.*, 2004). Only toxins of the STa class have been associated with human disease. The mature STa toxin is ~2kDa peptide containing 18 or 19 amino acid residues, six of which are cysteine that form three intramolecular disulphide bridges (Kaper *et al.*, 2004). The main receptor for STa is a membrane-spanning guanylate cyclase (Kuhnert *et al.*, 1999; Clarke, 2001; Kaper *et al.*, 2004). Binding of STa to guanylate cyclase stimulates guanylate cyclase activity leading to increased intracellular cGMP. This in turn activates cGMP-dependent and/or

cAMP-dependent kinases (Kaper *et al.*, 2004) ultimately increasing secretion (Kuhnert *et al.*, 1999; Clarke, 2001; Kaper *et al.*, 2004).

The STb toxin is associated with animal disease (Kuhnert *et al.*, 1999; Kaper *et al.*, 2004) and is a 48-amino-acid peptide with two disulphide bonds (Kaper *et al.*, 2004). It (STb) can elevate cytosolic Ca^{2+} concentration; stimulate the release of prostaglandin E2 and serotonin, all of which could lead to increased ion secretion (Kaper *et al.*, 2004).

2.5.4 Virulence determinants in EAEC

The major virulence factors are the aggregative adherence fimbriae (AAF/I and II), normally detected (Kuhnert *et al.*, 1999). Several toxins have been described for EAEC, two of which are encoded by the same chromosomal locus on opposite strands. The larger gene encodes an autotransporter protease called Pic having a mucinase activity and the opposite strand encodes the oligomeric enterotoxin known as *Shigella* enterotoxin 1 (ShET1), because of its presence in most strains of *Shigella flexneri* (Kaper *et al.*, 2004). Epidemiological studies implicate a package of plasmid-borne and chromosomal virulence factors similar to those of other enteric pathogens, indicating that several virulence factors are associated with EAEC virulence (Kaper *et al.*, 2004). Based on *in vitro* and animal data, the initial stage of EAEC pathogenesis involves adherence to the intestinal mucosa and/or the mucus layer using AAF/I and AAF/II proteins (Nataro & Kaper, 1998; Kaper *et al.*, 2004). This is followed by enhanced mucus production leading to deposition of a thick mucus-containing biofilm encrusted with EAEC (Nataro & Kaper, 1998).

A new protein, dispersin has been found to form a loosely associated layer on the surface of EAEC strains. This seems to counter the strong aggregating effects of the AAF adhesin facilitating spread of the organism across the mucosal surface or penetration of the mucous layer (Kaper *et al.*, 2004). The final stage, suggested from histopathologic and molecular evidence includes the elaboration of an EAEC cytotoxin which results in damage to intestinal cells (Nataro & Kaper, 1998). The mode of action of ShET1 is not yet understood but it might contribute to the secretory diarrhoea that accompanies EAEC and *Shigella* infection (Kaper *et al.*, 2004).

2.5.5 Virulence determinants in EIEC

The ability to invade, survive and multiply within colonic enterocytes is due to the presence of a 120-140 MDa plasmid encoding all the necessary genes (Clarke, 2001; Stenutz *et al.*, 2005). The major virulence determinants are the invasion plasmid antigens (Ipa) - IpaA, IpaB, IpaC and IpgD (Kaper *et al.*, 2004). These proteins mediate epithelial signaling events, cytoskeletal rearrangements, cellular uptake and lysis of the endocytic vacuole (Kaper *et al.*, 2004). Pore containing proteins IpaB and IpaC are inserted into the host cell membranes creating pores in the membrane. These proteins may be secreted using a type III secretion system apparatus encoded by *mxi* and *spa* genes (Kaper *et al.*, 2004). Protein IpaB also binds to the signaling protein CD44 triggering cytoskeletal rearrangements and cell entry, and to the macrophage caspase 1 resulting in apoptosis and release of IL-1 from macrophages (Kaper *et al.*, 2004). IpaC induces actin polymerization leading to the formation of cell extensions by activating GTPases, Cdc42 and Rac. Conversely, IpaA binds to vinculin and induces actin depolymerization thereby organizing the extensions induced by IpaC into a structure that enables bacterial entry (Kaper *et al.*, 2004). The translocated effector protein IpgD

is a potent inositol 4-phosphatase that helps to reorganise host-cell morphology by uncoupling the cellular plasma membrane from the actin cytoskeleton, which leads to membrane blebbing (Kaper *et al.*, 2004).

2.5.6 Virulence determinants in DAEC

This pathotype has been found to have a type III secretion system with a high degree of nucleotide and amino acid conservation, and with similar gene organization when compared to LEE of EHEC and EPEC. However, no *eae* gene and A/E lesion have been observed implying that LEE must be having a pathogenicity island with some differences when compared to the LEE of EPEC and EHEC (Kyaw *et al.*, 2002). A large proportion of DAEC strains produce a fimbrial adhesin called F1845 (Kaper *et al.*, 2004; Bouguenec & Servin, 2006) that binds to decay accelerating factor (DAF), a cell-surface glycosylphosphatidylinositol-anchored protein (Kaper *et al.*, 2004). Binding to DAF by an adhesin is accompanied by activation of signal transduction cascades including activation of PI-3 kinase (Kaper *et al.*, 2004). However, studies have reported that infection of an intestinal cell line by strains of DAEC impairs the activities and reduces the abundance of brush-border-associated sucrose isomaltase and dipeptidylpeptidase IV, an effect independent of DAF-associated pathway described above. This therefore provides a feasible mechanism for DAEC-induced enteric disease and indicates presence of other virulence factors (Kaper *et al.*, 2004).

In addition to the previously described pathotypes, several other potential *E. coli* pathotypes have been recorded. These include; Adherent-invasive *E. coli* (AIEC), Necrotizing enterocolitis (NEC), Necrotoxic *E. coli* (NTEC) and Cell-detaching *E. coli* CDEC (Kaper *et al.*, 2004).

2.6 Epidemiology of DEC in humans and animals

2.6.1 Epidemiology of EPEC

Infection occurs in children of less than 2 years mostly those younger than 6 months of age with infection dose presumed to be much lower. This pathotype has also been isolated from healthy infants of more than 2 years without significant correlation with disease. This reduced infection of older children and adults may be due to loss of specific receptors with age. However, several outbreaks of diarrhoea due to EPEC have been reported in healthy adults perhaps due to ingestion of a large dose from a common source. Sporadic outbreaks of the disease have been observed in some adults with diabetics, achlorhydria and the elderly (Nataro & Kaper, 1998).

2.6.2 Epidemiology of EHEC

The most famous and prioritized are the O157:H7 strains. Frequent infection of humans with O157:H7 serotype is food-borne. Such outbreaks are primarily restricted to North America and UK and are rare in South America, Japan, Oceania and Africa. The O157:H7 strains are carried by healthy cattle, sheep, goats and wild ruminants in their gastro intestinal tract (Nataro & Kaper, 1998; Mainil & Daube, 2005). Enterohaemorrhagic *E. coli* are well adapted to survive in faeces, soil and water (McGee *et al.*, 2001). It can survive in manure or slurry for weeks (McGee *et al.*, 2001) and its persistence in faecal matter of different farm animals varies with animal species (Williams *et al.*, 2008). Frequent sporadic cases of EHEC infection in humans are on the rise and appear to be geographically distributed (Nataro & Kaper, 1998; Mainil & Daube, 2005). In developing countries, EHEC is much less frequently isolated than other diarrhoeagenic *E. coli* strains such as ETEC or EPEC (Kaddu-Mulidwa *et al.*, 2001; Mainil & Daube, 2005).

2.6.3 Epidemiology of ETEC

This pathotype causes infant diarrhoea in developing countries and is a leading cause of traveler's diarrhoea in high-risk areas of Africa, Asia and Latin America. It can also be isolated from domestic animals and occur in diarrhoeal disease afflicting these animals (Qadri *et al*, 2005). The percentage of cases of sporadic endemic infant diarrhoea due to ETEC varies. Transmission is due to contaminated food and water, as sampling of both food and water sources from areas of endemic infection have demonstrated high rates of ETEC contamination (Nataro & Kaper, 1998; Mainil & Daube, 2005).

2.6.4 Epidemiology of EAEC

Studies have supported the association of EAEC with diarrhoea in the developing countries. The increasing number of such reports and the rising proportion of diarrhoeal cases in which EAEC are implicated suggest that EAEC are important emerging agents of pediatric diarrhoea. The association of EAEC with diarrhoeal disease appears to be geographic. EAEC may also be important agents of diarrhoea in industrialized countries as they have been frequently identified in stool samples of patients (Nataro & Kaper, 1998).

2.6.5 Epidemiology of EIEC

Outbreaks are usually food or water borne but person-to-person transmission does occur. In sporadic cases, many EIEC strains are probably misidentified as *Shigella* species or as nonpathogenic *E. coli* strains. Endemic sporadic disease occurs in some areas, generally where *Shigella* species is also prevalent but with different epidemiologic features. The incidence of EIEC in developed countries is thought to be low, with occasional outbreaks (Nataro & Kaper, 1998; Nataro *et al.*, 2006).

2.6.6 Epidemiology of DAEC

It has been implicated as a cause of diarrhoea in humans with studies showing increase of relative risk with age (from 1 - 5 years). Strains of DAEC also account for a large proportion of diarrhoeal cases among hospitalized patients suggesting that DAEC strains may be important diarrhoeal pathogens in the developed world (Nataro & Kaper, 1998).

2.7 Antibiotic drugs and drug resistance

2.7.1 Antibiotic drugs

Antibiotic drugs are used widely as food additives in modern animal husbandry to prevent infections and promote growth (Bonnet *et al.,* 2009). Most of these antibiotic drugs are designed to target essential parts or processes of bacteria (Bonnet *et al.*, 2009). These targets include; bacterial cell wall, synthesis of proteins, or replication and transcription of DNA (Greenwood and Whitley, 2002). Drugs such as nalidixic acid and other quinolones target alpha subunit of DNA gyrase. Aminoglycosides such as gentamicin target ribosomes. Tetracyclines attach to 30S ribosomal subunits preventing binding of incoming aminoacyltRNA to the A site of ribosome while drugs like chloramphenicol target peptidyl transferase enzyme that links amino acids to the growing peptide chain, halting chain elongation (Greenwood and Whitley, 2002).

2.7.2 Antibiotic resistance

Antimicrobial resistance is a natural phenomenon developed by bacteria so as to escape the effects of antimicrobial agents. The term usually refers to acquired resistance, which occurs when resistant strains emerge from previously susceptible bacterial populations usually after selective pressure is exerted by antimicrobial agents (WHO/FAO/OIE, 2003). *Escherichia coli* may be sensitive to many antibiotics.

However, isolates of *E. coli* from poultry are frequently resistant to one or more antibiotics especially when such antibiotics are overused (Salehi and Bonab, 2006). The use of antimicrobial agents can lead to emergence, prevalence and dissemination of antimicrobial resistance in bacteria (Harada and Asai, 2010), which in turn can find their way into human bodies through their entry routes. This resistance to antibiotic drugs reduces their efficacy in both veterinary and human medicine.

2.7.3 Origin and transfer of resistance genes

Origin of resistance genes may be naturally resistant bacteria or microorganisms that are producers of antibiotics, by a mechanism of gene picking-up and recombination. Animal gut and the human gut have been considered as the important location for genes exchanges. However, environmental bacterial niches, soil, river, pets, wild rodents, and fish, are also location where genes transfer occurs (WHO/FAO/OIE, 2003).

2.7.4 Pattern of usage of antimicrobial agents

The pattern of usage is extremely different between countries, regions and farms. Prophylaxis and metaphylaxis are administered with different rationales. Duration of administration of sub-therapeutic doses and time in the animal life are also diverse. To a lesser extent, therapeutic administration of antimicrobial agents can be variable (FAO/WHO /OIE, 2003).

Assuming 75% of the total collected faecal samples contained *E. coli*, then at 95% confidence,

$$N_0 = \frac{(1.96)^2 \times 0.75 \times 0.25}{0.05^2} = 288$$

It was then decided that a random total sample of 288 faecal samples be taken, consisting of 144 chicken faecal samples (72 from each region) and 144 cattle faecal samples from Kampala city abattoir.

In estimating the number of *E. coli* isolates to be used in multiplex PCR, Fleiss formula (1981) for calculating sample size for proportions was used:

$$n = C\frac{p_c q_c + p_e q_e}{d^2} + \frac{2}{d} + 2,$$

Where; n is sample size, for this case to be subjected to multiplex PCR

p_c is the pre-specified value based on existing studies or taken as 0

p_e is the postulated proportion, which in this case is assumed to be greater than 0. Assuming 20% of *E. coli* isolates are pathotypes, $p_e = 0.20$

$q_c = 1 - p_c = 1 - 0 = 1$ and $q_e = 1 - p_e = 1 - 0.20 = 0.80$

C is a constant that depends on values chosen for α and β. At $\alpha = 0.05$ and $1-\beta = 0.8$, the value of C = 7.85; and at $\alpha = 0.05$, $1-\beta = 0.9$, C = 10.51

d is the difference between p_c and p_e expressed as positive value.

Therefore, $n = 10.51 \frac{(0 \times 1 + 0.20 \times 0.80)}{(0.20)^2} + \frac{2}{0.20} + 2 = 54.04$

Based on this calculation, at least 54 *E. coli* isolates from each animal group (cattle and chicken) were subjected to multiplex PCR.

3.4 Ethical consideration

The research was approved by the college of Veterinary Medicine, Animal resources and Biosecurity after presentation of the proposal. Permission to collect samples was obtained from individual poultry farmers and also from the management of the abattoir.

3.5 Sample collection

A total of 144 fresh individual chicken faecal samples were collected from randomly selected medium and small-scale poultry farms in Kampala and Wakiso districts of Central region and, Jinja and Mbale districts of Eastern regions of Uganda. From each region, 72 faecal chicken samples were collected, 36 samples from each district. Cattle faecal samples (144) were collected from the City abattoir in Kampala. Fresh faecal cattle samples were aseptically collected from the rectum of individual slaughter animals while chicken samples where aseptically collected from fresh droppings of healthy birds, that had not contacted the ground, into sterile polythene bags and properly tied. They were then labeled and immediately placed in a cool box on ice and transported to the Microbiology laboratory, College of Veterinary Medicine, Animal Resources and Biosecurity for analysis. The samples were kept in a fridge at 4°C during preparation for analysis.

3.6 Bacterial cultures

A feacal swab was inoculated in peptone water (Oxoid, UK) and incubated at 37°C for 18 hours. Using a sterile wire loop, a bacterial culture was inoculated by the streak method on MacConkey (Himedia, India) agar plates. The plates were then placed agar side up in an incubator at 35-37°C for 18-24 hours for growth of the organism.

3.7 Isolation and identification of *E. coli*

To obtain pure cultures, at least five pink colonies suggestive of *E. coli* from each culture plate were transferred and sub-cultured on fresh MacConkey plates with incubation at 37°C for 18-24 hours. To confirm whether the pink colonies were *E. coli*, standard biochemical tests were carried out (Nataro and Kaper 1998). Briefly, each of the pink colonies was subjected to indole and citrate utilization tests. For each sample, 10 *E. coli* colonies were preserved in 10% glycerol at -20°C and later used in further studies.

3.8 Culturing and confirmation of *E. coli* isolates from glycerol stock

Using a sterile swab, *E. coli* strains from glycerol stock were transferred to MacConkey agar plates and incubated at 37°C for 18-24 hours. Pink colonies from the plate were then selected for biochemical identification using indole test positive for 99% of *E. coli* (Nataro & Kaper 1998). In this test, 10 pink colonies were suspended into 5ml of tryptone broth rich in amino acid tryptophan and incubated at 37°C for 24 - 48 hours. Kovac's reagent (5 drops or 0.5µl) was then added and appearance of a dark pink colour confirmed presence of *E. coli*.

3.9 Development of Multiplex PCR to detect the different pathotypes

3.9.1 Preparation of DNA template (DNA extraction) for PCR

Freshly grown overnight cultures of *E. coli* isolates on MacConkey Agar plate were suspended in 0.5ml of nuclease free water (Sigma Aldrich, USA). The mixture was boiled for 20 minutes, cooled immediately on ice followed by centrifugation at 12,000 x g for 10 minutes to pellet the cell debris. The supernatant was then used as template DNA in PCR reactions.

3.9.2 Primers for multiplex PCR

All Primers used in this study were procured from the Oligonucleotide synthesis unit of the University of Cape Town, South Africa. The following primer pairs; LT (*eltB* gene), ST (*estA* gene), Stx1 (stx_1 gene), Stx2 (stx_2 gene), Eae (*eaeA* gene), SHIG (*ial* gene) and EAG (*pCVD* gene) were used in this study (Table 1).

Table 1 Primers for detection of *E. coli* pathotypes

Primer	Target gene	Primer sequence	Product size (bps)	Reference
LT	*eltB*	5-TCTCTATGTGCATACGGAGC-3 5-CCATACTGATTGCCGCAAT-3	322	Nguyen *et al.*, 2004
ST	*estA*	5-GCTAAACCAGTAGAGTCTTCAAAA-3 5-CCCGGTACAAGCAGGATTACAACA-3	147	Nguyen *et al.*, 2004
Stx1	stx_1	5-GAAGAGTCCGTGGGATTACG-3 5-AGCGATGCAGCTATTAATAA-3	130	Nguyen *et al.*, 2004
Stx2	stx_2	5-ACCGTTTTTCAGATTTTGCACATA-3 5-TACACAGGAGCAGTTTCAGACAGT-3	298	Nguyen *et al.*, 2004
Eae	*eaeA*	5-CACACGAATAAACTGACTAAAATG-3 5-AAAAACGCTGACCCGCACCTAAAT-3	376	Nguyen *et al.*, 2004
SHIG	*Ial*	5-CTGGTAGGTATGGTGAGG-3 5-CCAGGCCAACAATTATTTCC-3	320	Nguyen *et al.*, 2004
EAG	*pCVD*	5-CTGGCGAAAGACTGTATCAT-3 5-CAATGTATAGAAATCCGCTGTT-3	630	Nguyen *et al.*, 2004

3.9.3 Multiplex PCR assay

A multiplex PCR was carried out in a 25µl reaction mixture containing 2µl of template DNA, 5µl of 5xPCR buffer, 0.5µl of 10mM deoxynucleoside triphosphates mixture, 2µl of 25mM $MgCl_2$, 0.125µl of 5U/µl AmpliTaq DNA polymerase and 0.5µl (0.2µM) of each primer pair. Apart from the primers, other ingredients of PCR reaction mixture were supplied from Promega (Madison, USA). Using a Techne thermocycler (TC-412, model FTC41H2D, UK Barloworld scientific ltd), the PCR run conditions were; 96°C

for 4 minutes, 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute for 30 cycles, with a 7 minute extension at 72°C.

To establish the optimal conditions, PCR reactions were initially performed on positive control *E. coli* isolates using a pair of primers specific for each pathotype. The multiplex targeting three pathotypes (EPEC, ETEC and EHEC) and another one for two pathotypes (EIEC and EAEC) were carried out in subsequent studies.

Escherichia coli used as positive and negative controls (Table 2) were kindly availed by Peter Nkurunziza Mumenya from the microbiology Medical Research Council laboratory, Uganda Virus Research Institute.

Table 2 Control *E. coli* strains for PCR

Category	Control strains	Target genes	Category	Control strain	Target genes
ETEC	H10407	*eltB, estA*	STEC	209PN	stx_1, stx_2
	90PN	*eltB*	EIEC	159PN	*Ial*
	67PN	*estA*	EAEC	122PN	*PCVD*
EPEC	186PN	*eaeA*	EC	130PN	-

3.9.4 Separation and visualisation of PCR products

Electrophoresis of PCR products was carried out on a 2.0 % agarose gel (Invitrogen life technologies, Paisley, PA4 9RF, Scotland UK) at 120 mV for 30 min, by loading 10µl of product. A molecular marker (100bp DNA ladder, Promega Madison, USA) was run concurrently. The buffer in the electrophoresis chamber and in the agarose gel was Tris-borate-EDTA (1xTBE) containing ethidium bromide (10mg/ml, Sigma Aldrich). The fragments of DNA in the gel were visualized by exposing the gel to UV light using U.V. transilluminator equipment and converted into a digital photographic image.

3.9.5 Interpretation of multiplex PCR data

The sample was considered positive for the pathotype if the result of gel electrophoresis showed the band of the right size as determined by the marker and negative if it gave no right band. The different *E. coli* isolates from different sources (cattle and chicken), positive for a given pathotype were tabulated for further use on antibiotic resistance profile.

3.9.6 Sequencing of Multiplex PCR products

3.9.6.1 Purification of PCR products for sequencing

Portions (8µl) of PCR Products of selected amplicons were purified using QIAquick PCR Purification Kit (QIAGEN). The purified products were then sent to Life Technologies Corporation (Applied Biosystems Inc. 850 Lincoln Centre Drive Foster City, CA 94404, USA) for sequencing.

3.9.6.2 Sequencing and sequence analysis

In sequencing the DNA fragments, the PCR products were cloned into pGEM-T plasmid (Promega, Mannheim, Germany) and the recombinant plasmids purified using the PureYield™ Plasmid Midiprep (Promega, Mannheim, Germany). Sequencing was done with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) on an automatic ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The sequencing data were analysed with Vector NTI.11.5 software (Invitrogen, Carlsbad, CA, USA).

The obtained amplicon sequences were analysed by carrying out a homology search with DNA database (GenBank) using Bioedit Sequence Alignment Editor Software.

This was done to confirm whether the amplicons corresponded to segments of amplified genes representing the different pathotypes of *E. coli.*

3.10 Antibiotic susceptibility tests

Escherichia coli isolates from the animal faecal samples were used to establish their response on selected common antibiotic drugs. This was done using the agar disc diffusion method on Mueller-Hinton (MH) agar (Oxoid) according to the recommendations reported by the Clinical and Laboratory Standards Institute (CLSI). Isolates were first cultured on MacConkey agar to obtain fresh colonies. Five well-isolated colonies were then picked using sterile wire loop and inoculated onto Mueller-Hinton broth. The broth was then incubated at 35°C until turbid, and turbidity adjusted to the proper density by comparing with 0.5 McFarland standards. Within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the suspension and rotated to remove excess liquid. Swab was then streaked over the entire surface of the Mueller-Hinton plate (prepared according to manufacturer's instruction) three times, rotating the plate approximately at 60 degrees after each application to ensure an even distribution of the inoculums.

Antimicrobial disks were individually applied to the plates using sterile forceps and then gently pressed down onto the agar and incubated at 35^0C for 16 to 18 hours, after which zone sizes were measured using a ruler and results interpreted. The antimicrobial agent discs used in this study were; tetracycline (30µg), ciprofloxacin (30µg), nalidixic acid (30µg), gentamicin (10µg) and chloramphenicol (30µg) (Himedia Laboratories Ltd, Mumbai, India).

3.11 Statistical analysis

For the antibiotics, contingency tables were created and data analysed using chi-squared test or Fisher's exact test (for expected values <5) and Cochran's Q test to compare the differences between the proportions of the isolates possessing different virulence gene markers that were resistant to various antimicrobial drugs. A value of $p < 0.05$ was considered statistically significant. The calculations were conducted using Genstat discovery software freely downloaded at www.vsni.co.uk. Microsoft excel was used to organize the data and for generation of graphs and charts.

CHAPTER FOUR

RESULTS

4.1 Bacterial cultures

Only four chicken faecal samples (2.8%) did not yield pink colonies on MacConkey agar plates. The rest, 140 of 144 (97.2%) chicken faecal samples yielded *E. coli* colonies on MacConkey agar. The trend was almost the same in cattle samples where 94.4% (136 of 144) of the samples were positive for *E. coli* (Table 3).

Table 3 Cattle and chicken faecal samples positive for *E. coli*

	Cattle faecal samples (n= 144)		**Chicken faecal samples (n= 144)**	
	Number	%	Number	%
E. coli positive	136	94.4	140	97.2
E. coli negative	8	5.6	4	2.8

4.2 Multiplex PCR for positive *E. coli* strains

Initial validation of the multiplex PCR was carried out using the positive control strains. With the first multiplex PCR amplification, amplicons of the expected band sizes were obtained with ETEC-H10407 for genes *eltB*, *estA*; ETEC-90PN for *eltB*; ETEC-67PN for *estA*; EPEC-186PN for *eaeA*; and EHEC-209PN for stx_1, stx_2 (Figure 4). The second multiplex PCR was done with EIEC-159PN for *ial*; and EAEC-122PN for *pCVD*. As expected no amplicon was obtained from the negative control. Hereafter, the validated multiplex PCR was applied to the samples for the detection of the various pathotypes of *E. coli*. In detecting the pathotypes with positive control strains, amplicons were obtained for stx_1, stx_2 and *eaeA* genes. Three amplicons; one for stx_1, stx_2 and another for *eaeA* were purified and sequenced (Section 3.9.6).

Analysis of the sequenced data confirmed the PCR products as portions of the respective genes (Section 4.3).

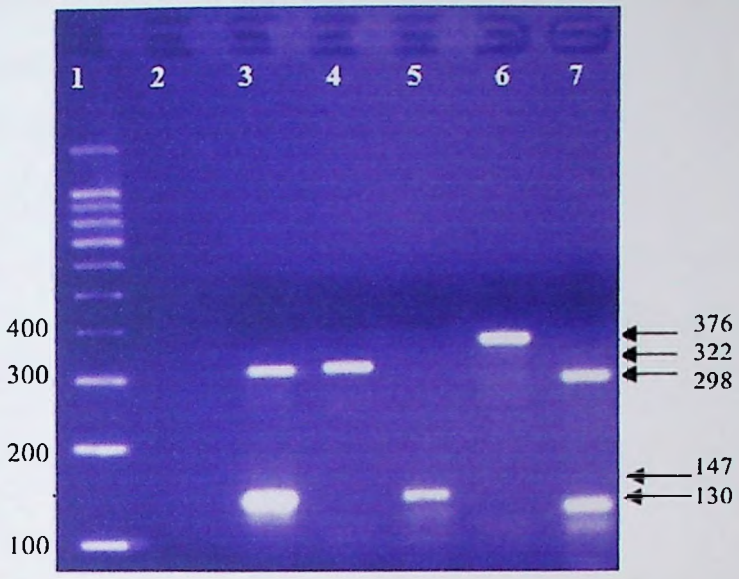

Figure 4 Optimization of first multiplex PCR with *E. coli* positive control strains
Lane 1, 100bp DNA ladder; lane 2, negative control, EC-130PN; lanes 3, ETEC-H10407 for genes *eltB, estA*; lane 4, ETEC-90PN for *eltB*; lane 5, ETEC-67PN for *estA*; lane 6, EPEC-186PN for *eaeA;* lane 7, STEC-209PN for stx_1, stx_2

4.3 Sequence analysis

The amplicon sequences obtained from sequencing were compared with the corresponding gene sequences in DNA database (GenBank) by carrying out a pairwise sequence alignment using BioEdit 5.0.6 Sequence Alignment Editor Software. Alignment was performed as follows; stx_1 amplicon sequence with shiga toxin 1 subunits A (stx_{1A}) and B (stx_{1B}) genes of *E. coli* FD930 (GenBank: AF461172.1), stx_2 amplicon sequence with shiga toxin 2 subunits A (stx_{2A}) and B (stx_{2B}) genes of *E. coli* O157: H- strain (GenBank: AY143337.1) and *eaeA* amplicon sequence with intimin gene (*eae*) of *E. coli* O157:H7 EDL933 (GenBank: AE005174:). The alignment confirmed amplicon sequences as portions of the corresponding amplified genes (Section 8.1 appendix I).

4.4 Detection of pathotypes of *E. coli* in bovine samples

PCR amplification was carried out using primers (Table 1) to detect the various *E. coli* pathotypes. Amplification products were obtained with the positive control strains corresponding to specific pathotypes (Figure 4). No PCR product was obtained with the negative control. Similarly, amplicons were obtained in 24 (B1 to B24, Table 4) of 60 (40%) of the bovine *E. coli*. All (24) pathogenic *E. coli* detected possessed at least stx_1, stx_2 genes or both of these genes (Table 4 and Figure 5). In addition, amplicons corresponding to *eaeA* gene were detected in three of the 24 (13%) isolates (Table 4). No PCR products corresponding to 4 genes (*estA, elB, ial* and *pCVD*) were amplified and these were recorded as negative (Table 4).

Table 4 Pathogenic *E. coli* isolates identified in bovine faecal samples

Bovine *E. coli* Isolates	Virulence genes targeted						
	stx_1	stx_2	*eaeA*	*estA*	*eltB*	*Ial*	*pCVD*
B1	+	+	-	-	-	-	-
B2	+	+	-	-	-	-	-
B3	-	+	-	-	-	-	-
B4	-	+	-	-	-	-	-
B5	-	+	+	-	-	-	-
B6	+	+	-	-	-	-	-
B7	-	+	+	-	-	-	-
B8	-	+	-	-	-	-	-
B9	+	+	-	-	-	-	-
B10	-	+	+	-	-	-	-
B11	-	+	-	-	-	-	-
B12	-	+	-	-	-	-	-
B13	-	+	-	-	-	-	-
B14	+	+	-	-	-	-	-
B15	+	+	-	-	-	-	-
B16	+	+	-	-	-	-	-
B17	+	-	-	-	-	-	-
B18	+	+	-	-	-	-	-
B19	-	+	-	-	-	-	-
B20	+	+	-	-	-	-	-
B21	+	+	-	-	-	-	-
B22	-	+	-	-	-	-	-
B23	-	+	-	-	-	-	-
B24	+	-	-	-	-	-	-
Total	12	22	03	00	00	00	00

B means bovine, +/- indicates presence and absence of the gene respectively.

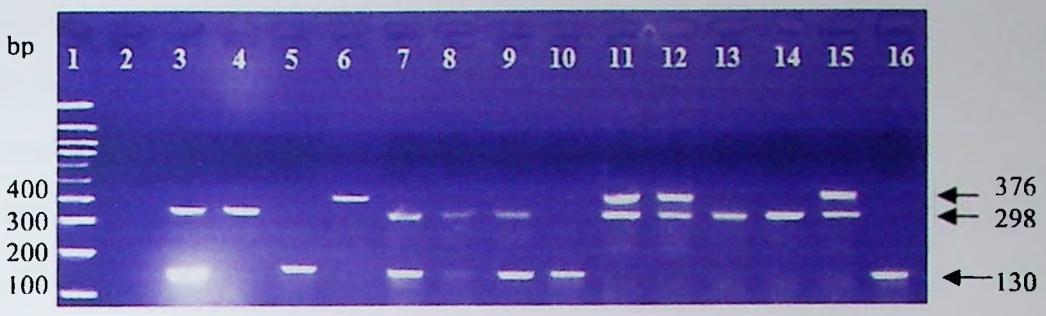

Figure 5 Results of multiplex PCR amplification of *E. coli* isolates from cattle samples. Lane 1, 100bp DNA ladder; lane 2, negative control, EC-130PN; lanes 3 to 7 are positive controls; lane 3, ETEC- H10407 for *eltB, estA;* lane 4, ETEC-90PN for *eltB*; lane 5, ETEC-67PN for *estA*; lane 6, EPEC-186PN for *eaeA* and lane 7, STEC-209PN for stx_1, stx_2. Lanes 8 to 16 are bovine isolates positive for STEC; lane 8, B2 (stx_1 and stx_2); lane 9, B9 (stx_1 and stx_2); lane 10, B17 (stx_1); lane 11, B5 (stx_2 and *eaeA*; lane 12, B7 (stx_2 and *eaeA*); lane 13, B3 (stx_2); lane 14, B8 (stx_2); lane 15, B10 (stx_2 and *eaeA*) and lane 16, B24 (stx_1).

4.4.1 Distribution of stx_1, stx_2 and *eaeA* genes in *E. coli* isolates from bovine

Majority of STEC isolates, 22 of 24 (92%) possessed stx_2, 12 (50%) possessed stx_1 while *eaeA* was detected in three (B5, B7 and B10), 13% of the isolates (Table 4). Out of the 24 STEC pathotypes identified, two (8 %) isolates; B17 and B24 (Table 4) had stx_1 gene only, nine (37.5%) had stx_2, 10 (42 %) had both stx_1 and stx_2 and three (12.5 %); B5, B7 and B10 had both stx_2 and *eaeA* genes (Figure 6). Isolates with both stx_1 and *eaeA* genes were not identified (Table 4, Figure 5).

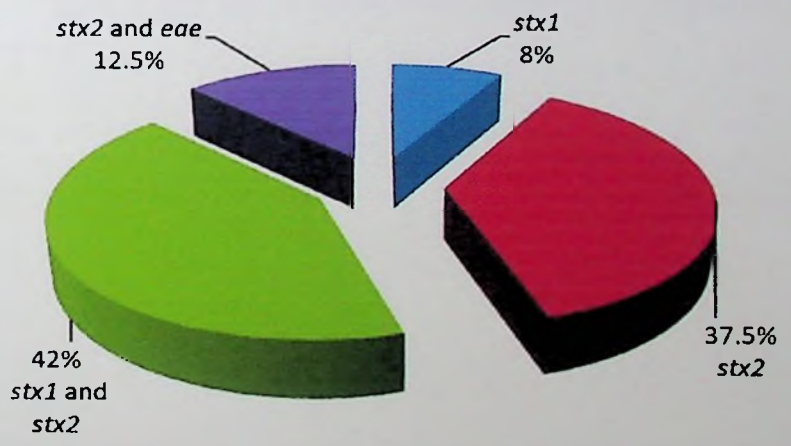

Figure 6 Distribution of stx_1, stx_2 and *eaeA* genes in bovine *E. coli* isolates

4.5 Detection of pathoypes of *E. coli* in chicken faecal samples

Similarly PCR amplification was carried out on 57 *E. coli* isolates from chickens. Amplicons corresponding only to *eaeA* gene were detected in four of 57 isolates (7%), (Table 5 and Figure 7). No additional PCR products were detected with the remaining primers for ETEC, EIEC, EHEC and EAEC and these were recorded as negative.

Table 5 Pathogenic *E. coli* isolates identified in chicken faecal samples

Chicken *E. coli* Isolates	Virulence genes targeted						
	stx$_1$	*stx*$_2$	*eaeA*	*estA*	*eltB*	*Ial*	*pCVD*
C1	-	-	+	-	-	-	-
C2	-	-	+	-	-	-	-
C3	-	-	+	-	-	-	-
C4	-	-	+	-	-	-	-

C means Chicken, +/- indicates presence and absence of the gene respectively

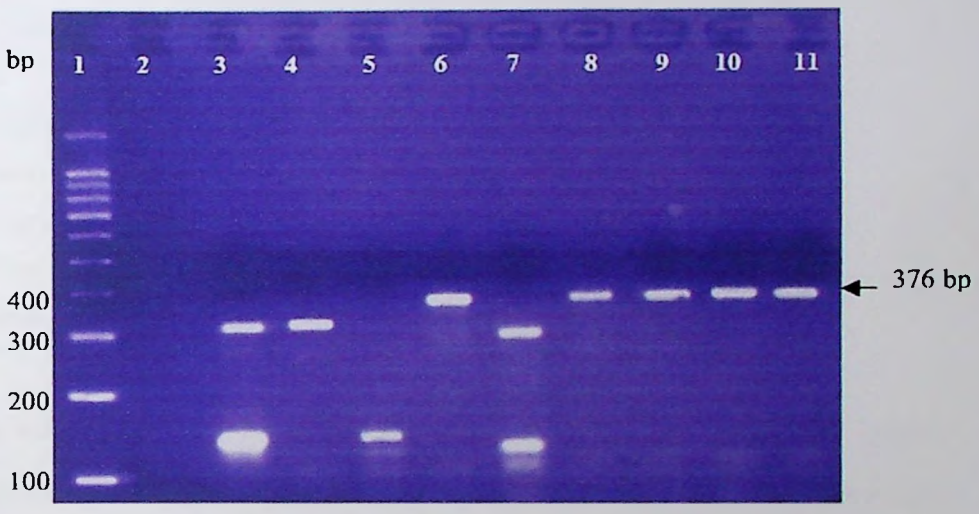

Figure 7 Results of multiplex PCR with *E. coli* isolates from chicken faecal samples. Lane 1, 100bp DNA ladder; lane 2, negative control, EC-130PN; lanes 3 to 7 positive control strains; lane 3, ETEC-H10407 for *eltB & estA;* lane 4, ETEC-90PN for *eltB;* lane 5, ETEC-67PN for *estA;* lane 6, EPEC-186PN for *eaeA;* lane 7, STEC-209PN for *stx*$_1$, *stx*$_2$*;* lane 8 -11, C1- C4 *eaeA*- (EPEC) positive chicken isolates.

4.6 Antibiotic susceptibility results

4.6.1 Resistance of the 24 STEC isolates from cattle to antibiotic drugs

Antibiotic susceptibility test was performed for the 24 STEC isolates from cattle using selected antibiotic drugs (Table 6). Of the 24 STEC isolates identified, 10 (41.7%) were resistant to at least one antibiotic drug used (Tables 7). Resistance of the 24 STEC isolates was observed (Tables 6 and 7) for ciprofloxacin (12.5%), chloramphenicol (8.3%), nalidixic acid (8.3%) and tetracycline (33.3%). There was no resistance of the 24 STEC isolates to gentamicin (0%,): Tables 6 and 7.

Table 6 Antibiotic resistance of the 24 STEC isolates from cattle faeces

Antibiotic drug	Resistant STEC		Susceptible STEC	
	Number	%	Number	%
Ciprofloxacin	3	12.5	21	87.5
Chloramphenicol	2	8.3	22	91.7
Tetracycline	8	33.3	16	66.7
Nalidixic acid	2	8.3	22	91.7
Gentamicin	0	0.00	24	100

Among the 10 resistant STEC pathotypes identified (Table 7), five (41.7%) carried only stx_2, three (25%) had stx_1 and stx_2 combination, two (16.7%) with stx_2 and *eaeA* combination. Of the two isolates carrying stx_1 gene, none was resistant to any antibiotic drug used. There was no significant difference in response to the five antibiotic drugs for the STEC isolates possessing; stx_1 and stx_2 combination ($p = 0.406$), stx_2 and *eaeA* ($p = 0.558$), and those with *eaeA* alone ($p = 0.588$). However, there was a significant difference in the way the *E. coli* isolates possessing stx_2 alone responded to the five antibiotics drugs ($p = 0.009$). Overall, there was a significant difference in the way the 24 STEC *E. coli* isolates responded to the five antibiotic drugs ($p = 0.001$).

Table 7 antibiotic resistance and virulence genes of STEC isolates from cattle

Pathotype positive	Virulence gene (no.)	Number (%) of isolates resistant to antibiotic drugs					Total No. (%)
		CIP	CHL	TET	NA	GM	
STEC (24)	*stx*$_1$ (2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	stx$_2$ (9)	2 (22.2)	1 (11.1)	5 (55.6)	1(11.1)	0 (0.0)	5 (41.7)
	stx$_1$+*stx*$_2$ (10)	0 (0.0)	1(10.0)	2 (20.0)	1(10.0)	0 (0.0)	3 (25.0)
	stx$_2$+*eaeA* (3)	1 (33.3)	0 (0.0)	1(33.3)	0 (0.0)	0 (0.0)	2(16.7)
		3 (30)	**2 (20)**	**8 (80)**	**2 (20)**	**0 (0.0)**	**10 (100)**

CIP, ciprofloxacin; CHL, chloramphenicol; TET, tetracycline; NA, nalidixic acid and GM, gentamicin

One STEC isolate carrying *stx*$_2$ gene only, was resistant to more than two antibiotic drugs (Table 8). All resistant STEC isolates with *stx*$_2$ and *eaeA* gene combination were resistant to only one antibiotic drug (Table 8). Two STEC isolates, one carrying *stx*$_2$ alone and another carrying both *stx*$_1$ and *stx*$_2$ were resistant to two antibiotic drugs. None of the STEC isolates was resistant to all antibiotic drugs used (Table 8).

Table 8 Antimicrobial resistance profiles of STEC pathotypes from cattle faeces

Profile	Gene (s)	Number of isolates
TET	*stx*$_1$, *stx*$_2$	1
	stx$_2$, *eaeA*	1
	stx$_2$	3
CIP	*stx*$_2$, *eaeA*	1
CHL	*stx*$_1$, *stx*$_2$	1
TET, NA	*stx*$_1$, *stx*$_2$	1
TET, CIP	*stx*$_2$	1
TET, CHL, NA, CIP	*stx*$_2$	1

4.6.2 Resistance of the four EPEC isolates from chicken to antibiotic drugs

Of the 57 *E. coli* isolates from chicken faeces, only four (7%) were identified as EPEC. No other pathotype other than EPEC was identified in chicken of this study. Of the four EPEC isolates identified, only two were resistant, one to ciprofloxacin and another to tetracycline (Tables 9 and 10).

Table 9 Antibiotic resistance of the four EPEC isolates from chicken faeces

Antibiotic drug	Resistant EPEC	
	Number	%
Ciprofloxacin	1	25
Chloramphenicol	0	0
Tetracycline	1	25
Nalidixic acid	0	0
Gentamicin	0	0

Table 10 Antimicrobial resistance profiles of EPEC pathotype from chicken faeces

Profile	Gene (s)	Number of EPEC isolates
TET	*eaeA*	1
CIP	*eaeA*	1

4.6.3 Prevalence (%) of Resistance of *E. coli* pathotypes and non-pathotypes to antibiotic drugs

Resistance of the 28 (24 STEC from cattle and 4 EPEC from chicken) pathogenic *E. coli* to antibiotic drugs was compared with that of the 89 non-pathogenic *E. coli* isolates from cattle and chicken faeces. Non-pathogenic *E. coli* were more resistant (79.8%, 71 of 89 isolates) than the pathogenic ones identified (42.9%, 12 isolates of 28). Prevalence of resistance to antibiotic drugs in both pathogenic and non-pathogenic

isolates was highest to tetracycline and lowest to gentamicin (Figure 8). Also prevalence of resistance of non-pathogenic *E. coli* to ciprofloxacin, tetracycline, and nalidixic acid was higher than that of pathogenic *E. coli*. However, prevalence of resistance to chloramphenicol was slightly higher in pathogenic than non-pathogenic *E. coli* isolates. Prevalence of resistance of pathogenic and non-pathogenic *E. coli* isolates did not significantly differ in their response to ciprofloxacin ($p = 0.153$), chloramphenicol ($p = 0.688$) and nalidixic acid ($p = 0.177$). However, there was a significant difference in resistance of *E. coli* isolates to tetracycline ($p < 0.001$).

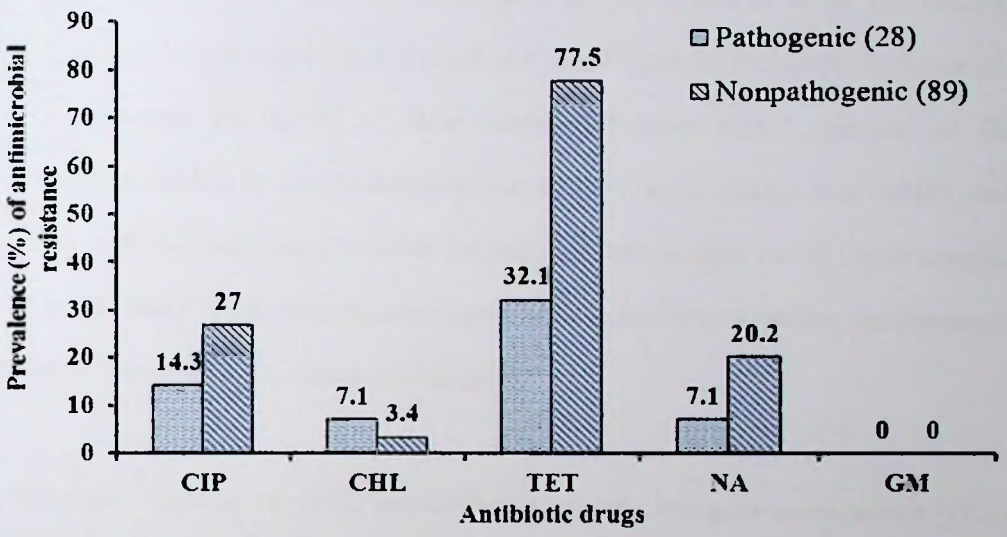

Figure 8 Prevalence of resistance of *E. coli* pathotypes and nonpathotypes

CHAPTER FIVE

DISCUSSION

5.1 Distribution of *Escherichia coli* pathotypes in cattle and chicken faeces

This study found STEC in cattle and EPEC in chicken faecal *E. coli* isolates. The percentage of STEC (40%) isolated in this study is higher than that from other studies on cattle from different settings in Uganda. For instance, STEC was isolated in 15 of 216 (6.9%) cattle reared on range (Majalija *et al.*, 2010) and in 45 of 159 (28.3%) faecal samples of zebu cattle from a ranch in Central Uganda (Kaddu-Mulindwa *et al.*, 2001). However, the ability of these cattle to harbour STEC depends on the environment. Studies by Kaddu-Mulindwa *et al* (2001) and Majalija *et al* (2010) used samples collected from animals within the same locality or same ranch. Cattle samples used in this study were collected from animals that came from different environments of Uganda brought to the abattoir for slaughter.

In this study, majority of STEC identified had stx_1 and stx_2 gene combination (42%) followed by stx_2 (37.5%), stx_2 and *eaeA* (12.5%) and lastly, stx_1 (8%). Of all STEC isolates identified in this study, none had both stx_1 and *eaeA* combination of genes. The results of this study are in agreement with that by Kaddu-Mulindwa *et al* (2001) where out of 45 STEC isolates identified, 27 produced stx_1 and stx_2 combination, 13 produced stx_2, and the rest produced stx_1. These results however, contrast those obtained by Majalija *et al* (2007, 2010) where the dominant *stx* subtype among STEC strains obtained from cattle of Western region was stx_1. Majalija *et al* (2010) observed almost a similar trend for *stx* genes carried by STEC in faeces of diarrhoeal children in a rural pastoral community within the same setting as 37% carried stx_1 alone, 32.1% with stx_2

and 30.9% with both *stx*$_1$ and *stx*$_2$. These results suggest that the different *stx* subtypes and combinations exist in healthy cattle of Uganda and presence and dominance of a given subtype and combination in particular animals depends on the variations in the environments within and among the different regions of the country.

The pattern of distribution of virulence gene markers observed in STEC isolates from cattle of this study is also similar to the virulence gene profile of STEC isolates in a study of cattle stool samples from a city abattoir in India (Khan *et al.*, 2002). However, no *stx*$_2$ and *eae* marker combination was identified instead *stx*$_1$ and *eae* combination was identified. In study by Osek (2003), both *stx*$_1$ and *eae*, and *stx*$_2$ and *eae* virulence gene combinations were identified in cattle isolates while Blanco *et al* (2003) identified *stx*$_1$ and *eae* combination more in calves than cows.

The *stx*$_2$ gene is the most commonly distributed gene among STEC isolates identified in this study with at least 92% of the isolates in possession of the gene (*stx*$_2$), either alone or in combination with *stx*$_1$ and *eaeA* genes. In other studies within and outside Uganda, *stx*$_2$ was also more common than *stx*$_1$ in cattle faeces (Kaddu-Mulindwa *et al.*, 2001; Blanco *et al.*, 2003; Oseki, 2003; Rigobelo *et al.*, 2011). However, these results contrast those by Majalija *et al* (2010) on cattle faeces, where *stx*$_1$-positive isolates were more than *stx*$_2$-positive isolates. Unlike the previous studies in Uganda, the present study involved use of animals collected from different regions of Uganda and at present, STEC possessing both *stx*$_1$ and *stx*$_2$ appear to be more common among healthy cattle of Uganda as well as *stx*$_2$ being the most widely distributed *stx* gene in healthy cattle of Uganda.

The STEC strains carrying only *stx*$_2$ gene are potentially more virulent than those carrying only *stx*$_1$ or even those carrying both *stx*$_1$ and *stx*$_2$ (Rodolpho *et al.*, 2007; Majalija *et al.*, 2010). The STEC carrying *stx*$_1$ or *stx*$_1$ and *stx*$_2$ are associated with mild diarrhoea whereas those carrying *stx*$_2$ alone are commonly associated with bloody diarrhoea and HUS (Majalija *et al.*, 2010). In this study, 37.5% of STEC isolates were positive for *stx*$_2$ gene alone. This therefore may present a serious threat to human health as these virulent isolates can enter the food chain finding their way into humans.

Also three of the *stx*$_2$-positive STEC were also positive for *eae* gene encoding intimin protein, indicating that STEC strains carrying *eae* gene are not common in healthy cattle. Presence of *eae* gene has also been found lower in other studies involving healthy cattle (Kaddu-Mulindwa *et al.*, 2001). However, *eae* presence may further present a serious hazard to humans in contact with these strains because presence of *eae* gene may be correlated with severe disease (Blanco *et al.*, 2003; Rodolpho *et al.*, 2007; Herrera-Hura *et al.*, 2009; Majalija *et al.*, 2010). For instance *eae* has been frequently isolated from diarrhoeic calves (Daube & Mainil, 2004; Herrera-Luna *et al.*, 2009) with studies indicating as high as 51.7% of the isolates being positive for *eae* gene (Daube & Mainil, 2004). Studies of STEC isolated from diarrhoeic children have also highly demonstrated presence of *eae* gene (Majalija *et al.*, 2010).

However, absence of *eae* gene in a potentially harmful STEC strain may not indicate its inability to cause disease especially in humans. For instance, *eae*-lacking STEC strains have been isolated in patients with HUS and haemorrhagic colitis (Khan *et al.*, 2002; Blanco *et al.*, 2003), which suggests presence of additional virulence factors. Type I fimbriae have been suggested (Nataro & Kaper, 1998). Also a mega plasmid-encoded

adhesin has been detected in bovine STEC strains lacking *eae* gene that may be an important virulence factor for *eae*-negative STEC strains capable of causing severe disease in humans (Blanco *et al.*, 2003).

In Uganda, this study seems to be one of the first studies on distribution of *E. coli* pathotypes in healthy chickens as there is limited information about *E. coli* pathotypes carried by these birds. Of the 57 chicken *E. coli* isolates investigated, four (7.0%) were EPEC. No other pathotypes other than EPEC were identified in this study. Absence of STEC in chicken of this study is in agreement with study by Farooq *et al* (2009) where no STEC pathotypes were observed in *E. coli* isolates from faeces of chicken in India. Dutta *et al* (2011) also reports absence of STEC in healthy chicken of India but indicates presence of STEC in diarrhoeal chicken, suggesting that healthy chicken may not be carriers of STEC but are carriers of EPEC. However, the relationship between pathotype carriage and host preference is not clear.

5.2 Resistance of *E. coli* pathotypes to antimicrobials

In this study, faecal samples were collected from older chickens as this stage presents contamination of eggs and poultry meat, and older cattle as it also presents higher chances of contamination of meat during slaughter. A total of 28 pathogenic *E. coli* isolates were identified and of these, 12 (42.9%) were resistant to at least one antibiotic drug used. Resistance of the pathotypes was lower to ciprofloxacin, chloramphenicol, and nalidixic acid as compared to tetracycline. The generally low resistance level may indicate less exposure of the pathotypes to the antibiotic drugs or source of resistance genes. The higher resistance to tetracycline reflects the level of drug overuse, misuse

and contamination in the environment either by sewage from humans or other animals. No resistance to gentamicin was observed.

Over use of antibiotic drugs corresponds directly to their ready availability on the market, low cost (Byarugaba *et al.*, 2011) and lack of professionalism on the side of the expert dealing in such drugs. To certain extent, crowding and poor sanitation also contribute to resistance of bacteria to antibiotic drugs (Salehi *et al.*, 2006). These factors are common especially in intensive farming and may be associated with greater disease potential, creating greater tendency for antibiotic use to control diseases (Kikuvi *et al.*, 2000). This in turn results into greater resistance to such greatly used antibiotic drugs.

No resistance to gentamicin was observed in this study and resistance to chloramphenicol and nalidixic acid was comparatively lower. Gentamicin has had little use in animals (Bywater *et al.*, 2004) and in Uganda no formulations seem to be available for use in animals. Because of this, less or no resistance to gentamicin is observed in animals. Also the level of contamination of the environment from which the animals pick resistant strains becomes more limited to humans. The same principle applies to chloramphenicol, which has not also been approved for use in animals in several countries including Uganda. However, this and other studies (Adelaide *et al.*, 2008; Bywater *et al.*, 2004) have shown resistance of *E. coli* to chloramphenicol in countries where it is not applied in animals. This could be a result of horizontal transfer of genes from sources like water contaminated with human sewage or co-resistance with other unrelated compounds (Bywater *et al.*, 2004). Indeed co-selection

of chloramphenicol resistance during selective pressure imposed by sulphonamides and streptomycin being due to linkage of genes has been reported (Kehrenberg *et al.*, 2001).

Both pathogenic and nonpathogenic bacteria can acquire resistance to antimicrobial drugs from the environment (Aarestrup, Wegener and collignon, 2008; Bogaard *et al.*, 2001; Salehi *et al.*, 2006). Of the 10 resistant STEC pathotypes identified in this study, the potentially virulent isolates carrying stx_2 alone were more resistant (41.7%) compared to those carrying both stx_1 and stx_2 (25%), or both stx_2 and *eaeA* (16.7%) . All resistant isolates that carried both stx_2 and *eaeA* genes and those carrying only *eaeA* (EPEC) were resistant to only one antibiotic drug.

In this study, resistance to at least two antibiotic drugs was taken to be multidrug. Three of the 12 pathogenic *E. coli* isolates were multidrug resistant and all were STEC isolates from cattle. These included two isolates carrying stx_2 gene only and another isolate carrying both stx_1 and stx_2. None of the isolates was resistant to all antibiotic drugs used in this study. However, one STEC isolate carrying stx_2 gene was resistant to four antibiotic drugs.

The resistance profile of the 28 pathotypes identified in this study was compared with that of the non-pathogenic strains (89 isolates) to establish whether significant response to drugs between the two groups existed. Non-pathogenic *E. coli* were more resistant (79.8%) than the pathogenic ones identified (42.9%). These non-pathogenic *E. coli* isolates could be serving as a source from which the pathogenic ones pick resistance genes by horizontal transfer. Prevalence of resistance to antibiotic drugs in both

pathogenic and non-pathogenic isolates was highest to tetracycline and lowest to chloramphenicol.

Study by Bettelheim *et al.*, 2003 also indicates high level of resistance among non-STEC than amongst STEC regardless of the source, agreeing with results of this study. However, STEC from diarrhoeic calves have been found to be more resistant to antibiotic drugs than those isolated from healthy animals (Bettelheim *et al.*, 2003). This higher antibiotic drug resistance in sick animals could be a result of intensive use of antibiotic drugs to control such infections which in turn results into a selective pressure favouring those able to survive, to multiply and constitute the majority.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Healthy cattle in Uganda are carriers of STEC and the genes or their combinations; stx_1 and stx_2, only stx_2, only stx_1 or both stx_2 and *eaeA* as identified in this study indicates that different *stx* sub-types and combinations exist in healthy cattle.

The fact that only EPEC pathotype was identified in healthy chicken and STEC in healthy cattle suggests presence of host preference factors for these pathotypes.

Presence of such virulent *E. coli* in cattle and chickens as observed in this study shows a potential threat in the human food chain. Therefore, farm authorities and the general public should be taught about the likely dangers of un-hygienically handling chicken and cattle, and their products.

Presence of resistant *E. coli* isolates in healthy animals indicates that use of antimicrobial drugs may be rampant and not following proper guidelines. Therefore, there is need to monitor the sale, storage and use of antibiotic drugs to minimize the selection of resistant bacteria.

6.2 Recommendations

In order to ensure public health protection, there is need for continued surveillance of emerging antimicrobial resistance among the pathogenic strains carried especially in the healthy animals.

Hygiene in slaughter places and dairy processing plants should be monitored to minimize the rate at which these pathotypes are transferred or spread in the human population.

In order to fully understand why healthy cattle are carriers of STEC and healthy chicken, EPEC, more studies should be carried out to establish the relationship between pathotype carriage and host preference.

Further studies should be carried out to establish the distribution of antibiotic resistance genes among *E. coli* pathotypes of chicken, cattle and humans.

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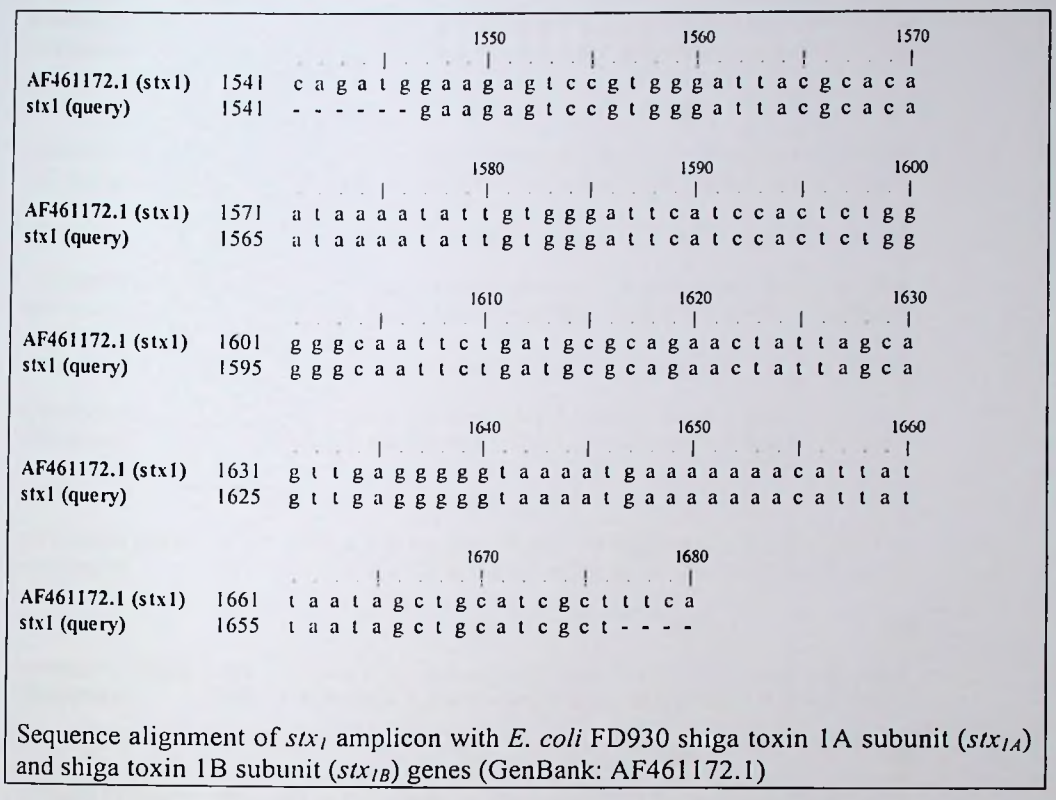
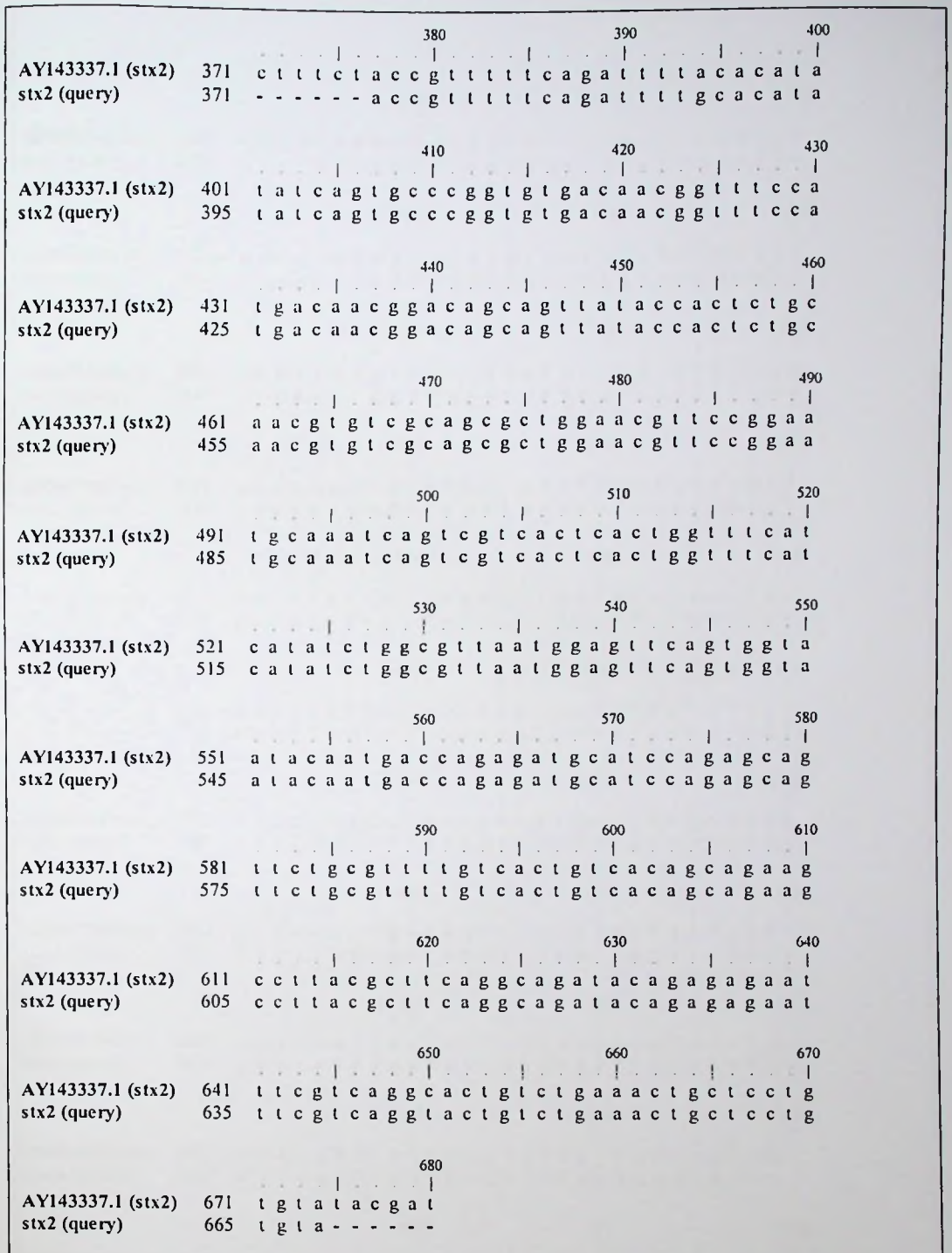
8.0 APPENDICES

8.1 Appendix I

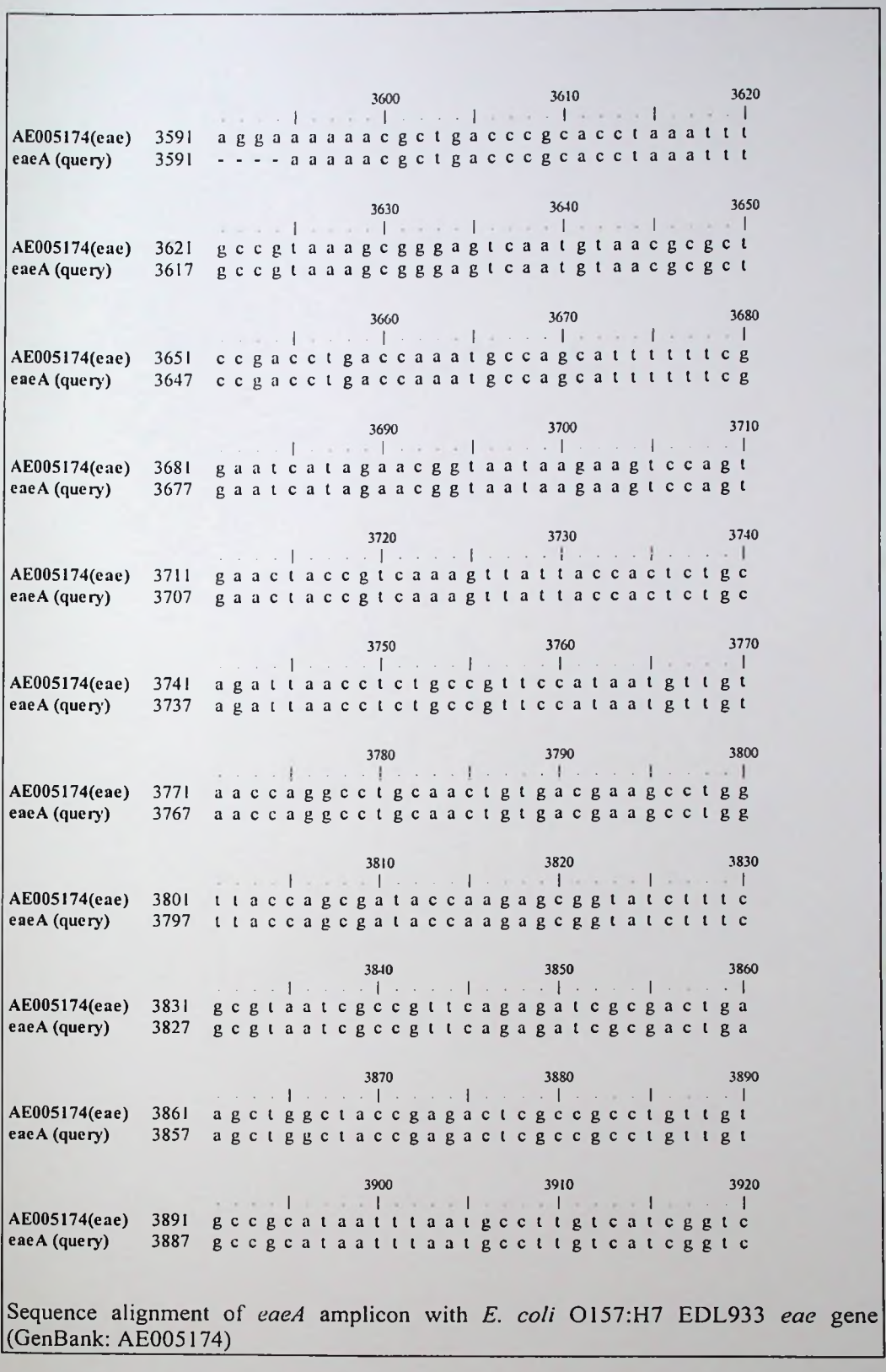
Sequence alignment results



Sequence alignment of stx_1 amplicon with *E. coli* FD930 shiga toxin 1A subunit (stx_{1A}) and shiga toxin 1B subunit (stx_{1B}) genes (GenBank: AF461172.1)



Sequence alignment of *stx*$_2$ amplicon with *E. coli* O157: H- strain 493/89 shiga toxin 2 subunit A (*stx*$_{2A}$) and shiga toxin 2 subunit B (*stx*$_{2B}$) genes (GenBank: AY143337.1



Sequence alignment of *eaeA* amplicon with *E. coli* O157:H7 EDL933 *eae* gene (GenBank: AE005174)

```
                        . . . . | . . . . | . . . . | . . . . | . . . . | . . . . |
AE005174(eae)   3891    g c c g c a t a a t t t a a t g c c t t g t c a t c g g t c
eaeA (query)    3887    g c c g c a t a a t t t a a t g c c t t g t c a t c g g t c

                                          3930                3940                3950
                        . . . . | . . . . | . . . . | . . . . | . . . . | . . . . |
AE005174(eae)   3921    a t g t t g c t t t t g g t c a c g t c c g g g g a c a t t
eaeA (query)    3917    a t g t t g c t t t t g g t c a c g t c c g g g g a c a t t

                                          3960                3970
                        . . . . | . . . . | . . . . | . . . . | . . . . |
AE005174(eae)   3951    t t a g t c a g t t t a t t c g t g t g a c c a g
eaeA (query)    3947    t t a g t c a g t t t a t t c g t g t g - - - - -
```

Continuation of sequence alignment results of *eaeA* amplicon with *E. coli* O157:H7 EDL933 *eae* gene (GenBank: AE005174)

8.2 Appendix II

Sequence data

Gene	Sequence	Size (bp)
eaeA	aaaaacgctgacccgcacctaaatttgccgtaaagcgggagtcaatgtaacgcgctccgacctgaccaaa tgccagcatttttcggaatcatagaacggtaataagaagtccagtgaactaccgtcaaagttattacca ctctgcagattaacctctgccgttccataatgttgtaaccaggcctgcaactgtgacgaagcctggttac cagcgataccaagagcggtatctttcgcgtaatcgccgttcagagatcgcgactgaagctggctaccgag actcgccgcctgttgtgccgcataatttaatgccttgtcatcggtcatgttgcttttggtcacgtccggg gacattttagtcagtttattcgtgtg	376
stx_2	accgtttttcagattttacacatatatcagtgcccggtgtgacaacggtttccatgacaacggacagcag ttataccactctgcaacgtgtcgcagcgctggaacgttccggaatgcaaatcagtcgtcactcactggtt tcatcatatctggcgttaatggagttcagtggtaatacaatgaccagagatgcatccagagcagttctgc gttttgtcactgtcacagcagaagccttacgcttcaggcagatacagagagaatttcgtcaggtactgtc tgaaactgctcctgtgta	298
stx_1	gaagagtccgtgggattacgcacaataaaatattgtgggattcatccactctgggggcaattctgatgcg cagaactattagcagttgagggggtaaaatgaaaaaaacattattaatagctgcatcgct	130