Isolation And Molecular Characterization Of Shiga Toxin Producing *Escherichia coli* In Cattle, Water And Diarrhoeal Children From The Pastoral Systems Of Southwestern Uganda

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Thesis presented for the degree of

DOCTOR OF PHILOSOPHY

Faculty of Health Sciences
UNIVERSITY OF CAPE TOWN

February 2009

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Typeset using PDFLaTeX as implemented in MIKTeX.
Printed in South Africa at:
University of Cape Town,
Private Bag,
7700, Rondebosch.
Cape Town.

Specially dedicated to

my parents

Methuselah and Glads Majalija

and wife

Mabel Majalija

Declaration

I..... declare that the work presented in this thesis is my original work, except where indicated, and that it has not been submitted before for any degree or examination at any university.

Signed: .....
Date:

Acknowledgements

This study was conducted under the supervision of associate Professor Gay B. Elisha and co-supervisor Dr Segal Heidi both of University of Cape Town, and associate Professor Francis Ejobi from Makerere University. I am greatly indebted to Professor Gay B. Elisha for the intellectual guidance, inspiration and patience during the entire study period. I am thankful to Dr Heidi Segal for equipping me with the needed skills in molecular biology and to associate Professor Francis Ejobi for the guidance during field work.

I am greatly indebted to Universities for Sciences, Humanities and Engineering for Partnership in Africa (USHEPiA) for the generous fellowship to pursue my Ph D studies. The administrators of USHEPiA, Ms. Nan Warner, Mrs. Masego Mogodu, Mrs. Zubaida Hattas and Ms. Norma Derby deserve a mention. Special thanks to Africa Institute for Capacity development (AICAD) for the additional financial support during data collection.

I appreciate Makerere University, my employer, for granting me a study leave and the generous research grant.

My work would not have been possible without the permission from the Director of Mbarara regional referral hospital and the cooperation of the medical staff of Rushere Hospital, Kiruhura and Nyakashashara health centres. Thank you very much for the participation and cooperation.

My family has been very dear to me; Martha, Jesse, Joel and Joshua, thank you for your prayers and patience during this time when you needed me most, but I was never around. My dear wife, Mabel Majalija, you are a special God given friend. Your constant love and encouragement motivated me. Thank you for being there for me.

The staff in the Department of Veterinary Microbiology, Faculty of Veterinary Medicine Makerere University were very helpful. I am particularly indebted to Mr. Lubowa Musiisi, the Chief technician, who cultured the bacterial isolates. I also thank Mr. Pascal Musoni, Manager Microbiology laboratory Groote Schuur Hospital at the University of Cape Town for providing me with a stock of *Escherichia coli* O157:H7.

Special thanks to my parents, Methuselah Majalija (RIP) and Glads Majalija; who treasured education and provided me the opportunity to study not available to themselves. Thank you very much. I can't repay you for what you did. *Mukama abahe omugisa*.

All Africa House has been my home for the past three years. I am grateful to the administrative managers of All Africa House for ensuring my comfort during this period. Similarly, I extend my appreciation to all USHEPiA Fellows, with whom I have shared both social and academic life. I thank Ms. Joanne Evans and Ms. Rachael Jacobs, two of my colleagues I closely worked with in the laboratory.

Lastly and most important, special thanks to God, my help and strength. I know it is by your power and favour that I have been able to accomplish this work. What *eyes have not seen, ears have not heard or even the mind perceived, that you have prepared for your children*. Your name be glorified through Jesus Christ our Lord and Saviour. *Amen*

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Abbreviations and Notations

B	
bp	Base pair
BD	bloody diarrhoea
BLAST	Basic Local Alignment Search Tool
C	
CHEF	Clamped homogeneous electric field
CTAB	Cetyltrimethylammonium bromide
D	
DNA	Deoxyribonucleic acid
E	
eae	Intimin gene
EAggEC	Enteroaggregative *Escherichia coli*
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
Esp	*Escherichia coli* secretory protein
EspA	*Escherichia coli* secretory protein A
EspB	*Escherichia coli* secretory protein B
EspD	*Escherichia coli* secretory protein D
G	
Gb_3	Globotriaosylceramide
Gb_4	Globotetraosylceramide
H	
HC	Haemorrhagic colitis
Hela	Henrietta Lacks's
HEp-2	Human epithelial cells
HUS	Haemolytic uraemic syndrome
I	
I.D	Identity

K

Kb	Kilo base

L

LEE	Locus of enterocyte effacement
LPS	Lipopolysaccharide

M

M	Molar
mM	millimolar
min	Minute(s)
mg	milligram
ml	millilitre

N

NCBI	National Center for Biotechnology Information
No.	Number
NC	needle complex
nt	Nucleotide

O

OD	Optical density

P

PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
PG	Phylogenetic group
PGs	Phylogenetic groups

R

RFLP	Restriction fragment length polymorphism

S

S	seconds
SF	sorbitol
SF^+	sorbitol fermenting
SF^-	non-sorbitol fermenting
SPT	Seropathotype
SPTs	Seropathotypes
STEC	Shiga toxin producing *Escherichia coli*
Stx	Shiga toxin protein
Stx1	Shiga toxin 1 protein
Stx2	Shiga toxin 2 protein
stx	Shiga toxin gene
stx_1	Shiga toxin 1 gene
stx_2	Shiga toxin 2 gene

T

TBE	Tris-borate EDTA buffer
TE	Tris-EDTA buffer
Tris	Tris(hydroxymethyl)aminomethane

TTSS	Type three secretion system

V

VTEC	vero(cyto)toxin producing *Escherichia coli*
V	Volts

W

WD	watery diarrhoea

Y

YT agar	Yeast tryptone agar

Symbols

α	alpha
β	beta
γ	gamma
δ	delta
ε	varepsilon
η	eta
ζ	zeta
θ	theta
ι	iota
κ	kappa
λ	lambda
μ	mu
μl	Microlitre
ν	nu
ξ	xi
ρ	rho
®	trade mark
%	percent
^{o}C	degrees centigrade
+	positive
-	negative

Abstract

This study describes the molecular characteristics of STEC isolated from the pastoralist community of Nyabushozi in Southwestern Uganda. Faecal samples or rectal swabs of children with diarrhoea obtained in phases 1 and 2 were investigated for the presence of STEC by PCR detection of *stx* genes. During phase 1, cattle reared on range which were associated with households of sick children were investigated in parallel to the children for STEC excretion. STEC was isolated from *E. coli* in 7 of 80 (8.8%) children and in 15 of 216 (6.9%) bovines in phase 1. Similarly, STEC was isolated from 11 of 142 (7.7%) *E. coli* carrying children and 3 of 45 (6.7%) water samples in phase 2.

Molecular characterization further ascertained the genetic relatedness of STEC. PFGE profiles of up to 10 colonies obtained from an individual source (child, bovine or water) and in total 185 STEC colonies were analysed. Nine profiles from 43 colonies (phase 1) and 15 profiles from 38 colonies (phase 2) obtained from children were not or were distally related, indicating the genetic diversity of clinical STEC. The intra-host analysis of STEC profiles revealed that strains from 11 of the 13 children exhibited multiple clonal subgroups. The 101 colonies from 15 bovines clustered in 18 different profiles. Clonal subgroups were observed in multiple STEC colonies from 11 of 12 bovines. Closely related profiles indicated that STEC isolated from two children (Hh2 and Hh4) was acquired from bovines or their environment. While none of the clinical or bovine STEC were related to 5 genetically diverse water strains.

A single isolate of STEC representing each PFGE profile in association with *stx* gene content was serotyped for the O antigen. Twenty four bovine STEC were typed into 10 O serogroups including O8, O76, O111 and O113, which were also identified among the clinical STEC. The 25 clinical STEC belonged to 15 serogroups of which O29, O149 and O176 are being reported for the first time as clinical STEC. STEC

O166 was isolated from a child and water during the same sampling, indicating the potential health hazard of drinking STEC-contaminated water. The production of Shiga toxin (Stx) investigated using Duopath Verotoxin detection kits showed that a majority of STEC from different sources produced Stx1 or Stx2 or both Stx.

Using PCR or PCR-RFLP assays, stx_2 and *eae* gene types were analysed. Variant stx_{2-vhc} was most prevalent and closely associated with stx_{2d-2} in clinical and bovine STEC. The frequency of *eae*-positive STEC among clinical and bovine STEC was 15 of 25 (60%) and 14 of 24, (58.3%), respectively. *eae*-$\gamma 2/\theta$ was predominant among the bovine STEC, *eae*-κ/δ in clinical STEC, while *eae*-$\beta 1$ was associated with STEC from different sources including water. Previously undescribed *eae*-positive serogroups O28ac, O113, O142 and O158 were identified.

Studies of the genetic background showed that both clinical and bovine STEC obtained in phase 1 predominantly belonged to phylogenetic group A and B1, while phase 2 clinical and water STEC belonged to group D and A, respectively. Seropathotype classification of clinical STEC, separated most strains (20 of 24 strains) into seropathotype D. These STEC belonged to phylogenetic groups A, B1 and D.

Thus, the characterised genetic attributes of STEC from Nyabushozi suggests that the pathogens have the potential to cause a wide spectrum of childhood illnesses ranging from mild to bloody diarrhoea and haemolytic uraemic syndrome.

Chapter 1

General Introduction

1.1 Aims of this work: background

1.1.1 Geographic, demographic and climate characteristics of the cattle corridor in Uganda

Uganda is located in the great lakes region of East Africa. The country is bordered by Kenya in the East, Tanzania and Rwanda in the South, the Democratic Republic of Congo in the West and Sudan in the North (Figure 1.1). The landmass covers an area of about 241,500 sq. km, of which 15.3% is open water, 3.0% permanent wetlands and 9.4% seasonal wetlands (National Environmental Management Authority, 2002). More than two-thirds of the country, located on a plateau, 1000-2500m above sea level is available as agricultural arable land (Kamanyire, 2000; National Environmental Management Authority, 2002). Over 87% of the 28.4 million inhabitants are rural based (United Nations Population Fund, 2007), engaged in livestock and crop agricultural activities (Kamanyire, 2000).

Stretching across the country is the cattle corridor, a typical savannah grassland, which extends from the border with Rwanda in the south-west to the Sudan and Kenya borders in the North-east, dividing the country into two distinct crop and cattle keeping regions (Asiimwe, 2000; Kamanyire, 2000). The region covers 43% of the country (National Environmental Management Authority, 2002), receives erratic rainfall (500-1000mm annually), has prolonged droughts, poor soil and scarce

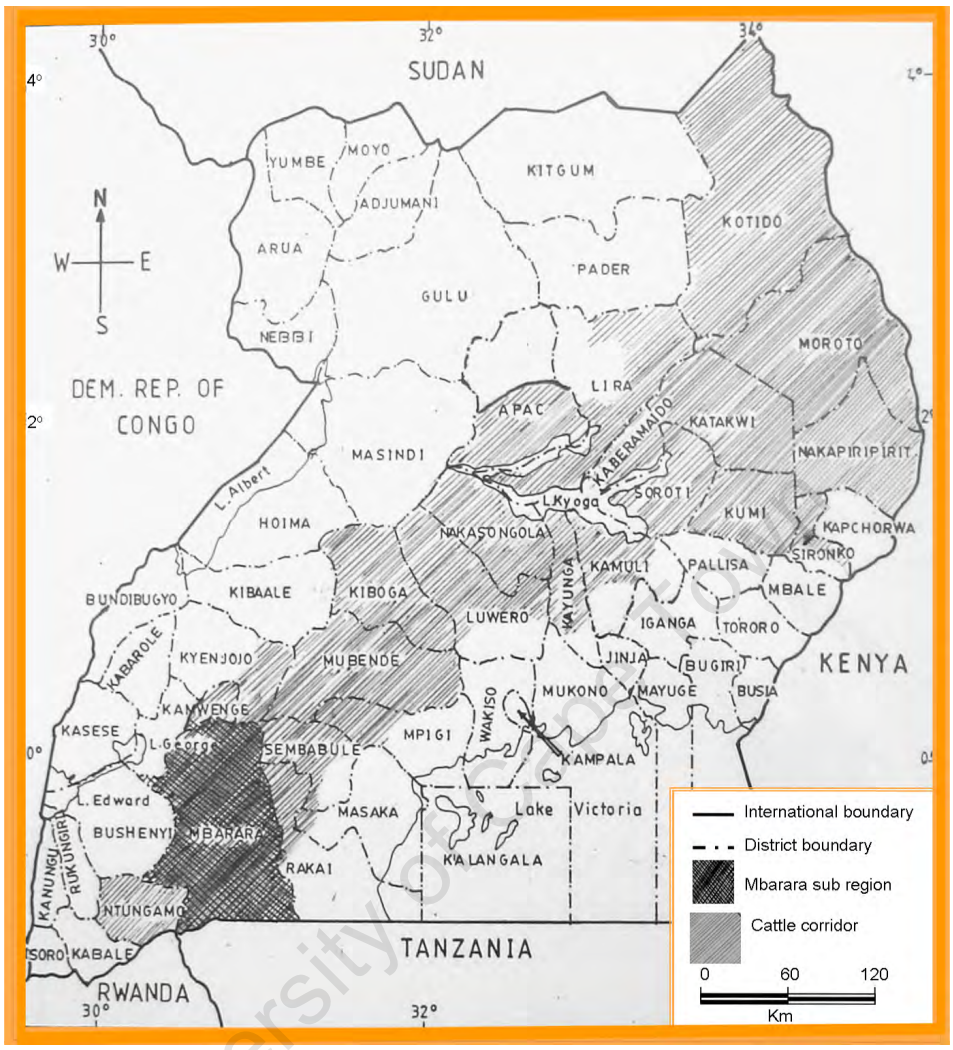

Figure 1.1: Cattle corridor region in Uganda.

permanent surface reservoirs (Kisamba-Mugerwa and Nuwagaba, 1996). The pastoral and agro-pastoral communities in the cattle corridor rangelands support more than 95% of the national cattle population (Kisamba-Mugerwa, 2001; Oloya, 2006), and produce most (85%) of the beef and milk in the country (Kisamba-Mugerwa, 2001). The longhorn Ankole cattle, an indigenous dual purpose animal, dominate the cattle corridors of central and western Uganda. As the area is beset by severe water shortage, which in turn affects the availability of green pasture, large herds of cattle are reared under pastoralism where resources are utilised commonly by the pastoralists (Kisamba-Mugerwa and Nuwagaba, 1996).

1.1.2 Pastoral livestock system in Mbarara subregion

Mbarara subregion in south-western Uganda (Figure 1.1) is composed of 4 districts; Ibanda, Isingiro, Kiruhura and Mbarara that form part of the corridor. The region has approximately 1 million inhabitants and boasts the highest cattle population in the country; almost every adult owns cattle (Uganda Bureau of Statistics, 2007). The region is commonly referred to as the "land where milk and honey flows along the paths" because of abundant milk supply to the country (Kamanyire, 2000). Over 92% of its population is rural and less than 5% of the population have access to clean potable water (Uganda Bureau of Statistics, 2007). The drier eastern district of Kiruhura is predominantly under pastoralism (Mwambutsya, 1991). Valley dams, constructed in the region provide permanent water reservoirs for humans and their livestock.

For centuries, a dominant Bahima ethnic group in Mbarara subregion have kept longhorn Ankole cattle and practised pastoralism (Mwambutsya, 1991), their animals sharing both grass and water (Mugasi *et al.* 1999). Cattle form the central focus of their cultural, spiritual and economic existence (Kisamba-Mugerwa and Nuwagaba, 1996). Tradition dictates that the number of cattle owned by an individual is a measure of wealth and a symbol of social status in the community (Mugasi *et al.* 1999). Pastoralism is further promoted by a tradition that regards modern livestock management systems as imprisonment of the animal (Aveling *et al.* 1998). In pastoral communities of Uganda, including the Bahima, consumption of raw milk and milk products is a strong tradition (Oloya, 2006).

1.1.3 Cattle: the source of one of the pathogenic groups of *Escherichia coli*

Escherichia coli (*E. coli*) is part of the normal gut flora of man and animals (Donnenberg and Whittam, 2001). Certain strains possess virulence factors enabling them to cause gastrointestinal diseases, or infections at other sites (Kaper *et al.* 2004). *E. coli* strains that cause diarrhoeal diseases have been categorised into pathogenic groups [1.2] based on their virulence properties (Nataro and Kaper, 1998; Kaper *et al.* 2004). One of these groups, enterohaemorrhagic *E. coli* (EHEC), is defined

by the presence of Shiga-toxin genes (Nataro and Kaper, 1998). Such strains are frequently termed Shiga toxin-producing *E. coli* (STEC), of which some, are highly pathogenic for humans, particularly children (Nataro and Kaper, 1998; Paton and Paton, 1998b; Kaper *et al.* 2004; Thorpe, 2004; Tarr *et al.* 2005; Gyles *et al.* 1998).

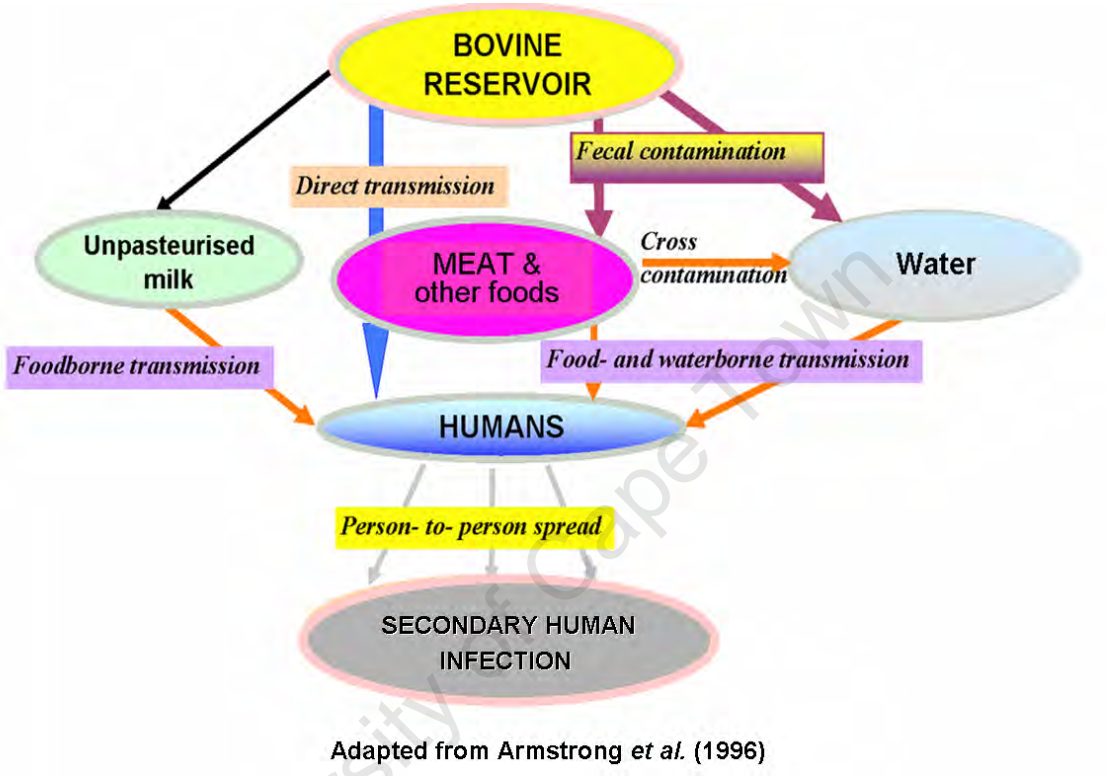

Figure 1.2: Reservoirs and transmission routes of STEC.
STEC is a food and water-borne pathogen acquired orally. Cattle are the primary source of STEC, shed in faeces to contaminate the carcass. Other domestic ruminants also shed STEC. Undercooked ground beef is the main source of infection. Water and fresh vegetables contaminated with bovine faeces are additional sources. Direct human-to-human transmission occurs but human to cattle transmission has not been reported.

The gastrointestinal tracts of ruminants, particularly cattle are the major reservoirs of STEC, which is shed in faeces of healthy animals (Nataro and Kaper, 1998; Gyles *et al.* 2007; Armstrong *et al.* 1996; Caprioli *et al.* 2005; Hussein and Sakma, 2005; Hussein, 2007; Mora *et al.* 2007). STEC is the only true pathogenic group of *E. coli* with a zoonotic origin (Blanco *et al.* 2005).

From cattle, the primary host, STEC is transmitted to humans via several routes as shown in Figure 1.2. First the carcasses may be contaminated at slaughter;

the subsequent beef grinding processes distribute the organisms throughout the beef (Boyce *et al.* 1995). Undercooked ground beef is a primary source of STEC infection (Riley *et al.* 1983; Griffin and Tauxe, 1991; Armstrong *et al.* 1996; Caprioli *et al.* 2005). Additionally, milk, especially unpasteurized milk and milk products (Hussein and Sakuma, 2005; Hussein, 2007), are sources of STEC. Other foods, such as salami, sausages, contaminated vegetables and fruits have also been associated with similar infections (CDC, 1995,2006a; Michino *et al.* 1999; Breuer *et al.* 2001; Caprioli *et al.* 2005). Person-to-person transmission of STEC occurs, promoted by close contact or poor hygienic conditions (Gouveia *et al.* 1998; Parry and Salmon, 1998). More frequently, STEC infections are associated with human-cattle contact or contact with the environment of cattle (Crampin *et al.* 1999; O'Brien *et al.* 2001), especially in rural farming communities (Strachan *et al.* 2001). Children are at particular risk of infection from the farm environment contaminated with cattle faeces (Crump *et al.* 2002; Grif *et al.* 2005). Surface drinking water supplies, contaminated with manure-containing STEC, have also been implicated in STEC infection (Olsen *et al.* 2002; Yatsuyanagi *et al.* 2002).

There is abundant information on the epidemiology of STEC in developed nations; however, very little is known about the epidemiology of this organism in sub-Saharan Africa. STEC infection is considered a health hazard in developed nations where it is associated with fast-food restaurants (Karmali *et al.* 1983; Grimm *et al.* 1995 Armstrong *et al.* 1996; CDC, 1995,2006a) and intensive livestock systems (Fegan *et al.* 2004; Vali *et al.* 2005; Sanderson *et al.* 2006). Although foodborne STEC infections are relatively frequent in North America and Japan, (Michino *et al.* 1999; Breuer *et al.* 2001; Kassenborg *et al.* 2004; CDC, 2006b), in rural communities contamination of the environment with high-density cattle population, water in particular, has become a major source of infection (Strachan *et al.* 2001; Olsen *et al.* 2002). The few studies on the epidemiology of STEC in sub-Saharan Africa did not consider human populations in close contact with cattle (Kaddu-Mulindwa *et al.* 2001; Gascon *et al.* 2000; Rappelli *et al.* 2005). One study of STEC in cattle from a farm in central Uganda identified STEC, of diverse serotypes, in 42 of 159 cattle. Interestingly, some of the serotypes, such as O74:H42 and O76:H2, were isolated for the first time, (Kaddu-Mulindwa *et al.* 2001; http://www.microbionet.com.au/vtectable.htm), which could suggest that cattle in Uganda harbour distinct serotypes of STEC.

As part of the study by (Kaddu-Mulindwa *et al.* 2001), diarrhoeal children from Mulago referral hospital in Kampala were investigated for STEC infection. The children had not had contact with cattle included in the study and were not part of the pastoralist community. STEC was not isolated from the children (Kaddu-Mulindwa *et al.* 2001).

Nothing is known about the occurrence of STEC in cattle in the pastoralist community of the cattle corridor of Uganda. Further, the extent of transmission of STEC from cattle, or their environment, to children, who live in close contact with cattle is unknown. Thus, the aims of my study were to investigate and characterise STEC from cattle and water sources in Mbarara region (Figure 1.1). Additionally, these organisms were sought in diarrhoegenic-children in the pastoralist community of this region.

1.2 *E. coli* associated with gastrointestinal infections

Initially, all diarrhoea-associated *E. coli* were termed enteropathogenic *E. coli* (Neter *et al.* 1955). An increase in knowledge of pathogenicity led to the designation of six specific groups: Enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (Nataro and Kaper, 1998; Clarke, 2001; Donnenberg and Whittam, 2001; Huang and DuPont, 2004; Kaper *et al.* 2004; Nguyen *et al.* 2005).

1.2.1 Enteropathogenic *E. coli*

Since it was first recognised more than 6 decades ago (Bray, 1945), EPEC remains a major pathogen of children in developing countries (Nataro and Kaper, 1998; Clarke, 2001; Naylor *et al.* 2005). It is associated with poor hygienic conditions and a major cause of high childhood mortality (Chen and Frankel, 2005). EPEC is the prototype of the locus of enterocyte effacement (LEE) carrying *E. coli* pathogens,

which intimately attach to the intestinal epithelial cells causing attaching and effacing (A/E) lesions (Jerse *et al.* 1990; McDaniel *et al.* 1995; Jores *et al.* 2004). The subsequent pathophysiological changes cause electrolyte imbalance and impaired absorption from, and increased secretion into, the intestinal lumen cells leading to diarrhoea (Field, 2003).

1.2.2 Enteroinvasive *E. coli*

Enteroinvasive *E. coli* is the only group of *E. coli* known to cause diarrhoea by invasion of epithelial cells (Nataro and Kaper, 1998). It causes watery diarrhoea and dysentery which are clinically similar to the corresponding illness caused by Shigella (Nataro and Kaper, 1998; Lan *et al.* 2004).

As EIEC and Shigella organisms have the same phenotypic characteristics (Lan *et al.* 2004), they are believed to have evolved from the same lineage but diverged into distinct pathovars (Pupo *et al.* 1997; Lan *et al.* 2004).

1.2.3 Enterotoxigenic *E. coli*

Enterotoxigenic *E. coli* (ETEC) produce plasmid-encoded heat labile toxins (LT) and heat stable-toxins (Nataro and Kaper, 1998). ETEC is the major cause of weaning diarrhoea, a watery type of diarrhoea among infants in developing countries (Wennerås and Erling, 2004). The disease is usually self limiting but fatal cases have been reported in infants, however, the organisms are frequently isolated from healthy people (Nataro and Kaper, 1998). Since individuals in developed nations lack immunity to ETEC, they frequently develop diarrhoea following their first exposure to ETEC infection referred to as travelers' diarrhoea (Sack, 1990; Lima, 2001).

1.2.4 Enteroaggregative *E. coli*

Enteroaggregative *Escherichia coli* is the major cause of a persistent diarrhoea (14 days) in children and adults worldwide (Huang and DuPont, 2004; Weintraub,

2007). The water- and food-borne pathogen is promoted by poor sanitary conditions (Adachi *et al.* 2002). In recent times, EAEC has become a major cause of diarrhoea in patients infected with human immunodeficiency virus (HIV) (Durrer *et al.* 2000). The pathogens derived their name from the characteristic aggregative adherent pattern they form on human epithelial (HEp-2) cells (Durrer *et al.* 2000; Huang and DuPont, 2004). They posses an adhesive pili, (Salyers and Whitt, 1994) which enable EAEC to colonise the intestinal epithelial cells (Czeczulin, *et al.* 1997; Huang and DuPont, 2004). The organism initiates mucus secretion by the enterocytes (Hicks *et al.* 1996) as well as inflammation leading to diarrhoea (Huang and DuPont, 2004).

1.2.5 Diffusely adherent *E. coli*

Diffusely adherent *Escherichia coli* (DAEC) causes diarrhoeal disease in humans of all age groups, worldwide (Jallat *et al.* 1993). Characteristically this pathogen forms a diffuse adherence (DA) pattern on cultured epithelial cells (Nataro and Kaper, 1998; Scaletsky *et al.* 2002). It is the surface fimbriae proteins that are major adherence factors responsible for the DA phenotype (Bilge *et al.* 1996), encoded by genes located on a conjugative plasmid or in the chromosome (Czeczulin *et al.* 1997; Lopes *et al.* 2005).

1.2.6 Enterohaemorrhagic *E. coli*

Enterohaemorrhagic *E. coli* (EHEC) is a subgroup of Shiga toxin (Stx)-producing *E. coli* (STEC) that causes a range of diseases from mild watery diarrhoea, to bloody diarrhoea (haemorrhagic colitis) and haemolytic-uraemic syndrome (Levine, 1987; Levine *et al.* 1987; Tarr *et al.* 1996,2005 ; Nataro and Kaper, 1998; Thorpe, 2004). The genes encoding Shiga toxins (stx_1 and stx_2) are phage mediated and strains may contain one or both of the genes (O'brien and Holmes, 1987). Since these toxins have activity on Vero cells, they are also called Verotoxins (Konowalchuk *et al.* 1977). As this group is the subject of my research, a more detailed description of EHEC is presented [1.3].

1.3 Shiga toxin containing *E. coli*

1.3.1 Biology

STEC are non-spore forming Gram negative rods which produce catalase and indole, but lack oxidase. They are non-fastidious organisms which grow readily on peptone, meat extract, blood and MacConkey agar media (Ryan, 1990). The majority of strains are motile (Ryan, 1990); a notable exception is STEC O157:NM which lost its flagellae during evolution (Feng *et al.* 1998; Karch and Bielaszewska, 2001).

A feature of STEC O157:H7 strains is that they lack the ability to ferment sorbitol (March and Ratnam, 1986). As STEC belonging to this serogroup is most frequently associated with haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS), most diagnostic laboratories use sorbitol MacConkey agar (SMAC) to distinguish STEC O157:H7 from non-O157:H7 strains (March and Ratnam *et al.* 1986; Mead, and Griffin, 1998; Bielaszewska and Karch, 2000). Clinically important sorbitol fermenting non-O157 STEC serotypes such as O111, O113, O26 and as well as O157:NM are frequently undetected in diagnostic laboratories (Tarr *et al.* 2005). For this reason, the extent of non-O157:H7 STEC in human illness is not known. As such, clinical infections with non-157:H7 STEC are likely to be misdiagnosed and under reported (Mead and Griffin, 1998; Thorpe, 2004; Thompson *et al.* 2005; CDC, 2007; Gilmour *et al.* 2007a).

1.3.2 Serotypes

Serotyping of STEC is based on the expression of the somatic (O) lipopolysaccharide, capsular (K) and flagellar (H) antigens according to Kauffmann (1947) classification. Presently, 472 STEC serotypes have been identified (Blanco *et al.* 2004c; *http://www.lugo.usc.es/ecoli*). Apart from STEC O157:H7 and NM, other serotypes of public health importance are O26:H-, O26:H11, O91:H-, O103:H2, O111:H-, O113:H21, O118:H16, O128:H2, O145:H-, O145:H28 and O146:H21 (Beutin *et al.* 2004; Karch *et al.* 2005).

1.3.3 Virulence factors of Shiga toxin-producing *E. coli*

1.3.3.1 Shiga toxin: Structure and biological properties

Shiga toxins belong to a family of closely related toxins, the AB_5 hexameric type of molecules (Merritt and Hol 1995; Sandvig, 2001; Fraser *et al.* 2004). The family includes Shiga elaborated by *Shigella dysenteriae* type1 and Stx from STEC (Fraser *et al.* 1994). In addition, other pathogens, *Citrobacter rodentium*, *Aeromonas ceviae* and *Enterobacter cloacae* contain Stx (Nataro and Kaper, 1998).

The hexameric molecule (Figure 1.3), consists of five B-subunits (7.7kDa) each linked to a single 32kDa A-subunit via a disulphide bond (O'brien and Holmes, 1987; Donnenberg and Whittam, 2001; Sandvig, 2001). The enzymatic A-subunit has an internal disulphide bond which is proteolytically cleaved to form the A_1 and A_2 fragments (Sandvig, 2001; Fraser *et al.* 2004). It is the B-pentamer that recognises and binds to the host cells in the target organs (Merritt and Hol, 1995).

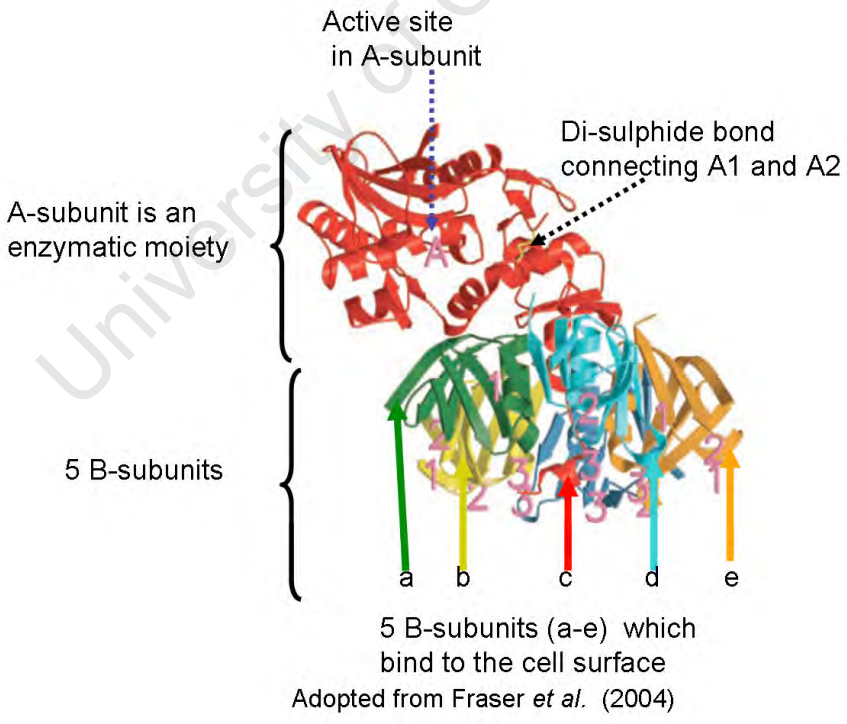

Figure 1.3: The crystal structure of Shiga toxin.

The biological activities of Shiga toxin have been well characterised. Purified Shiga toxin is unequivocally enterotoxigenic to the small intestine of rabbits and lethal to mice (O′Brien *et al.* 1982). Further, its cytotoxicity to African-Green monkey kidney (Vero) cells (Konowalchuk *et al.* 1977), Hela cells and partial toxicity to baby hamster cells are well established (O′Brien *et al.* 1983; O′Brien and LaVeck, 1983).

One or both λ-like bacteriophage mediated Shiga toxins (Stx1 and Stx2) and their variants are the main virulence factors of STEC (O'Brien and Holmes, 1987). It has been shown using epidemiological and experimental studies that the potency of Stx2 is higher (400 times) than Stx1 (Tesh *et al.* 1993). Also, STEC containing Stx2 is associated more with severe human illness than STEC containing Stx1 or Stx1 and Stx2 (Tesh *et al.* 1993; Boerlin *et al.* 1999). Since the amino acid sequence identity between Stx1 and Stx2 is only 50% (Tesh *et al.* 1993; Fraser *et al.* 2004), they differ in their immunological properties (O'Brien and Holmes, 1987).

A study by Strockbine *et al.* (1986) showed Stx1 is immunologically similar to Shiga from *S. dysenteriae* type 1. Further, they have identical amino acid sequences in the B-subunits and 98% identity in the A-subunit (O′Brien *et al.* 1982). Less heterogeneity has been observed in Stx1 than in Stx2 (Paton *et al.* 1993; Nataro and Kaper, 1998). Three variants encoded by stx_{1-OX3} (Koch *et al.* 2001), stx_{1c} (Zhang *et al.* 2002a; Brett *et al.* 2003b) and stx_{1d} (Girardeau *et al.* 2005) have been described.

In contrast, greater amino acid sequence variation is exhibited by Stx2 (Schmitt *et al.* 1991; De Baets *et al.* 2004; Zhang *et al.* 2005). Presently, 15 distinct stx_2 variants (Table 1.1) have been identified (Melton-Celsa *et al.* 1996; Pierard *et al.* 1998; Bertin *et al.* 2001; Schmidt *et al.* 2000; Eklund *et al.* 2002; Brett *et al.* 2003a; Gobius *et al.* 2003; Leung *et al.* 2003; Zhang *et al.* 2005). Following the chronological order suggested by Scheutz *et al.* (2001), the variants are designated: stx_2, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f} and stx_{2g} (Calderwood *et al.* 1996; Acheson, 1998). STEC may contain more than one stx_2 variant (Bertin *et al.* 2001; Scheutz *et al.* 2001; Eklund *et al.* 2002). Stx-EDL993, Stx2e and Stx2d have distinct antigenic and biological characteristics (Caprioli *et al.* 2005); however, other Stx2 variants which share greater amino acid identity have similar biological and immunologic properties (Melton-Celsa *et al.* 1996).

Table 1.1: Variants of stx_2

Variant	Associated disease in man
$stx_{2-EDL933}$	HUS
stx_{2c}	HUS
stx_{2-O48}	HUS
stx_{2-OX3a}	sudden infant death
stx_{2-OX3b}	sudden infant death
stx_{2-O111}	HUS
stx_{2-O118}/stx_{2d}	abdominal cramps
$stx_{2-NV206}$	none
stx_{2-vhc}	none
stx_{2d-1}/stx_{2-vha}	mild diarrhoea/ HUS
stx_{2d-2}/stx_{2-vhb}	mild diarrhoea/ HUS
stx_{2e}	asymptomatic infection/mild diarrhoea
stx_{2ev}	none
stx_{2f}	none
stx_{2g}	none

Source: Bertin *et al.* (2001); Leung *et al.* (2003) and De Baets *et al.* (2004)

Variants of stx_{2d} encode the activatable group of toxins (Melton-Celsa *et al.* 1996,2002) because the protease elastase in the intestinal mucus increases their cytotoxicity (Kokai-Kun *et al.* 2000). Stx2, a prototype toxin from O157:H7 EDL933 and Sakai strains and the presence of Stx2c variants are closely related with severe human illness (Friedrich *et al.* 2002). Conversely, proteins encoded by stx_1, stx_{2-O118} (non activatable stx_{2d}) and stx_{2e} cause no illness or mild watery diarrhoea in humans (Friedrich *et al.* 2002; Jenkins *et al.* 2003b; Cleary, 2004). Currently, stx_{2f} (Schmidt *et al.* 2000) and stx_{2g} (Leung *et al.* 2003), which were recently identified among feral parrots and healthy cattle, respectively, have not been linked to human illness.

Studies have shown that the distribution of Stx glycolipid receptors, globotrioa-

sylceramide (Gb_3) in the various tissues and organs determines the toxic effects (Lingwood, 1996). The Gb_3-rich endothelial cells of the kidney and brain are prone to severe cytopathic effects (Lingwood, 1996; Kaper *et al.* 2004; Noris and Remuzzi, 2005). Stx2e, preferentially binds to globotetrasylceramide (Gb_4) than to Gb_3 (DeGrandis *et al.* 1989; Boyd *et al.* 1993; Paton and Paton, 1998b; Sonntag *et al.* 2005).

1.3.3.2 Systemic uptake of Shiga toxin

Subsequent to colonizing the intestinal mucosa, EHEC releases Shiga toxins into the gut lumen (Paton and Paton, 1998b). Although it is evident that Stx translocates across the epithelial cell barrier without causing cellular damage (Acheson *et al.* 1996; Paton and Paton, 1998b), it alters the integrity of the epithelial tight junctions (Sears, 2000) and underlying endothelial cells (Noris and Remuzzi, 2005). The presence of STEC as well as its toxin evokes a local cellular immune response mediated by tumour necrosis factor alpha (α) and interleukin-8 (IL-8) (Hurley *et al.* 2001). This is preceded by increased neutrophils which traverse the compromised tight junctions into the intestinal lumen (Parkos *et al.* 1991; Hurley *et al.* 2001). Concurrently, Stx transmigrate in the opposite direction into the microvascular circulation (Hurley *et al.* 2001). Besides the tight junction, Stx1 crosses the epithelial cells by intracellular receptor-mediated endocytosis (Hurley *et al.* 1999,2001).

Recent studies have clearly shown that Stx is transported in the blood stream bound to polymorphonuclear leukocytes (PMNs) (te-Loo *et al.* 2000). Although erythrocytes (Bitzan *et al.* 1994) and platelets (Cooling *et al.* 1998) were initially thought to transport Stx, recent findings down play their role (te-Loo *et al.* 2000). The role of PMN in the transport of Stx has been unequivocally shown by the increased blood count of Stx-neutrophil bound cells in patients with HUS (te-Loo *et al.* 2000). Further evidence shows that the Gb_3 expressed by glomerular endothelial cells of target organs has 100-fold higher affinity for Stx than the non-Gb_3 Stx-receptor on PMNs (Ruggenent *et al.* 2001). As such, Stx is transferred to the capillary endothelial cells of glomerular of renal tubules and central nervous system (Kaper *et al.* 2004; Noris and Remuzzi, 2005). Once the toxin has been internalised by the cell and transported to the Golgi apparatus (Tesh and O'Brien, 1991; Kaper *et al.* 2004),

the A-subunit is proteolytically nicked into two fragments, A_1 and A_2 (O′Brien and Holmes, 1987; Sandvig, 2001; Robert *et al.* 2006). The enzymatic portion, A_1, inactivates the ribosome by enzymatically depurinating a single adenine base from the 28S eukaryotic rRNA of the 60S ribosome, to inhibit protein synthesis and eventual cell death (O′Brien and Holmes, 1987; Nataro *et al.* 2004; Noris and Remuzzi, 2005).

It has been reported that intestinal mucosa epithelial cells express Gb_3 receptors with higher affinity for Stx1 than Stx2 (Tesh *et al.* 1993; Jacewizc *et al.* 1999). Consequently, the systemic translocation of Stx1 is minimised and its cytopathic effects are localised to the intestinal areas which manifest as bloody diarrhoea (Tesh *et al.* 1993). However, Stx2 is known to cross the intestinal epithelial cell into the blood (Jacewizc *et al.* 1999) and preferentially bind to the Gb_3-rich cells of kidney and brain tissues (Tesh *et al.* 1993).

1.3.3.3 Role of Shiga toxin in the induction of watery diarrhoea

It was noted that the presence of STEC in the intestinal areas stimulates inflammation of the mucosa (Kandel *et al.* 1989), which interferes with electrolyte balance (Field, 2003). Accordingly, the reduced water and sodium ion intake, as well as increased chloride ion secretion into the intestinal lumen, lead to watery diarrhoea (Li *et al.* 1993; O′loughlin and Robins-Browne, 2001; Field, 2003). Earlier workers, Keusch *et al.* (1972) demonstrated that purified Shiga toxin elicited diarrhoea through fluid accumulation in a segment of a ligated rabbit ileal loop. However, there are contrasting results with recent findings. STEC-negative mutants were able to induce watery but not bloody diarrhoea in rabbits (Fontaine *et al.* 1998; O′loughlin and Robins-Browne, 2001). Thus, Stx plays a major role in inducing bloody diarrhoea (Fontaine *et al.* 1998; Schmidt *et al.* 1999; O′loughlin and Robins-Browne, 2001; Kaper *et al.* 2004; Karmali, 2004).

1.3.3.4 Role of Shiga toxin in the induction of haemorrhagic colitis in humans

Although EHEC infection may be asymptomatic or self-limiting watery diarrhoea with abdominal cramps (Proulx *et al.* 2001; Karch *et al.* 2005), in 30-70% of pa-

tients, infection may progress to HC (Mead and Griffin, 1998; Banatvala *et al.* 2001; Andreoli *et al.* 2002; Noris and Remuzzi, 2005). The presence of STEC and Stx elicit a colonic inflammatory response (Thorpe, 2004), a major factor in the induction of localised intestinal capillary endothelial damage (King *et al.* 2002). A number of studies have shown Stx induces a chemokine response from human intestinal epithelial cells, characterised by interleukin-8 (IL-8) and tumour necrosis factor and PMNs activation (Thorpe *et al.* 2001; Harrison *et al.* 2004). The activated PMNs causes endothelial cell injury (Bitzan *et al.* 1998). Recent studies have demonstrated further that Stx interferes with production of endothelial-derived vasoactive mediators, endothelin and nitric oxide, which in turn promote oxidant microvascular injury seen in cases of HC and HUS (Li *et al.* 1993; Andreoli *et al.* 2002; Vareille *et al.* 2007). Additionally, Stx causes selective apoptosis of Gb_3 receptor-rich villi (Kandel *et al.* 1989). This results in increased exudation and decreased absorption by the intestinal epithelial cells (Field, 2003). Essentially, the toxin causes marked angiopathy of the microvasculature in the mesentery (Sakiri *et al.* 1998; Tarr *et al.* 2005), mucosal and submucosal areas of the colon resulting in haemorrhage, necrosis and sloughing of the cells into the lumen (Sakiri *et al.* 1998).

1.3.3.5 Role of Shiga toxin in the pathogenesis of haemolytic uraemic syndrome

It is apparent that the direct effects of Stx as well as the host immune response to the toxin are responsible for the clinical manifestations in HUS patients (Paton and Paton, 1998b; Banatvala *et al.* 2001; Proulx *et al.* 2001; Andreoli *et al.* 2002; Karpman, 2002). Inflammation of renal parenchymal cells and release of nitric oxide (Vareille *et al.* 2007) are evident. The toxin is associated with upward regulation of proinflammatory cytokines, TNF (Sakiri *et al.* 1998; Ruggenent *et al.* 2001). The latter increases the expression of Gb_3 receptors, binding and uptake of Stx by the glomerular endothelial cells (Tesh *et al.* 1993). The overall effect is severe renal damage in patients (Tesh *et al.* 1993; Noris and Remuzzi, 2005). Additionally, renal endothelial injury is a result of activated neutrophils, which damage endothelial cell fibronectin (Forsyth *et al.* 1989). Further, Stx2-neutrophil-bound cells exhibit delayed spontaneous apoptosis, which is thought to aggravate further neutrophil-mediated tissue damage (Liu *et al.* 1999).

The direct cytotoxicity caused by Stx appears as glomerular endothelial and tubular cell death (Sears and Kaper, 1996; Taguchi *et al.* 1998; Schuller *et al.* 2004). There is obstruction to capillary blood flow and renal insufficiency (Noris and Remuzzi, 2005). Stx-induced renal cellular damage involves the renal endothelial, glomerular, mesangial, proximal and distal renal tubular epithelial cells (Hughes *et al.* 1998; Kevin and Kaplan, 2000; Karpman, 2002). The extensive tissue damage is due to higher expression of Gb_3 and higher affinity for Stx2 (Kevin and Kaplan, 2000). Essentially, Stx-induced HUS is marked by thrombotic microangiopathic haemolytic anaemia, thrombocytopenia, and renal failure (Ruggenent *et al.* 2001; Andreoli *et al.* 2002). Histopathologically, the glomerular endothelial cells are swollen and detached from underlying basement membranes (Sakiri *et al.* 1998; Ruggenent *et al.* 2001; Karpman, 2002) and thrombotic occlusion of capillary lumens (Thorpe, 2004; Noris and Remuzzi, 2005).

Similarly, postdiarrhoeal central nervous signs in STEC infection results from Stx stimulated production of tissue necrosis factor-α by endothelial cells (Harrison *et al.* 2004), resulting in cell damage in the brain (Andreoli, 1999; Andreoli *et al.* 2002). Histopathologically, brain lesions are characterised by haemorrhagic brain infarcts, and cerebral oedema, the main cause of death in Stx HUS (Andreoli *et al.* 2002).

Based on recent epidemiological studies, 3-20% of STEC infected patients, mainly children under 5 years, develop postdiarrhoeal HUS (Fitzpatrick, 1999; Karch *et al.* 2005; Noris and Remuzzi, 2005). Fifty percent of all patients experience a life long noticeable degree of renal impairment (Thorpe, 2004). Further, 10% develop life long renal complications or die (Thorpe, 2004).

1.3.3.6 The effect of age on the pathogenesis of haemolytic uraemic syndrome

Children under 5 years are three times more likely to develop HUS and HUS-related renal failure than adults (Paton and Paton, 1998; Noris and Remuzzi, 2005; Tarr *et al.* 2005). An explanation is due to the increased expression of Gb_3 and selective Stx-binding in the renal glomeruli and juxtaglomerular capillaries in children (Paton and Paton, 1998).

1.3.3.7 Locus of enterocyte effacement: functional analysis

The locus of enterocyte effacement (LEE), which supports the intimin gene (*eae*), enhances the ability of STEC to elude the local cellular protective mechanisms and colonise the intestinal epithelial cells (Nataro and Kaper, 1998). Localised destruction of microvilli and intimin-mediated intimate attachment of STEC to the intestinal epithelial cells (Moon *et al.* 1983; Jerse *et al.* 1990) is followed by delivery of the Shiga toxins (Kaper *et al.* 2004). Several proteins including transcriptional regulators, type III secretion system (TTSS) proteins, translocators, type III-specific chaperons, secreted effectors, intimin and translocator secretion control proteins (Dean *et al.* 2005; Ogino *et al.* 2006) are involved in signal pathways of LEE (Nataro and Kaper, 1998).

Sufficient evidence shows that the LEE-containing intimin was inserted adjacent to either *selC, pheU*, or *pheV* tRNA loci depending on the lineage of the ancestral clones (Whittam *et al.* 1993; Feng *et al.* 1998; Zhang *et al.* 2002b). Genomic studies have revealed that LEE is composed of 41 core genes conserved in STEC, EPEC and *Citrobacter rodentium* (Knutton *et al.* 1998; Donnenberg and Whittam, 20012001). However, 13 additional genes located in a P4 family prophage are present in the LEE of EHEC (Hacker and Kaper, 2000; Schmidt and Hensel, 2004). These genes are thought to have been acquired by *E. coli* O157:H7 after the acquisition of LEE by this organism (Donnenberg and Whittam, 2001).

Functionally, five polycistronic operons, LEE1, LEE2, LEE3, LEE4 and LEE5 encode the core genes (Clarke *et al.* 2003; Hao-Jie and Wan-Jr, 2005). Located at the 5'-end of LEE (Figure 1.4) are LEE1, LEE2 and LEE3 which encode TTSS, (Mellies *et al.* 1999; Gal-Mor and Finlay, 2006). TTSS spans the bacterial cell membrane, translocates and injects the bacterial effector proteins into the host cells (Hacker and Kaper, 2000; Donnenberg and Whittam, 2001; Caprioli *et al.* 2005; Gal-Mor and Finlay, 2006). These functions are mediated by its needle complex (NC) structure composed of a basal body, a central long needle (Sekiya *et al.* 2001) and an expandable sheath-like EspA (Ogino *et al.* 2006). TTSS is assembled from at least 20 known proteins which are encoded by three categories of genes; namely, *E. coli* secretion genes (*esc*), secretion of *E. coli* protein genes (*sep*) and *E. coli* secreted protein genes (*esp*) as well as the chaperone for *E. coli* secretion (*ces*) (Creasey *et al.*

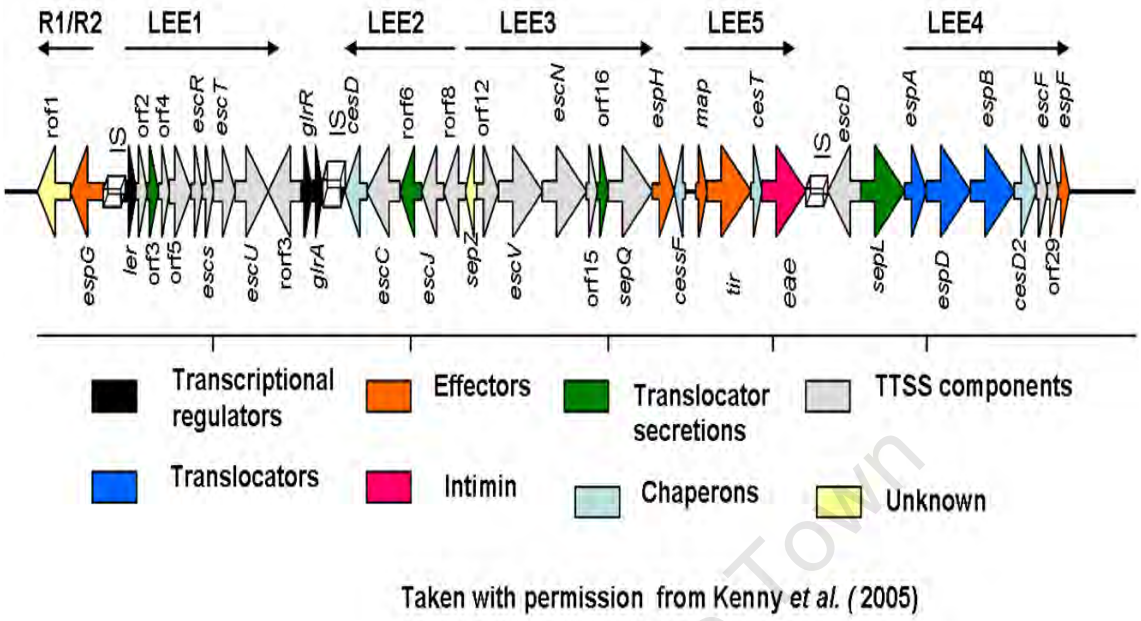

Figure 1.4: Structure of the conserved 41 open reading frames in the LEE.

2003). The recently described *esc* genes encode conserved proteins in TTSS (Hacker and Kaper, 2000; Clarke *et al.* 2003) which include *escC*, *escD*, *escF*, *escJ*, *escN*, *escR* *escS*, *escT*, *escU*, and *escV* (Hacker and Kaper, 2000; Clarke *et al.* 2003). Further studies have shown the EscF protein polymerises to form the thin needle core of the NC (Ogino *et al.* 2006). The EscC and EscJ constitute the outer ring and inner ring proteins of the basal body respectively (Gauthier *et al.* 2003; Ogino *et al.* 2006).

The secreted effector proteins (Sep), EspA, EspB and EspD are encoded by the 4^{th} operon located at the 3'-end of the LEE (Deng *et al.* 2004; Dean *et al.* 2005). Studies have shown EspA, a 25kDa protein forms a syringe-like filament component which associates with TTSS to deliver EspB and EspD into the host cell (Knutton *et al.* 1998; Clarke *et al.* 2003; Ogino *et al.* 2006). In addition, EspA plays a role in attaching EHEC to the host cells (Cleary *et al.* 2004). Similarly, the 37kDa EspB protein modulates the filamentous EspA activity during bacteria-host cell interaction and changes its function from adherence to translocating effector proteins (Deng *et al.*2004). Moreover, it is thought to initiate A/E histopathology by interfering with the cytoskeleton arrangement (Clarke *et al.* 2003; Shaw *et al.* 2005). EspD is

involved in pore formation in the host cells (Knutton *et al.* 1998).

The central portion of LEE, LEE5 operon encodes intimin, an outer membrane adhesin protein (Jerse *et al.* 1990), the translocator intimin receptor (Tir) (Kenny *et al.* 1997; Caprioli *et al.* 2005) and the chaperone, CesT (Schmidt and Hensel, 2004). Intimin mediates intimate attachment of STEC to the enterocytes (Jerse *et al.* 1990; Frankel *et al.* 2001). Tir is inserted into the host cell with the intimin biding site exposed and accessible for the interaction with this protein (Kenny *et al.* 1997; Clarke *et al.* 2003), which may also interact with receptors other than Tir (Hartland *et al.* 1999; Sinclair and O'Brien, 2002). It has been suggested that CesT is crucial in stabilising Tir (Schmidt and Hensel, 2004). Apart from binding intimin, Tir plays a role in the signal transduction process leading to A/E lesions (Dean *et al.* 2005). It has been shown that intimin-Tir binding initiates pedestal-like cytoskeleton changes in the intestinal epithelial cells (Clarke *et al.* 2003).

Additional effector proteins involved in signal transduction pathways include EspB, Map, EspF, EspH, EspG and EspZ (Shaw *et al.* 2005; Gal-Mor and Finlay, 2006), but they do not seem to play a role in the formation of A/E lesions (Ritchie and Waldor, 2005). EspF causes cell death and disrupts intestinal epithelial tight junctions (Donnenberg and Whittam, 2001; Guttman *et al.* 2006). Recent studies demonstrated that EspF plays an additional role in the inhibition of tissue inflammation and suppression of the PMN response, which enhance STEC colonisation of the small intestine (Ritchie and Waldor, 2005). Map (mitochondrial-associated protein) promotes mitochondrial death, stimulates filopodia formation (Dean *et al.* 2005) and together with EspG initiates colonisation of the small intestine as well (Ritchie and Waldor, 2005).

Non-LEE-encoded A (NleA) effector protein, which is encoded in three pathogenicity islands outside of LEE, was recently characterised, and together with effector proteins from LEE, mediate the A/E lesions (Deng *et al.* 2004; Garmendia *et al.* 2005).

The regulation of LEE is more complex than was originally thought, both global and specific regulation are involved (Deng *et al.* 2004). The global regulatory proteins, GrLA and GrLR activate and repress the activity of LEE, respectively (Deng *et al.* 2004; Gal-Mor and Finlay, 2006). Regulation involves also the plasmid-encoded reg-

ulator (Per) (Mellies *et al.* 1999), DNA biding protein H-NS, integration host factor (IHF) and quorum sensing (QS) (Clarke *et al.* 2003), and a LEE-encoded positive regulator (Ler) (Elliot *et al.* 1998), localised in LEE1 (Castillo *et al.* 2005). Per concurrently regulates intimin and activates Ler; the latter regulates the four LEE (LEE2-LEE5) cistron (Schmidt and Hensel, 2004). Further, transcription of LEE2 and LEE3 are regulated by QS (Sperandio *et al.* 1999) and QS *E. coli* regulator, (Qse)A (Clarke *et al.* 2003).

1.3.3.8 Locus of enterocyte effacement and its role in diarrhoea

LEE carrying pathogens such as EHEC, *Citrobacter rodentium*, rabbit diarrhoegenic *E. coli*1 (RDEC-1) and EPEC (Kaper and Nataro, 1998) have been associated with diarrhoea for many years (Frankel *et al.* 1996a; Hacker and Kaper, 2000; Clarke *et al.* 2003). The organisms colonise the enterocytes and enhance their virulence by localised destruction of microvilli and adhering intimately to the intestinal epithelial cells (Sherman *et al.* 1988; Jerse *et al.* 1990; Nataro and Kaper, 1998). Within the cellular matrix, cytoskeletal modification occurs characterised by increased polymerisation of actin-rich pedestal and formation of pseudopod-like extensions (Hacker and Kaper, 2000), histopathological changes referred to as A/E lesions (Moon *et al.* 1983; Kresse *et al.* 1998; China *et al.* 1999; Kaper *et al.* 2004).

Besides the A/E lesions, LEE triggers diarrhoea by other mechanisms (Guttman *et al.* 2006). Effacement of microvilli leads to an electrolyte imbalance associated with reduced sodium ion uptake resulting in net secretion of fluids in the intestinal lumen (Field, 2003). Secondly, there is impaired digestion and absorption of nutrients (Kenny *et al.* 1997; Kenny, 2002). Further, the disruption of intestinal epithelial tight junctions increases exposure of the underlying tissues to materials from the intestinal lumen which cause inflammation and secretion of fluid (Guttman *et al.* 2006).

1.3.3.9 Variants and characteristics of intimin

The outer membrane adhesin protein, intimin, was first described in EPEC eighteen years ago (Jerse *et al.* 1990). Two years later a similar protein was identified

in EHEC O157:H7 (Yu and Kaper, 1992). Structurally, intimin has a mosaic-like appearance (McGraw *et al.* 1999; Frankel *et al.* 2001) and is heterogenous (Ramachandran *et al.* 2003; Blanco *et al.* 2004c; Mora *et al* 2007). Its N'terminal is highly conserved across the variants, whereas the C′ terminal is heterogenous (Phillips and Frankel, 2000; Frankel *et al.* 2001; Oswald *et al.* 2000; Zhang *et al.* 2002b). The C' terminal of intimin, referred to as the 280-amino-acid sequence (Int280) is the cell binding region (Adu-Bobie *et al.* 1998b; Frankel *et al.* 2001), exhibiting specific host tissue cell tropism (Philips *et al.* 2000; Chong *et al.* 2007).

To date, 18 distinct variants have been described namely; α1, α2, β1, β2, ξ or β2B, $\delta(\kappa,\beta$2O), γ1, γ2(θ), ε1, ε2(νR), ζ, ι1, ι2(μR), λ, μB, νB, ξB and ρ (Adu-Bobie *et al.* 1998a; Oswald *et al.* 2000; Tarr and Whittam, 2002; Zhang *et al.* 2002b; Jenkins *et al.* 2003a; Ramachandran *et al.* 2003; Blanco *et al.* 2004b; Mora *et al.* 2007; Cookson *et al.* 2007). The nomenclature, using the Greek alphabet, for the assignation of intimin variants was proposed by Zhang *et al.* (2002b). Any intimin gene with a nucleotide sequence of less than 95% homology to the current intimin gene types is classified as a new variant (Zhang *et al.* 2002b). On the other hand, intimin genes with more than 95% nucleotide sequence identities are given the subtype name of the corresponding almost identical intimin type (Zhang *et al.* 2002b). It appears that the diversity of intimin genes is due to the acquisition and recombination of foreign bacterial DNA (McGraw *et al.* 1999).

1.3.3.10 Phylogenetic diversity of intimin

It is believed that *eae* entered the genome of the ancestral *E. coli* O55 before *stx* (Feng *et al.* 1998). Rather than being random, *eae* type distribution among the serotypes of STEC has an evolutionary basis (Tzipori *at al.* 1995; Oswald *et al.* 2000; Reid *et al.* 2000). *eae*-β1 appears to be widely distributed among diverse serotypes of STEC (McGraw *et al.* 1999; Oswald *et al.* 2000; Ramachandran *et al.* 2003; Blanco *et al.* 2004a,2004b; Mora *et al.* 2007). On the other hand, *eae*-γ1 is associated with EHEC O157:H7 and *eae*-γ2 with STEC O111 (Oswald *et al.* 2000), the two serogroups belong to EHEC1 and EHEC2 respectively (Donnenberg and Whittam, 2001). *eae*-α is more restricted to EPEC O55:H6 than STEC (Oswald *et al.* 2000). It has also been shown that O157:H7 containing *eae*-γ1 has a specific cell

tropism, binding to the follicle-associated epithelium of Peyers patches, other than colonising the entire small intestinal epithelium (Phillips *et al.* 2000; Fitzhenry *et al.* 2002).

The variant *eae-*ϵ is commonly detected in STEC strains of serogroups O8, O11, O45, O103, O121, O136, O163 and O165 (Oswald *et al.* 2000; Blanco *et al.* 2004c); while *eae-*ξ is associated with O84 and *eae-*ζ with serogroups O84, O138, O150 and O156 (Blanco *et al.* 2004c). Like O157:H7, *eae-*ϵ positive O103 strains has selective tropism for Peyer's patches (Fitzhenry *et al.* 2002).

1.4 Evolution of enterohaemorrhagic *E. coli*

Escherichia coli and *Salmonella typhimurium*, two important members of Enterobacteriaceae family, are believed to have diverged from a common lineage between 120 and 160 million years ago (Ochman and Wilson, 1987). Subsequently, along the evolutionary path, about 10-15 million years ago, commensal and pathogenic *E. coli* strains evolved and eventually diverged from a common ancestor (Whittam *et al.* 1993; Feng *et al.* 1998; Donnenberg and Whittam, 2001).

The evolutionary studies of pathogenic *E. coli*, particularly STEC gives insights on the possible origins of the virulence markers present in diverse pathovars of *E.coli* (Baumler, 1997). The recently sequenced *E. coli* genomes, (Blattner *et al.* 1997; Hayashi *et al.* 2001; Perna *et al.* 2001), undeniably revealed a highly dynamic *E. coli* genome (Donnenberg and Whittam, 2001) characterized by gains, losses and rearrangements of DNA regions (Whittam *et al.* 1993; Ochman *et al.* 2000; Johansen *et al.* 2001; Perna *et al.* 2001; Welch *et al.* 2002; Caprioli *et al.* 2005; Dobrindt, 2005), giving rise to diverse strains and pathotypes of *E. coli* seen today (Lawrence and Ochman, 1998; Donnenberg and Whittam, 2001; Schmidt and Hensel, 2004; Caprioli *et al.* 2005).

Plausible evolutionary theories delineate a stepwise evolutionary ladder for the emergence of STEC (Feng *et al.* 1998; Donnenberg and Whittam, 2001; Wick *et al.* 2005). Soon, after diverging from *Shigella flexnori* (Ochman *et al.* 2000), the SF^+, β-glucuronidase positive (GUD^+) ancestor *E. coli* (A2) evolved into a O55:H7-like

ancestral *E. coli* (A3) (Whittam *et al.* 1993; Feng *et al.* 1998) as depicted in Figure 1.5. Approximately 5 million years ago (Armstrong *et al.* 1996), a LEE carrying *eae*-γ1 (Donnenberg and Whittam, 2001) was inserted at the *selC* site of the genome of O55:H7-like ancestor (Wieler *et al.* 1997).

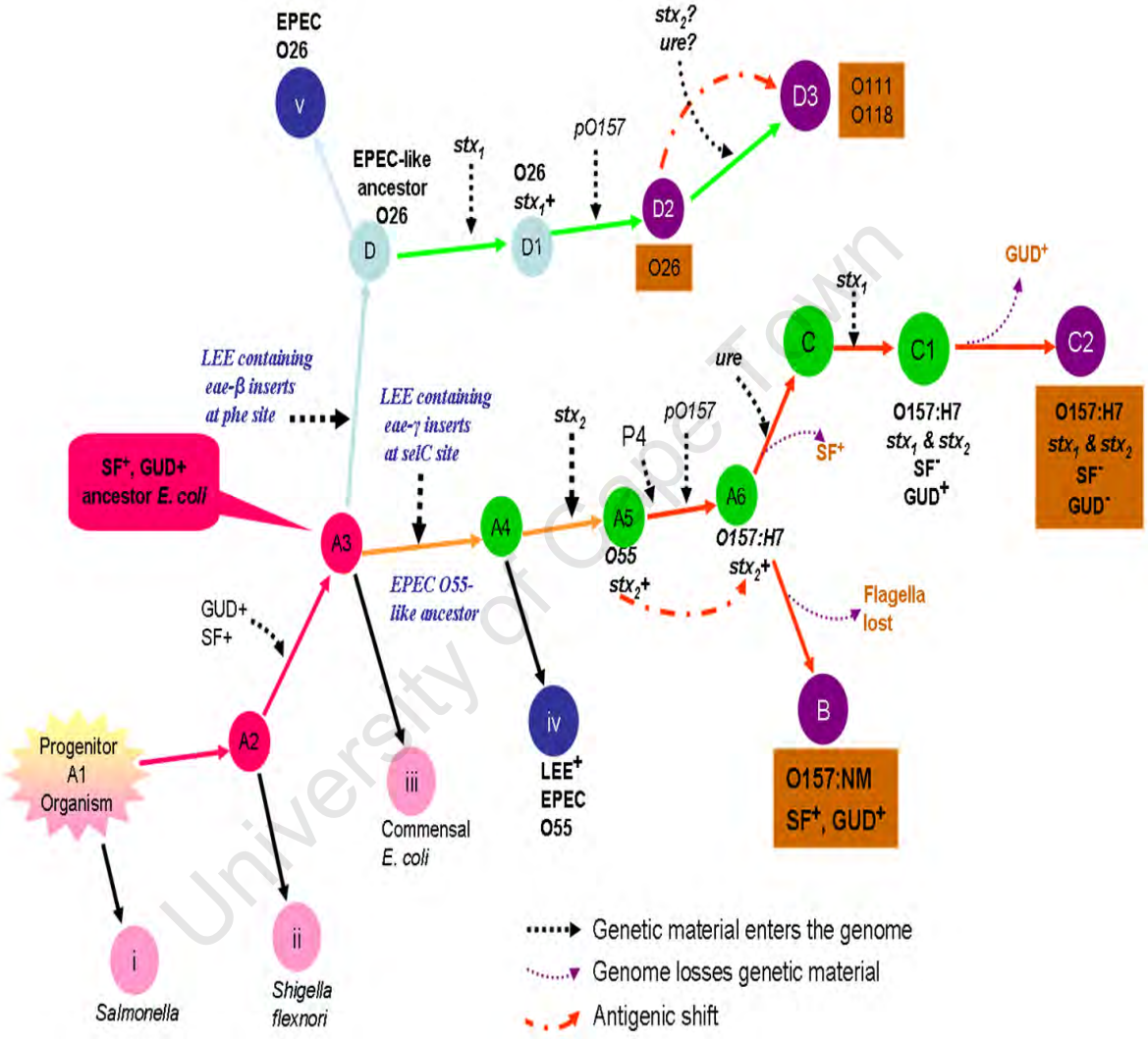

Figure 1.5: A model for the evolution of EHEC.

Thereafter, it is thought that a bacteriophage containing *stx*$_2$ entered the LEE-containing O55:H7-like ancestor (A4) (Wick *et al.* 2005; Zhang *et al.* 2006). This was a major evolutionary landmark in the emergence of EHEC (A4-A5) and diverging from EPEC (A4-iv) (Reid *et al.* 2000). Furthermore, the *stx*$_2$-carrying O55:H7-like progenitor (A5) gained a virulence plasmid (pO157) and simultaneous antigenic drift (A5-A6) resulted in O157-like *E. coli* (Feng *et al.* 1998; Donnenberg

and Whittam, 2001; Wick *et al.* 2005). At about the same time, a prophage P4 containing 13 genes was integrated into the LEE of EHEC progenitor (Ochman *et al.* 2000; Johansen *et al.* 2001; Perna *et al.* 2001).

At this evolutionary step (A6), two parallel evolutionary pathways occurred; one lineage lost the flagellae but retained the ability to ferment sorbitol and β-glucuronidase to become (SF^+, GUD^+) O157:NM lineage (B), referred to as the German clone (Feng *et al.* 1998; Wick *et al.* 2005). The other lineage lost the ability to ferment sorbitol but gained urease gene cluster (C) (Friedrich *et al.* 2005) and stx_1 to give rise to O157:H7-like clone (C1) (Wick *et al.* 2005). Eventually the clone lost its GUD activity and emerged as the SF^-, GUD^- EHEC O157:H7 (C2) which carries stx_1 and stx_2 (Whittam *et al.* 1993; Feng *et al.* 1998; Donnenberg and Whittam, 2001; Monday *et al.* 2001, Wick *et al.* 2005; Zhang *et al.* 2006). Recent evidence showed that EHEC O157:H7, O157:NM and EPEC O55:H7 belonged to same clonal group (EHEC 1) (Donnenberg and Whittam, 2001).

Besides EHEC O157:H7, the evolution of other STEC, especially non O157:H7 EHEC, is not clearly understood. Although non-O157:H7 STEC posses *stx* and *eae*, two genes associated with HC and HUS infections in humans (Karmali, 1989; Paton and Paton, 1998b, Nataro and Kaper, 1998, Law, 2000; Karmali, 2004; Beutin *et al.* 2004, Caprioli *et al.* 2005), they are only distantly related to EHEC O157:H7 (Whittam *et al.* 1993; Feng *et al.* 1998; Donnenberg and Whittam, 2001; Wick *et al.* 2005). It is thought they emerged when their SF^+, GUD^+ ancestral *E. coli* (A3), (Figure 1.5) acquired LEE containing eae-(β) at the *phe* site and thereafter evolved to O26:H11-like (D) ancestor (Donnenberg and Whittam, 2001; Wick *et al.* 2005). A stx_1-containing bacteriophage entered the genome of O26-like *E. coli* (D1), followed by a plasmid, pO157, giving rise to O26:H11 (D2) (Donnenberg and Whittam, 2001; Wick *et al.* 2005). Subsequent antigenic shifts ensued (D2-D3), leading to the emergence of serotypes such as O26:H-, O111:H8, O111:H- and other EHEC (Donnenberg and Whittam, 2001; Wick *et al.* 2005). These non-O157:H7 EHEC, with LEE inserted at the *phe* (Sperandio *et al.* 1998), form the EHEC 2 cluster (Donnenberg and Whittam, 2001). It is probable that the bacteriophage-containing stx_2 and the urease gene cluster entered the genome at the time of antigenic shift (D2-D3).

Although the evolution of the majority of STEC is still unknown, it could be hypothesized that they also emerged by horizontal gene transfer, as described for EHEC. These STEC are distantly related to each other and to EHEC 1 and EHEC 2 clusters (Whittam *et al.* 1993).

It is clear now that O157:H7 and other STEC evolved and adapted to the gastrointestinal tract (GIT) of ruminants, particularly the bovine (Schmidt *et al.* 2000). The bovine GIT is the single most important gene pool (Gilmour *et al.* 2006) with a complex microbiome of genetically diverse bacterial species (Kaper and Sperandio, 2005) typified by horizontal transfers of virulence genes (Ochman *et al.* 2000; Donnenberg and Whittam, 2001; Feil and Spratt, 2001). It is not surprising, therefore, that most of the 470 STEC serotypes identified today are normal microbiota of the gut of healthy cattle (http://www.lugo.usc.es/ecoli). For this reason, cattle have attracted worldwide public health attention because they excrete STEC and human infections with this zoonose (Mead and Griffin, 1998; Hacker and Kaper, 2000) may be characterised by fatal sequelae in children and the elderly (Proulx *et al.* 2001).

1.5 Epidemiology of Shiga toxin-producing *E. coli*

1.5.1 Emergence of enterohaemorrhagic *E. coli*

In 1982 investigations of outbreaks of diarrhoeal illness in Oregon and Michigan, USA identified O157:H7 as the causative organism of the illness (Riley *et al.* 1983). In both outbreaks, hamburgers from fast-foods restaurants were suspected, and EHEC O157:H7 was isolated from hamburgers (Riley *et al.* 1983; Wells *et al.* 1983).

Further studies identified a Shiga-like toxin elaborated by O157:H7 (O′Brien *et al.* (1983). Interestingly, these workers had previously characterised a similar toxin from an *E. coli* of unknown serotype, associated with bloody diarrhoea (O′Brien *et al.* 1982). As the toxin was immunologically identical to Shiga toxin produced by *Shigella dysenteriae* type 1 (O′Brien *et al.* 1982, 1983), it was named Shiga-like toxin (SLT) or Shiga toxin (O'Brien and LaVeck, 1983).

Concurrent investigations of an outbreak of bloody diarrhoea and HUS in Canada,

unequivocally linked *E. coli* to the outbreak (Karmali *et al.* 1983). Further, the *E. coli* elaborated toxins that were cytotoxic to African Green monkey kidney cells (Vero cells) (Karmali *et al.* 1983). The name Verocytotoxin (VT), suggested by Konowalchuk *et al.* (1977), who observed and described similar activity of *E. coli* toxins on Vero cells was given. Later, it was confirmed that Verocytotoxins (Karmali *et al.* 1983) and Shiga toxins (O'Brien and LaVeck, 1983) were identical; nevertheless, parallel nomenclature continues to be used interchangeably in literature today.

It is strongly believed that O157:H7 emerged as a human pathogen in the last half of the 20th century (Armstrong *et al.* 1996; Mead and Griffin, 1998). Retrospective investigations on the prevalence of O157:H7 among diarrhoegenic patients between 1975 and 1982 in the USA and United Kingdom, detected only one O157:H7 isolate from each of the countries (Riley *et al.* 1983; Day *et al.* 1983). Moreover, the recent past has seen an advent of STEC serogroups such as O26, O91, O103, O111, O118, O145 and O166, which may cause severe human illness, similar to O157:H7 (Levine, 1987; Beutin *et al.* 2004; Kaper *et al.* 2004; Caprioli *et al.* 2005).

1.5.2 Worldwide occurrence of Shiga toxin-producing *E. coli*

Since its first recognition as a food-borne pathogen 25 years ago (Riley *et al.* 1983), outbreaks of EHEC O157:H7 have occurred on a global scale, reported in Latin America, (Vaz *et al.* 2006), Africa (Effler *et al.* 2001), Europe, including Germany (Zhang *et al.* 2002b; Beutin *et al.* 2004), France (Pradel *et al.* 2000) and the United Kingdom (Fitzpatrick, 1999), Asia, in Japan (Izumiya *et al.* 1997; Michino *et al.* 1999), Australia (Paton *et al.* 1996; Elliot *et al.* 2001; Bettelheim *et al.* 2002) and North America (Karmali *et al.* 1983; Thorpe, 2004). Several studies have shown that STEC serotypes have different geographical distributions, O157:H7 predominantly causes human infections in USA (Slutsker *et al.* 1997), Canada (Thorpe, 2004), Japan (Izumiya *et al.* 1997; Michino *et al.* 1999), United Kingdom (Vali *et al.* 2004) and Argentina (Parma *et al.* 2000). Annual infections rates of 1-30 cases per 100 000 investigations have been reported. It is children that are most at risk of the consequences of infection with EHEC (Fitzpatrick, 1999).

Prevalence of O157:H7 over other STEC serotypes is probably related to the fact that most diagnostic laboratories screen only for O157:H7 strains (Tarr *et al.* 2005; Thompson *at al.* 2005; CDC, 2007). However, recent studies using molecular techniques show increased prevalence of non-O157:H7 STEC-bloody diarrhoea related illnesses in Europe (Beutin *et al.* 1998,2004) and Australia (Elliot *et al.* 2001); caused by O26, O121, O103, O111, O113 and O145 serogroups (Bettelheim, 2003; Beutin *et al*; 2004; Karch *et al.* 2005; Vanselow *et al.* 2005).

1.5.3 Shiga toxin producing *E. coli* in sub-Sahara Africa

Notwithstanding the paucity of information on STEC in cattle and the role of these organisms in diarrhoea from sub-Saharan Africa, major outbreaks of O157:H7 have been reported in this region. The first outbreak occurred in Swaziland, in 1992, and involved over 20,000 cases (Effler *et al.* 2001). The source of EHEC was surface water contaminated with bovine faeces (Effler *et al.* 2001). Five years later, another outbreak, associated with eating smoked meat pie (*kanda*) of cattle origin, occurred in the remote rural population of Central Africa Republic (Germani *et al.* 1997). Over 290 cases were involved, including four fatalities (Germani *et al.* 1997). A third outbreak occurred in Cameroon, involving over 275 cases and 45 deaths (Cunin *et al.* 1999). The source of infection was not established but STEC-contaminated water and food were possible sources of the organisms (Cunin *et al.* 1999). In all cases cattle were implicated as the source of O157:H7 infection (Germani *et al.* 1997; Cunin *et al.* 1999; Effler *et al.* 2001). A recent outbreak of severe diarrhoea was reported in the Democratic Republic of Congo (DRC) in 2003, which involved more than 463 children (Koyange *et al.* 2004). Stool sample screened from 32 of them contained O157:H7 (Koyange *et al.* 2004).

Sporadic cases have also been reported on the continent; O157:H7 was isolated from a 2-year old boy with severe diarrhoea in Kenya (Sang *et al.* 1996). Additionally, outbreaks have occurred in Cote d'Ivoire (Dadie *et al.* 2000), South Africa (Galane and Roux, 2001) and Nigeria (Okeke *et al.* 2000,2003). This organism has also been isolated from animal food products (Abdul-Raouf *et al.* 1996; Benkerroum *et al.* 2004).

In contrast to developed nations, STEC outbreaks in Africa have occurred in the rural communities causing high morbidity and mortality (Germani *et al.* 1997; Cunin *et al.* 1999; Effler *et al.* 2001). Thus, STEC infection is a potential health problem to a disease-burdened population (Rutstein, 2000; WHO, 2003; Stein *et al.* 2004), particularly when it occurs in association with *S. dysenteriae type 1* (Cunin *et al.* 1999).

1.5.4 Epidemiology of Shiga toxin producing *E. coli* in Uganda

Although several studies have identified a variety of bacterial pathogens associated with childhood diarrhoea (Nasinyama, 1996; Kaddu-Mulindwa *et al.* 2001; Tumwine *et al.* 2002, 2003; Mbonye, 2004), none of the studies has reported STEC. In one particular study, Kaddu-Mulindwa *et al.* 2001 investigated STEC in cattle from a farm in central Uganda, and among diarrhoeal children in the urban district of Kampala. Of the 159 cattle sampled, STEC was isolated in 43(28%), however, none of the children had STEC. Since the children and cattle investigated for STEC were in different locations, it was not possible to correlate STEC infections in cattle and children. Of special concern, however, are the pastoralist communities that live in close contact with their cattle, drink raw milk and share drinking water sources with their cattle, which are risk factors associated with STEC infection (Wilson *et al.* 1996; Valcour *et al.* 2002; Schets *et al.* 2005).

Generally little is known about the epidemiology of STEC in Uganda, and to the best of my knowledge, nothing is known about STEC serotypes of human and cattle origin in the pastoralist communities of Kiruhura district, Mbarara sub-region in southwestern Uganda. Since surveillance of STEC is not done routinely in developing countries (Park *et al.* 2002; Tarr *et al.* 2005; WHO, 2005), its incidence remains unknown in these countries. Thus, the aims of this study were to investigate and characterise STEC from cattle, water sources and diarrhoegenic-children in the pastoral community of Nyabushozi county, Kiruhura district.

Chapter 2

Isolation and detection of STEC strains from humans, cattle and water sources in the pastoral systems of Nyabushozi county, Kiruhura district

2.1 Introduction

Numerous studies carried out over the last 25 years have confirmed cattle as primary reservoirs of STEC for human infections (Thorpe, 2004; Caprioli *et al.* 2005; Tarr *et al.* 2005; Hussein and Sakuma, 2005; Hussein, 2007). These organisms may be transmitted from healthy, STEC-excreting cattle to humans via the food chain (Hussein and Sakuma, 2005). In recent times, cases of STEC infection acquired from direct contact with cattle, or their environment (O'Brien *et al.* 2001; Crump *et al.* 2002; Heinikainen *et al.* 2007), and the consumption of water contaminated with bovine faeces (Olsen *et al.* 2002; Grif *et al.* 2005) have increased among rural farming communities (Wilson *et al.* 1996; Olsen *et al.* 2002; Valcour *et al.* 2002).

Notwithstanding numerous worldwide studies on the prevalence of STEC, including EHEC strains, in cattle (Hornitzky *et al.* 2002; Pearce *et al.* 2006; Mora *et al.* 2007), there is little information on the presence of these organisms in cattle in Uganda. This chapter describes the isolation and identification of STEC in children, bovines

and water in Nyabushozi county, Mbarara subregion in southwestern Uganda.

2.2 Experimental design

2.2.1 Study area and population

The study was carried in Nyabushozi county, Kiruhura district in Mbarara subregion, which until 2006 was part of Mbarara district. Kiruhura is a remote district which shares its western border with Mbarara, Ibanda and Isingiro districts, Rakai and Sembabule districts are on the eastern border, and Kyenjojo district lies on the North (Figure 2.1).

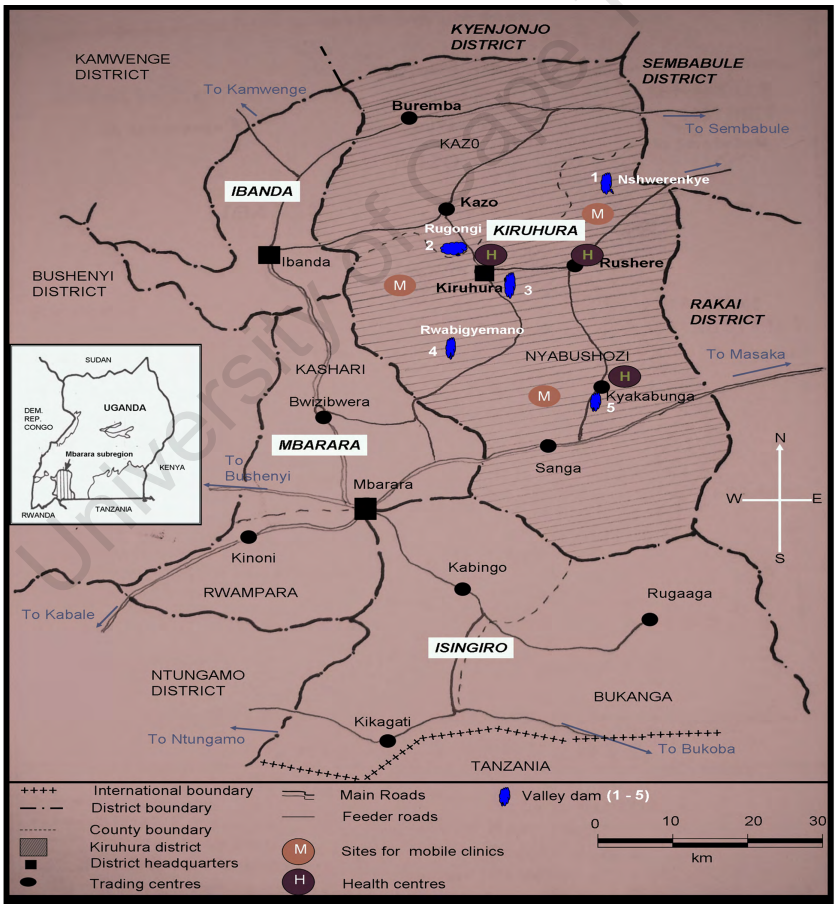

Figure 2.1: Kiruhura district in Mbarara sub-region.
Valley dams (1-5) are indicated.

Kiruhura is divided into two administrative units, Nyabushozi and Kazo counties, that are connected by all-weather gravel roads and seasonal access roads. Government health care in Nyabushozi county is provided within the three health clinics of Kyakabunga, Rushere and Kiruhura in addition to two mobile vaccination centres.

Nyabushozi county with an estimated human and cattle population of 110,000 and 65,000 respectively, is predominantly a cattle keeping area. As the area experiences severe water shortage, valley dams (Figure 2.1) scattered in the county provide water for the pastoralists and their livestock.

2.2.2 Period of sample collection

The study was carried out in 2 phases: October 2004 to January 2005 (phase 1) and October 2005 to January 2006 (phase 2). During the 4 months of phase 1, faecal samples from cattle and the first group of children were collected. In phase 2, additional faecal samples were collected from children and also, water samples were collected from November 2005 to January 2006. The study periods (phase 1 and 2) coincided with the dry season when people and their livestock inevitably share the water from and at the valley dams.

2.2.3 Ethical considerations

The study was approved by the Research and Ethics Committee of the Uganda National Council for Science and Technology (Appendix A). An informed consent was sought from each parent or guardian of the sick child after explaining the rationale of study. Only children from adults who willingly signed the consent forms were included in the study (Appendix B).

2.2.4 Human study population

The target population was children of 12 years and below experiencing episodes of diarrhoea and reporting for treatment at any of the three health clinics (Kyakabunga, Rushere, Kiruhura) or mobile vaccination centres between October 2004

and January 2005 or October 2005 to January 2006. This age group was chosen because a majority of the diarrhoeal cases recorded at the three health clinics prior to this study belonged to this age category. During these periods, stool samples were collected Monday to Wednesday of each week from children with diarrhoea. No duplicate stool samples were included in the study. An episode of diarrhoea was defined as the passing of three or more loose or watery stools in the 24-h period (Baqui *et al.* 1991), prior to presentation at a clinic.

2.2.5 Demographics and clinical information of diarrhoeal children

Pre-coded questionnaires were completed at the time of recruitment to record demographic details of the children (Appendix C). A medical worker fluent in Runyankore, the language used in the community, examined and recorded the physical signs and clinical histories of the children. In order to ascertain the age of the children their health cards, baptismal or birth certificates were used. In cases where these records were absent, the age was derived from recalling previous dates of major local events such as the last elections of local leaders in the county.

2.2.6 Inclusion and exclusion criteria

For inclusion in the study, children must have resided in the area for at least one month prior to the study. Additionally, the children's families were asked whether they had used the selected valley dams, during the time of the study. A positive reply was necessary for inclusion. Only children from parents or guardians who consented to the study were included.

Children suffering from diseases other than diarrhoea, from homesteads not using the selected valley dams, and those visiting the locality for less than 4 weeks were excluded. Further, children older than 12 years were excluded.

2.2.7 Collection of stool samples

A medical worker assisted parents and guardians of children to collect stool samples or rectal swabs in cases where there was no stool. A total of 256 stool samples were collected during phase 1 and phase 2 of the study. All stools/rectal swabs were placed in sterile plastic bottles containing 20ml of Stuart's transport media (BBL®) and transported on ice in a cooler box to the Microbiology Laboratory, Faculty of Veterinary Medicine, Makerere University where they were placed at 4^oC.

2.2.8 Selection of cattle and faecal collection

Cattle were purposefully selected based on the homestead of diarrhoeal children recruited during the study. A guide assisted with the identification of cattle associated with the homestead of the child. Children were followed to their homesteads where fresh faecal samples were collected from every n^{th}-cattle where 1/n samples represented 10% of the sampled cattle in a homestead. A total of 300 cattle from 90 herds (homesteads) were sampled. Diarrhoeal cattle were purposely included in the study. About 50gm of cattle faeces was collected by a veterinary surgeon (Dr Samuel Majalija) from the rectum and placed in sterile plastic bottles containing 20ml of Stuart's transport media (BBL®) and transported as described [2.2.7].

2.2.9 Selection of valley dams

Selection of communal valley dams was determined based on information from local leaders of shared use of dams by humans and their livestock (Figure 2.2). The 5 selected dams were Nshwereenkye (1), Rugongi (2), Kiruhura (3), Rwabigyemano (4) and Kyakabunga (5), as indicated in Figure 2.1. The sizes of the dams varied from about 8m by 15m for the smallest Kiruhura dam to about 20 x 80m for Rugongi and Rwabigyemano. These provide water for the community and their livestock all year round. Cattle had direct access to water since the valley dams were not barrier-protected, and their movements were not restricted to a particular valley dam.

Figure 2.2: Rwabigyemano valley dam.
Children and cattle share the same water source.

2.2.10 Water sample collection

From each dam, three sampling sites used by humans as water collection points and cattle as watering spots were selected. Every month, 300ml water samples were collected in sterile 500ml polypropylene bottles, from each site, for three consecutive months. From November 5 2005 to January 6 2006, 45 water samples were collected. Immediately after collection, water samples were placed on ice in a cooler box and transported to the microbiology laboratory [2.2.7].

2.2.11 Media and buffers

Media and buffers used in the study are presented in Appendix D.

2.2.12 Bacteriological culture

The isolation, identification and preparation of *E. coli* glycerol stocks was carried out by Mr. Lubowa Musiisi, Chief Technician, Veterinary Microbiology Laboratory

Makerere University. He was similarly responsible for the isolation and identification of *Salmonella* and *Shigella* strains.

Bacterial cultures for the isolation of *E. coli* were prepared within 24 and 48 hours of obtaining water and faecal samples, respectively.

Faecal swabs were inoculated on MacConkey (MAC) and sorbitol MacConkey (SMAC) agar (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and incubated at 37^oC for 18-24 h. Presumptive *E. coli* lactose fermenting pink and non-sorbitol fermenting (SF^-) colourless colonies on MAC and SMAC respectively, were confirmed using standard biochemical methods, including API 20E test strips (Biomerieux Vitek, Inc., Hazelwood, Mo.) where necessary.

Bacteriological culture and enumeration of *E. coli* from water samples was carried out using standard techniques (World Health Organization, 1971). Briefly, the 300ml water sample was poured through a cellulose ester membrane filter. The filter was placed on m-FC agar (Difco) and incubated at 44.5^0C for 24 hours. Blue or partially blue colonies suggestive of *E. coli* were individually tested and confirmed by standard biochemical methods (Ewing, 1986). *E. coli* count was recorded as number of colony forming units (cfu) per 100ml of water. Prior to preparation of glycerol stocks, *E. coli* were separately sub cultured on MAC.

In order to increase the possibility of isolating STEC strains, glycerol stocks containing multiple isolates of bovine, humans and water *E. coli* populations were prepared. A sweep of 30-40 *E. coli* colonies from MAC and/or SMAC agar plates, were each inoculated in 2-YT broth (10ml) and incubated at 37^oC for 18-24 h with shaking. Thereafter, glycerol stocks of the cultures were prepared and stored at -70^oC. Bacterial stocks were packed and transported according to the guidelines for shipping infectious substances category A (http://www.idph.state.il.us/about/laboratories/manual08/app_G.pdf), to the Division of Medical Microbiology, Faculty of Health Sciences, University of Cape Town, South Africa where further analyses was carried out. Colonies taken from SMAC and MAC cultures were used as primary cultures.

2.2.13 Preparation of genomic DNA

Two methods were used to extract DNA: the boiling method and the cetyltrimethylammonium bromide (CTAB) method (Ausubel *et al.* 1987; Sambook, 1995). With respect to the former, a loopful of bacteria, or single colonies of *E. coli*, was suspended in 20μl and 5μl ultra-pure water respectively. DNA was released by boiling for 10 minutes. Cellular debris was removed by centrifugation and lysate was transferred to an Eppendorf tube. In addition, genomic DNA was extracted using the CTAB method (Sambook, 1995). DNA concentration was determined by spectrophotometric analysis (UV-Visible Spectrometers Biomate 5, Thermo Electron Corporation, UK), in which the absorbance at a wavelength of 260nm was measured.

2.2.14 Detection of STEC by polymerase chain reaction (PCR) amplification

2.2.14.1 Primers and PCR detection of *stx*

Oligonucleotide primers used for the detection of *stx* genes (Table 2.1) were synthesized by the DNA synthesis laboratory at the University of Cape Town. Initial detection of *stx*-containing *E. coli* was carried out using DNA extracted by boiling a sweep of colonies cultured from glycerol stocks [2.2.12].

Table 2.1: Primers and PCR amplification conditions for the detection of *stx* genes

Primer	Target gene	Primer ($5' \rightarrow 3'$) sequence	Product size (bp)	PCR cycle conditions*	Reference
stx1F	stx_1	CGCTGAATGTCATTCGCTCTGC	302	94^{o}C, 60 s;	Blanco
stx1R		CGTGGTATAGCTACTGTCACC		55^{o}C, 60s;	*et al.*
				72^{o}C, 60 s.	(2003b)
stx2aF	stx_2	CTTCGGTATCCTATTCCCGG	516	94^{o}C, 60 s;	Blanco
stx2aR		CTGCTGTGACAGTGACAAAACGC		55^{o}C, 60 s;	*et al.*
				72^{o}C, 60 s.	(2003b)
stx2bF	stx_2	GATGGCGGTCCATTATC	1493	94^{o}C, 60 s;	Paton
stx2bR	A-subunit	AACTGACTGAATTGTGA		47^{o}C, 60 s;	*et al.*
				72^{o}C, 90 s.	(1993)

*Extension at 72 °C for 5 min completed the amplification process.

Multiplex PCR assays were used: primers stx1F/stx1R (Blanco *et al.* 2003b) were used for the detection of stx_1, while one of 2 sets of primers stx2aF/stx2aR (Blanco *et al.* 2003b) and stx2bF/stx2bR (Paton *et al.* 1993) was used for the detection of stx_2 (Table 2.1). Amplification was carried out in a thermocycler (GeneAmp 2720, Perkin Elmer) in a final volume of 30μl using the conditions presented in Table 2.1. The reactions contained 50ηg of genomic DNA, 20ρmol of each primer, 200μM of each dNTP (Fermentas) and 5U *Taq* DNA polymerase (GoTaq Flexi DNA Polymerase, Promega) in the buffer supplied. Amplifications proceeded according to parameters shown in Table 2.1. Extension at 72 °C for 5 min completed the amplification. Assays were repeated 3 times using freshly prepared template DNA from a sweep of colonies. EHEC O157:H7 was included as a positive control. This was kindly provided by Mr. Pascal Musoni, Manager Microbiology laboratory Groote Schuur Hospital, University of Cape Town.

For each of the *stx*-positive sweeps, the presence of *stx* genes was determined in 10 individual colonies for each isolate using PCR as described above.

2.2.14.2 Agarose gel electrophoresis

Amplicons obtained were separated by electrophoresis using 0.8-2.5% agarose gel immersed in 1X TAE buffer. Gels were stained with ethidium bromide (1μg/ml), visualised under ultraviolet light and photographed with Kodak EDAS 290 Camera. Thereafter, the digital images were captured by Kodak I.D program (Scientific Imaging System, USA).

2.2.14.3 Purification of PCR products and quantification of DNA

When necessary, PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Southern Cross Biotechnology, Cape Town) according to the manufacturer's manual. To determine the concentration of DNA in the purified PCR product; 1μl of DNA was mixed with 1μl gel tracking dye (Bioline, UK) and separated on 1% agarose. The intensity of the DNA band was visually compared to the intensities of standard bands of molecular weight marker, HyperLadder I (Bioline, UK) and the corresponding DNA concentration was expressed as ηg/μl.

2.2.14.4 DNA sequencing and computer analysis of sequencing data

Automated DNA sequencing was carried out at the core sequencing facility, University of Stellenbosch, South Africa, using an ABI 3130 Genetic analyzer. DNA sequences were analyzed using DNAMAN (version 4.0, Lynnon Biosoft). To search the Genbank (National Center for Biotechnology Information (NCBI)) for homologous nucleic acid sequences and to deduce amino acid sequence similarity with existing sequences, the basic local alignment search tool (BLAST) at http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi was used.

2.3 Results

2.3.1 Clinical information on children and isolation of *E. coli*, *Salmonella* and *Shigella* from their faeces

Of the 256 children from whom faecal samples were collected, 90 and 166 were sampled in phase 1 and 2, respectively. Bloody diarrhoea was observed in 57 of the 256 children (Table 2.2) and predominantly in children aged 2-12 years (54 children). *E. coli* was isolated from the stools of 41 of the 57 children (Table 2.2).

Table 2.2: Types of diarrhoea in children and the isolation of *E. coli*, *Salmonella* and *Shigella*

	Age group (years)										
	< 2		2-5		6-12			Isolation of			
	P1	P2	P1	P2	P1	P2		*E.c*	*S*	*Sh*	*S, Sh*
Diarrhoea							Total				
Bloody	0	3	12	18	8	16	57	41	0	0	0
Watery	16	29	26	46	22	38	177	164	4	6	1
Mucoid	3	5	4	6	1	3	22	17	0	0	0

P1, phase 1; P2, phase 2 of the study.
E.c, *E. coli*; *S, Salmonella*; *Sh, Shigella*

The majority of the children (177), representing all the age groups, presented with

watery diarrhoea (Table 2.2). *E. coli* was isolated from 164 of the stools obtained from these children. In addition, *Salmonella* or *Shigella* was isolated from 4 and 6 of these children (aged 2-12 years). One child was infected with both pathogens. The remaining children (22) had mucoid diarrhoea. *E. coli* was isolated from 17 of the stools (Table 2.2).

Based on verbal reports to medical personnel, 22 of the 256 children, had received antibiotics, usually co-triamoxazole. Some of the children were on treatment at the time a stool sample was taken.

2.3.2 Age and health status of cattle and isolation of *E. coli*

A total of 300 rectal faecal samples were obtained from 235 adult bovines, 50 heifers and 15 calves, representing 90 different herds. *E. coli* was isolated from 196 of 235 (83%) adult bovines, 10 of 50 (20%) heifers and 10 of 15 (66%) calves. Seven of these calves had diarrhoea. In total, 216 (72%) of the 300 faecal samples contained *E. coli* (Table 2.3).

Table 2.3: Age of cattle and isolation of *E. coli*

	Calves	Heifers	Adults	Total
Age in months	0-6	>6-24	> 24	
No. of cattle sampled	15	50	235	300
Isolation of *E. coli* No. (percentage)	10(66)	10(20)	196(83)	216(72)

2.3.3 Information on valley dams

For each of the valley dams, (Figure 2.2) triplicate water samples were collected for 3 consecutive months (November 2005 to January 2006) from 5 dams. Where possible, sampling was carried out at water collection points used by inhabitants of the community and where there was evidence of cattle (cattle pats) using the same site.

E. coli was isolated from all the water samples (45) and in numbers greater than 100cfu/100ml of water (Table 2.4). All dams had *E. coli* counts higher than the maximum acceptable limit (<10 coliforms per 100ml) for drinking water in rural water sources (WHO, 1971).

Table 2.4: *E. coli* counts in water samples from 5 valley dams

Sample period		Sampled valley dams				
		Kyakabunga	Kiruhura	Rwabigyemano	Nshwereenkye	Rugongi
		E. coli cfu/100ml of water				
Nov'05	A	700	1100	950	600	240
	B	450	800	950	525	120
	C	180	750	670	465	150
Dec'05	A	380	950	950	535	780
	B	180	620	1700	400	610
	C	720	450	400	335	145
Jan'06	A	550	700	740	400	400
	B	260	800	480	320	215
	C	300	700	800	230	200

A, B and C represent 3 sampling sites per valley dam

2.3.4 Detection of *stx* genes in human, bovine and water *E. coli* isolates using PCR and DNA extracted from a sweep of colonies

PCR assays to detect the presence of *stx* genes were carried out. *E. coli* glycerol stocks from MAC and/or SMAC isolates from children and cattle [2.2.12] collected during phase 1 of the study [2.2.2] were inoculated on 2-YT agar and incubated at 37^{o}C for 18 hours [2.2.12]. *E. coli* strains from 62 children were recovered from MAC stocks and the remainder (from 18 children) were obtained from SMAC preparations. The bovine isolates were cultured from MAC (190) and SMAC (26) glycerol stocks. *E. coli* from one bovine (Bb7) was isolated from both MAC and SMAC stocks.

PCR amplification was carried out using primers (Table 2.1) to detect stx_1 and stx_2 with DNA released by boiling a sweep of colonies [2.2.13]. Amplification products of 302 bp and, 512 bp or 1493 bp, representing stx_1 and stx_2, respectively (Table

2.1) were obtained with *E. coli* O157:H7. No PCR product was obtained with the negative control, *E. coli* K-12. Appropriately sized amplicons were obtained from 7 of 80 (8.8%) human, and 15 of 216 (6.9%) cattle, faecal *E. coli* isolates. To confirm whether the obtained PCR products contained *stx* genes, amplicons representative of stx_1 and stx_2, from 1 human and 2 bovine *E. coli* isolates were extracted from agarose, purified [2.2.14.3] and sequenced [2.2.14.4]. Analysis of the sequencing data confirmed them as portions of stx_1 and stx_2, respectively.

E. coli from children in phase 2 of the study and from the water samples were cultured on MAC only. The glycerol stocks were inoculated as described [2.2.12] and DNA from sweeps of colonies were used in PCR assays to detect *stx* genes. Of the 142 human *E. coli* isolates, products consistent with the presence of stx_1 or stx_2 were obtained from 11 *E. coli*. None of the 11 *E. coli* were obtained from the same homestead as the bovines in phase 1.

Amplicons were obtained from 3 of 45 *E. coli* strains from the valley dams. The *stx*-positive strains were from Rwabigyemano (November 2005), Kyakabunga (November 2005) and Kiruhura (January 2006).

2.3.5 PCR detection of *stx* genes in individual *E. coli* colonies from humans, bovines and water samples

In order to obtain single STEC colonies for further studies and to determine whether the *stx* gene content was uniform in the individual STEC populations, 10 colonies from each of the STEC-positive sweeps were investigated for the presence of *stx* genes as previously described [2.2.14].

2.3.5.1 Detection of *stx* genes in human isolates

Of the 7 human STEC-positive sweeps representing phase 1 [2.2.2], 10 *stx*-positive colonies were identified in 3 isolates from children designated Hh4, Hh5 and Hh6 (Table 2.5). In each of these isolates the *stx* gene content was uniform. *E. coli* from Hh4 contained stx_1, whereas stx_2 was identified in *E. coli* from Hh6 (Table 2.5). Both *stx* genes were detected in *E. coli* from Hh5 (Table 2.5). The *stx* gene content

from the remaining humans (Hh1, Hh2, Hh3 and Hh7) is shown in Table 2.5 and Figure 2.3A.

Table 2.5: Types of diarrhoea in children from phase 1 and *stx* gene content of individual colonies

Types of diarrhoea	Isolate I.D	stx_1	stx_2	stx_{1+2}
		(No. of colonies)		
WD	Hh1	-	-	+(1)
WD	Hh2	-	-	+(2)
WD	Hh3	-	+(5)	+(3)
WD	Hh4	+(10)	-	-
WD	Hh6	-	+(10)	-
WD	Hh7	-	+(2)	-
BD	Hh5	-	-	+(10)

WD, watery diarrhoea; BD, bloody diarrhoea; I.D, identity
+, detected *stx* gene(s); -, no *stx* gene was detected

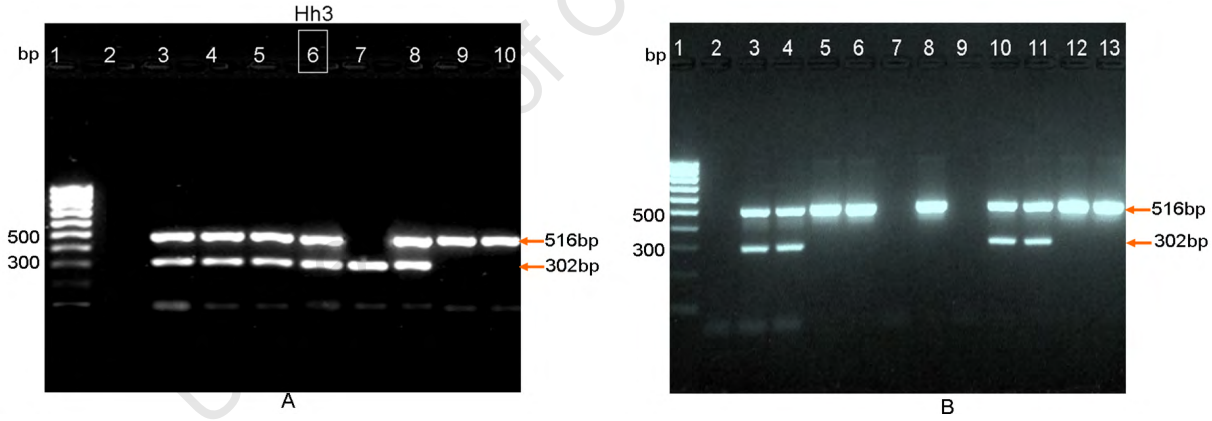

Figure 2.3: Detection of *stx* gene in *E. coli* strains from children in phase 1.

Figure A: Detection of *stx* following PCR using DNA extracted from a sweep of colonies. The presence of stx_1 and stx_2 is indicated by the presence of 302 bp and 516 bp fragments, respectively. *E. coli* O157:H7 was included as a positive control. Lane 1, hyperladder IV (Bioline); lane 2, no DNA; lane 3, O157:H7; lane 4, Hh1; lane 5, Hh2; lane 6, Hh3; lane 7, Hh4; lane 8, Hh5; lane 9, Hh6; lane 10, Hh7.

Figure B: Detection of *stx* in 10 individual *E. coli* colonies from Hh3. Lane 1, hyperladder IV (Bioline); lane 2, no DNA; lane 3, O157:H7; lanes 4, 10 and 11, individual colonies containing stx_1 and stx_2 genes; lanes 5, 6, 8, 12 and 13, individual colonies containing stx_2 gene. Lanes 7 and 9, individual colonies lacking *stx* gene.

Only *E. coli* from Hh3 demonstrated variable *stx* gene content (Figure 2.3B and Table 2.5). None of these children were co-infected with *Salmonella* and/or *Shigella* (Table 2.2).

A similar approach was used to study *E. coli stx*-positive sweeps from children in the 2^{nd} phase. The results are presented in Table 2.6.

Only the strains from Hh15 (Figure 2.4A and B) and Hh18 contained both *stx* genes; for the remaining strains, there was almost an equal split between stx_1 and stx_2-containing strains (Table 2.6).

Table 2.6: Types of diarrhoea in children from phase 2 and *stx* gene content of individual colonies

Types of diarrhoea	Isolate I.D	stx_1	stx_2	stx_{1+2}
		(No. of colonies)		
WD	Hh10	-	+(1)	-
WD	Hh12	+(1)	-	-
WD	Hh13	+(8)	-	-
WD	Hh14	+(6)	-	-
WD	Hh15	-	-	+(6)
WD	Hh16	-	+(5)	-
BD	Hh8	+(1)	-	-
BD	Hh9	+(4)	-	-
BD	Hh11	-	+(1)	-
BD	Hh17	-	+(2)	-
BD	Hh18	-	-	+(3)

WD, watery diarrhoea; BD, bloody diarrhoea; I.D, identity
+, detected *stx* gene(s); -, no *stx* gene was detected

As with the humans in phase 1, none of the humans from phase 2 were co-infected with STEC, *Salmonella* and/or *Shigella*.

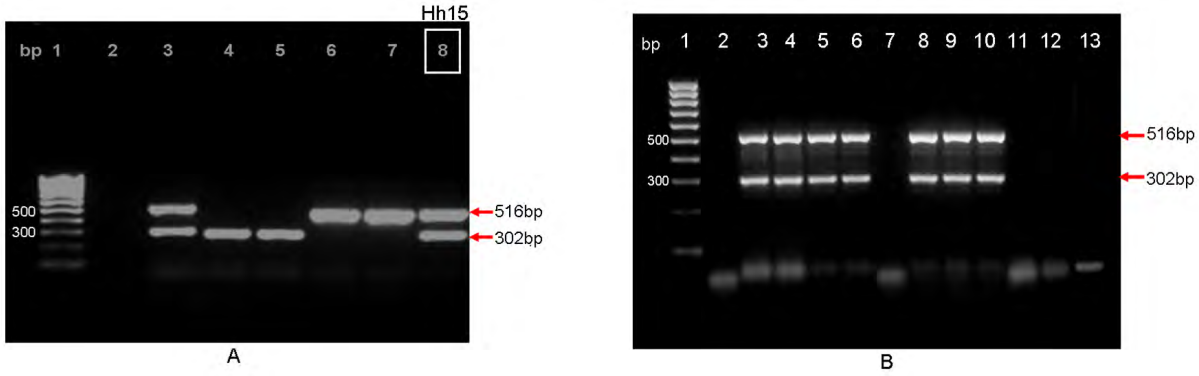

Figure 2.4: Detection of *stx* gene in *E. coli* strains from children in phase 2.

Figure A: Detection of *stx* following PCR using DNA extracted from a sweep of colonies. The presence of stx_1 and stx_2 is indicated by the presence of 302 bp and 516 bp fragments, respectively. *E. coli* O157:H7 was included as a positive control. Lane 1, hyperladder IV (Bioline); lane 2, no DNA; lane 3, O157:H7; lane 4, Hh8; lane 5, Hh9; lane 6, Hh10; lane 7, Hh11; lane 8, Hh15.

Figure B: Detection of *stx* in 10 individual colonies of *E. coli* from Hh15. Lane 1, hyperladder IV (Bioline); lane 2, no DNA template; lane 3, O157:H7, positive control; lanes 4-6, 8-10; individual colonies containing stx_1 and stx_2 genes. Lanes 7, 11-13, individual colonies lacking *stx* genes.

In summary, of the 180 colonies screened for *stx* genes from 18 STEC isolates from children, 81 (45%) were *stx*-positive colonies. Thirty of the colonies (37%) contained stx_1 alone, 26 (32.1%) contained stx_2 alone and 22 (30.9%) contained both *stx* genes (Table 2.5 and 2.6). There was no further attempt to increase the number of *stx*-containing colonies and these results cannot be used to extrapolate the extent of STEC infections.

2.3.5.2 PCR detection of *stx* genes in individual *E. coli* colonies from bovines and water samples

Using *E. coli* colony sweeps, *stx* genes were detected in 15 bovines [2.3.4]. The results of the PCR detection of *stx* genes in individual *E. coli* colonies are shown in Table 2.6.

Two strains (SF^+ and SF^-) containing both *stx* genes were identified in Bb7 (Table 2.6). Most *E. coli* contained stx_1 alone or in combination with stx_2 genes. *stx* gene

variation was detected in strains from 4 bovines (Bb1, Bb6, Bb8 and Bb14).

The distribution of the *stx* genes among *stx*-containing colonies from bovine isolates was as follows; stx_1 gene was detected in 29 (28.7%), stx_2 gene in 21 (20.8%) and stx_1/ stx_2 in 51 (50.5%) of the *stx*-containing colonies as shown in Table 2.7.

Table 2.7: *stx* gene content of individual colonies

Isolate I.D	stx_1	stx_2	stx_{1+2}
	(No. of colonies)		
Bb1	-	+(2)	+(8)
Bb2	+(10)	-	-
Bb3	-	-	+(10)
Bb4	-	-	+(1)
Bb5	-	-	+(7)
Bb6	-	+(1)	+(8)
Bb7^a	-	-	+(4)
Bb7^b	-	-	+(10)
Bb8	-	+(8)	+(2)
Bb9	-	+(10)	-
Bb10	+(3)	-	-
Bb11	+(1)	-	-
Bb12	+(3)	-	-
Bb13	+(1)	-	-
Bb14	+(9)	-	+(1)
Bb15	+(2)	-	-

Bb7^a SF$^-$; Bb7^b SF$^+$ strains; I.D, identity

+, detected *stx* gene(s); -, no *stx* gene was detected

Three water samples containing STEC strains were identified in the preliminary studies. stx_2-positive STEC strains were isolated from Kyakabunga (1 colony) and Rwabigyemano (3 colonies) valley dams, while a stx_1-positive colony was obtained from Kiruhura [2.3.4].

2.4 Discussion

Cattle play a central role in the socio-cultural and economic activities of the rural pastoral communities in the remote cattle keeping areas of Nyabushozi county. Traditional practices promote close human-cattle contact, which inevitably expose the community, children in particular, to STEC infection. Children, as young as 4 years old, have been assigned to tend calves putting themselves at risk of infection with STEC from cattle and their environment in the kraal.

To facilitate the isolation of STEC, faeces was collected directly from the rectums of bovines which is usually associated with higher recovery rates of STEC than isolation from faecal swabs and pats (Lahti *et al.* 2003). Fifteen (15) of 216 (7.85%) *E.coli* carrying cattle carried STEC, lower than the corresponding rate (28.3%) for cattle from a farm in central Uganda (Kaddu-Mulindwa *et al.* 2001). The differences in husbandry practices could explain the variation in the shedding of STEC observed between the 2 studies. In my study, the cattle were entirely reared on range; they were free to move and graze in the vast pastureland, minimizing their contact with STEC contaminated pastures. Such vast grazing land also reduces the possibility of STEC transmission to other cattle, as cattle sharing the same pastures is remote. The prevalence of STEC in cattle in my study accords with other prevalence rates (0.9-15%) recorded in cattle under similar management practices from a number of countries including India (Khan *et al.* 2002b), Australia (Cobbold and Desmarchelier, 2000) and the USA (Sargeant *et al.* 2000; Renter *et al.* 2003; Cobbold *et al.* 2004; Bollinger *et al.* 2005).

Apart from husbandry practices and faecal sampling, geographical location and seasonal variation may influence the prevalence of STEC in bovines (Caprioli *et al.* 2005), as illustrated by the wide variations in prevalence rates of 0.2 to 75% in Europe (Pradel *et al.* 2000; Bonardi *et al.* 1999; Blanco *et al.* 2003a; Urdahl *et al.* 2003), 18-48% in Australia (Hornitzky *et al.* 2002), 46-69% in Japan (Kobayashi *et al.* 2001) and 53-82% in Brazil (Cerqueira *et al.* 1999, Vicente *et al.* 2005).

Just over half (8 of 15 isolates; 53.3%) of bovine STEC isolates carried stx_1, while only 6.7% contained stx_2. Six of 15 isolates, (40%) carried both genes. These data contrast with the results of an earlier study in Uganda in which the majority of STEC

possessed both *stx* genes (Kaddu-Mulindwa *et al.* 2001). The literature indicates that, as with STEC prevalence in cattle, a number of factors, including geographical location (Aktan *et al.* 2007) and possibly cattle management may influence *stx* gene content of STEC (Cobbold *et al.* 2004). In India and the USA STEC from free-range managed cattle carried stx_1 in combination with stx_2 (Wani *et al.* 2007; Renter *et al.* 2003). On the other hand, stx_1-containing STEC were prevalent in cattle reared on pastures in Norway (Urdahl *et al.* 2003), whereas, cattle reared under extensive grazing from Brazil, were mostly infected with stx_2-containing strains (Farah *et al.* 2007).

Notwithstanding the poor quality of the water, valley dams have remained the most reliable source of water for the inhabitants of Nyabushozi and their livestock, especially during the prolonged dry seasons. On a daily basis, over 600 head of cattle used each of the 5 dams during the study period. Cattle defaecated directly into the water and together with the run-off water flowing from the surrounding pastureland, inevitably, *E. coli* was discharged into the dams. Therefore, the high *E. coli* counts recorded in all the dams was not surprising. In a study by McBride *et al.* (2000), *E. coli* was isolated from 99% of surface water reservoirs (ponds and dams) accessed by livestock in New Zealand (Ministry of Agriculture and Forestry, NZ, 2004). As *E. coli* can survive in the faeces of bovines for several weeks (Wang *et al.* 1996; Kudva *et al.* 1998) after being discharged into water, high *E. coli* counts in the valley dams are consistently maintained. Of course, other animals and birds may also contaminate the surface water in the valley dams (Hagedorn *et al.* 1999).

Surface water sources prone to faecal contamination in rural cattle farming communities, are a major source of STEC infection to humans (Jackson *et al.* 1998; Michel *et al.* 1999; Olsen *et al.* 2002). As expected, all the dams used by the Nyabushozi community were highly contaminated with *E. coli*. Three of the 5 dams contained STEC (3 of 45 water samples; 7.3%), cattle faeces being the likely source of these organisms. The presence of STEC in the dams can be attributed to the absence of protective barriers enabling direct access of cattle to the dams. Of 54 water samples collected from water sources within dairy farms in Brazil 1.9% contained STEC (Vicente *et al.* 2005). Similarly, Sargeant *et al.* (2000) detected STEC in 1.5% (3 of 199 samples) of water sources in range cattle farms in the USA. As the water sources were fenced, the source of the STEC is assumed to result from the drainage of water

contaminated with bovine faeces into the dams (Vicente *et al.* 2005). In another study from the Netherlands, 2.7% of private drinking water sources for humans and cattle in the high density cattle farming areas contained STEC (Schets *et al.* (2005). Although the water sources were fenced, molecular typing of STEC strains from water, implicated cattle as the source of STEC (Schets *et al.* 2005). Evidence from different countries including Canada (Jackson *et al.* 1998), Scotland (Solecki *et al.* 2007) and Japan (Yatsuyanagi *et al.* 2002) has shown that consumption of water contaminated with bovine faeces is associated with STEC infection, particularly in children.

Although *E. coli* is part of the main microflora of intestinal tracts of humans (Nataro and Kaper, 1998), these organisms were not isolated from the stools of 34 of 256 children. One possible explanation for the absence of *E. coli* is the use of medicinal herbs in the treatment of diarrhoea, which could have interfered with the proliferation of gut microflora, including *E. coli*. It is a common practice among rural poor communities in Africa (Yousif, 2002) to use medicinal plants with antibacterial properties in the treatment of diarrhoeal diseases (Olukoya *et al.* 1993; Iwalokun *et al.* 2001; Kamanzy *et al.* 2002; Samie *et al.* 2005). In addition, self medication with antibiotics has been previously reported (Byarugaba, 2004; Awad *et al.* 2007; Planta *et al.* 2007) and this could not be not ruled out, despite the fact that most of the study population had indicated the opposite.

STEC was isolated from 8.1% (18 of 222) of the *E.coli* carrying diarrhoeal children. This frequency rate is similar to the corresponding rate (8.4%) of STEC in diarrhoeal children living in a low-income rural community in Nigeria without access to treated water (Okeke *et al.* 2000). Although interaction between cattle and the children was not mentioned, it was likely that the rural folk kept livestock which was the source of STEC in the children.

On the other hand, in two earlier studies in Uganda (Nasinyama 1996; Kaddu-Mulindwa *et al.* 2001) STEC was not isolated from 87 and 237 diarrhoeal children, respectively. Similarly, in two previous studies, one from Tanzania in 1997 (Gascon *et al.* (2000) and another one from Mozambique in 1998-99 (Rappelli *et al.* 2005), none of the 103 and 548 *E. coli* strains from diarrhoeal children respectively, contained *stx* genes as determined by PCR (Gascon *et al.* 2000; Rappelli *et al.* 2005).

A notable difference between the groups of children is that unlike the children in the Nyabushozi pastoralist community, the children from Uganda, Tanzania and Mozambique (Nasinyama, 1996; Gascon *et al.* 2000; Kaddu-Mulindwa *et al.* 2001; Rappelli *et al.* 2005), lived close to urban centres and had no contact with cattle. Significantly, Nyabushozi county supports one of the highest populations of cattle in Uganda (Uganda Bureau of Statistics, 2007). Previous studies have reported a direct correlation between cattle density and human infection with STEC in the rural farming communities of Canada (Wilson *et al.* 1996; Valcour *et al.* 2002). Further, unlike the residents in central Uganda in particular, consumption of locally fermented raw milk, known as *eshabwe* and ghee, is a long time tradition among the inhabitants of Nyabushozi county, including their children. Unequivocally, unpastuerised milk has been previously linked to STEC infection (Hussein and Sakuma, 2005).

With respect to the clinical isolates, 27.8% (5 of 18) contained stx_1, 33.3% (6) stx_2 and 38.9% (7) contained both *stx* genes. Available clinical and epidemiological data indicate that the clinical outcome of STEC infection varies with the type of *stx* content; STEC carrying stx_1 or stx_1 and stx_2 are associated with mild disease (watery diarrhoea). On the other hand, strains carrying stx_2 alone frequently cause bloody diarrhoea and HUS (Boerlin *et al.* 1999; Friedrich *et al.* 2002). That two-thirds of the STEC carried stx_1 or both genes, suggests that most of the strains from the children are of low virulence (may cause watery diarrhoea). However, no association between the *stx* gene content and nature of diarrhoea can be established from the preliminary results.

STEC infection may occur concurrently with other diarrhoeal pathogens. However, none of the STEC infected children was co-infected with *Salmonella*, *Shigella* or both of these pathogens. Undeniably, numerous bacterial pathogens cause childhood diarrhoea in rural communities, nonetheless, these were outside the focus of this thesis.

Chapter 3

Molecular typing of STEC strains

3.1 Introduction

To identify STEC for further characterisation and to gain an understanding of the ecology of the strains, the genetic profiles of the STEC were determined.

A number of molecular typing tools have been described; however, pulsed field gel electrophoresis (PFGE) is regarded as the 'gold standard' (Tenover *et al.* 1995; Olive and Bean, 1999; Rios *et al.*, 1999; Preston *et al.* 2000; Avery *et al.* 2002) because of its high discriminatory power (Bohm and Karch, 1992; CDC, 1995, 2006, Izumiya *et al.* 1997) and reproducibility (PulsenetUSA, 1998; Welinder *et al.* 2002).

3.2 Experimental design

3.2.1 Bacterial strains

A total of 187 STEC strains from children (n=81), bovines (n=101) and water (n=5) were investigated (Tables 3.1 and 3.2).

Table 3.1: *stx*-positive colonies from children

Origin and number of STEC isolates					
Child	No. of colonies	Child	No. of colonies	Child	No. of colonies
Hh1	1	Hh7	2	Hh13	8
Hh2	2	Hh8	1	Hh14	6
Hh3	8	Hh9	4	Hh15	6
Hh4	10	Hh10	1	Hh16	5
Hh5	10	Hh11	1	Hh17	2
Hh6	10	Hh12	1	Hh18	3

Hh1-Hh7, children in phase 1
Hh8-Hh18, children in phase 2

Table 3.2: *stx*-positive colonies from bovines and water

Origin and number of STEC isolates					
Bovine	No. of colonies	Bovine	No. of colonies	Bovine/ water	No. of colonies
Bb1	10	Bb7^b	10	Bb13	1
Bb2	10	Bb8	10	Bb14	10
Bb3	10	Bb9	10	Bb15	2
Bb4	1	Bb10	3	Ww1	1
Bb5	7	Bb11	1	Ww2	3
Bb6	9	Bb12	3	Ww3	1
Bb7^a	4				

Bb and Ww, STEC-positive colonies from bovines and water, respectively
Bb7^a, SF$^-$; Bb7^b, SF$^+$ strains

3.2.2 Pulsed field gel electrophoresis

3.2.2.1 Preparation of DNA

Previously described PFGE methods (Gautom, 1997 and PulseNet 1998), were modified. Briefly, bacterial colonies from an overnight culture on 2-YT agar were emul-

sified in 400μl cell suspension buffer (100mM Tris, 100mM EDTA, pH 8.0) to an optical density of 1.3-1.4 at 610nm (UV-Visible Spectrometers Biomate 5, Thermo Electron Corporation, UK). To deproteinate the cells, proteinase-K and lysozyme were added, each to a final concentration of 1 mg/ml, mixed thoroughly and incubated for 15 min at 37^{o}C. An equal volume (1% low density agarose gel (Seakem gold®) and 1% sodium dodecyl sulphate) was added and the mixture dispensed into non-disposable plastic moulds and allowed to set at 4 °C for 5 min.

3.2.2.2 Cell lysis and restriction enzyme digest of genomic DNA

Plugs for each isolate, were immersed separately in 5ml cell lysis buffer (50mM Tris, 50mM EDTA, pH 8.0, 1% sarcocyl® 0.5 mg/ml proteinase-K) and incubated with shaking, at 55^{o}C for 90 min. Subsequently, the plugs were washed twice at 50^{o}C, for 15 min each time, in 15ml preheated ultrapure water (Milli-Q) (50 °C) and then four times at 50^{o}C, for 15 min each time, in 10ml of preheated (50 °C) TE Buffer (10mM Tris, 1mM EDTA, pH 8.0) with shaking.

Pre-digest incubation was carried out on 2-4mm plugs at 37^{o}C in the supplied enzyme buffer (Biolab, New England) for 15 min. Restriction digest ensued at 37^{o}C with 50 U of *Xba*I and bovine serum albumin at a final concentration of 0.1 mg/ml (Promega) for 16 hours.

3.2.2.3 Electrophoresis conditions

Electrophoresis was carried out with a contour-clamped homogeneous electric field (CHEF-DR II) apparatus (Bio-Rad, La Jolla, California). Plugs were loaded and sealed into a 1% agarose gel (SeaKem® Gold Lonza Rockland, Inc. USA) made of 0.5X TBE, prior to submerging the gel in 2.5 litres of 0.5X TBE. Electrophoresis was carried out at a pulse time of 2.2 to 54.8 sec for 20 hours at 240v, and the TBE buffer was maintained at 12 °C. PFGE lambda ladder marker (New England Biolabs) and a control strain O157:H7 were included in each gel.

3.2.2.4 Visualization and photography of the gel

Following electrophoresis, gels were stained in ethidium bromide (1.0μg/ml) for 30 min, followed by 2 washes in ultra-pure water at 30 minutes interval, before examining them under UV transilluminator. PFGE profiles were captured by Kodak ID imaging program (Scientific Imaging System, USA) and stored as TIFF files.

3.2.2.5 Analysis of PFGE data and interpretation of results

The resulting images were converted into a digital form, processed and analysed using GelCompar II software package (version 4.6 Applied Maths BVBA Belgium). Lanes were assigned to each profile according to the GelCompar II instruction manual. The TIFF images were normalized in comparison to the control strain, O157:H7.

Calculation of similarity matrix between STEC fingerprints was carried out using Dice similarity coefficient (Dice, 1945), which was generated by the GelCompar II software package (version 4.6 Applied Maths BVBA Belgium). DNA fragments between sizes 48.5 and 582 kb were considered for analysis. To determine the genetic relatedness of STEC isolates, cluster analysis of PFGE patterns was performed by the unweighted-pair group arithmetic averaging (UPGMA) method, at a maximum band position tolerance of 1.0% and an optimisation of 1%. Dendrograms describing the relationship among PFGE profiles were generated.

STEC strains exhibiting PFGE profiles with 70-99% were considered closely related; 65-69%, distally (possibly) related and less than 65% similarity, unrelated (Welinder-Olsson *et al.* 2002). PFGE profiles of STEC isolates within and across sources were analysed. PFGE patterns with 70% and more similarity were assigned the same PFGE profile number. Isolates from individual hosts (child, bovine) and water clustering in the same PFGE pattern (70-99%, similar), but with distinguishable PFGE banding patterns were assigned the same profile number and different small letters representing clonal subgroups (Zhang *et al.* 2000; Renter *et al.* 2003; Geue *et al.* 2002). Strains belonged to the same cluster if their similarity level was 64% or more (Vaz *et al.* 2006).

3.2.2.6 Optimisation of PFGE assays

For the optimisation of PFGE conditions, 5 DNA assays of O157:H7 prepared on different days within 2 weeks were digested with restriction enzyme *Xba*I and run on the same gel. For each of the 5 assays, a consistent number of bands (18) which migrated similar distances were produced (Figure 3.1).

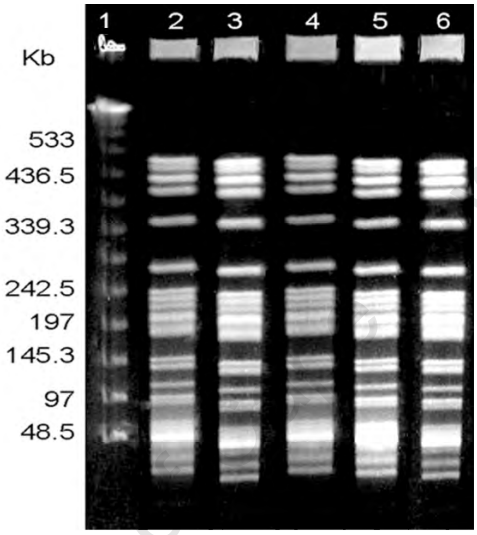

Figure 3.1: PFGE of *Xba*I-digested genomic DNA from STEC O157:H7.

Plugs were prepared on different days but digested at the same time. Lane 1, PFGE lambda ladder marker (New England Biolabs); lane 2, O157:H7-day 1; lane 3, O157:H7-day-3; lane 4, O157:H7-day-7; lane 5, O157:H7-day-10; lane 6, O157:H7-day-12.

Additionally, to determine inter gel reproducibility, similarity coefficients of PFGE profiles of O157:H7 obtained from 8 gels were analysed and a dendrogram generated as shown in Figure 3.2. The similarity among the strains run in 8 gels ranged from 96-100%, showing high degree of intergel reproducibility.

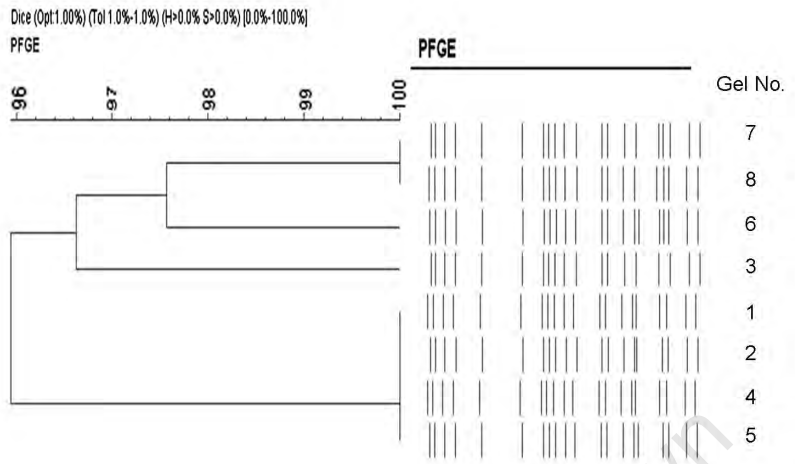

Figure 3.2: Dendrogram showing the relatedness of STEC O157:H7.

Genomic DNA was digested with *Xba*I, followed by PFGE using 8 different gels. As defined at 100% similarity levels, profiles of STEC O157:H7 obtained from gels 1, 2, 4 and 5 were identical and 96% similar to profiles obtained from the other 4 gels (3, 6-8).

3.3 Results

3.3.1 Genetic fingerprints of individual clinical STEC colonies from phase 1

Following PFGE, profiles were obtained for 43 of 46 colonies from the children (7) in phase 1. Although DNA assays were repeated 3 times, profiles were not obtained for 2 colonies and 1 colony for Hh3 and Hh4, respectively (Table 3.3).

The PFGE profile of the single colony from Hh1 was designated 1. Analysis of the dendrogram of the 2 profiles of STEC from Hh2 showed a coefficient similarity of 94.1% (Table 3.3). The profile was designated 2 with clonal subgroups *2a* and *2b*. Hh4 was co-infected with unrelated strains (profiles 4, 5 and 6) (Figure 3.3, Table 3.3). Strains with profile 5 or 6 are possibly related with a similarity of 67.3% (Figure 3.3). To accommodate these differences Hh4 was designated Hh4i, Hh4ii and Hh4iii. Similar assignations were applied to multiple STEC from the same source.

Table 3.3: PFGE profiles and clonal subgroups of individual STEC colonies from each child in phase 1

Child	No. of colonies	PFGE profile	Clonal subgroups	Similarity coefficient (%)
Hh1	1	1		
Hh2	2	2	*2a, 2b*	94.1
Hh3/1	3	3	*3a, 3b, 3c*	90.7-97.7
Hh3/2	3	3	*3d, 3e, 3f*	92.7-97.7
Hh4i	3	4	*4a, 4b, 4c*	88.2-96.3
Hh4ii	1	5		
hh4iii	5	6	*6a, 6b, 6c(2*), 6d*	88.9-100
Hh5	10	7	*7a, 7b, 7c(3*), 7d, 7e(2*), 7f, 7g*	91.2-100
Hh6	10	8	*8a(2*), 8b, 8c, 8d(2*), 8e(2), 8f, 8g*	79.2-100
Hh7	2	9	*9a, 9b*	97

*Number of identical colonies

Hh3/1, stx_2; Hh3/2, stx_1 and stx_2-containing STEC

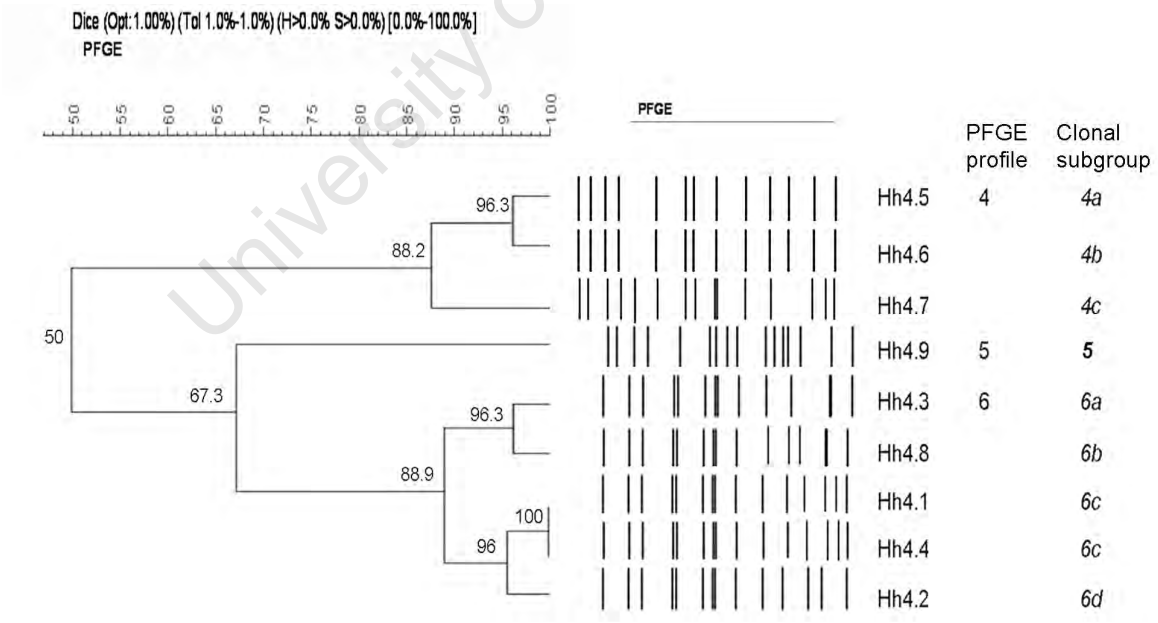

Figure 3.3: Dendrogram showing the relatedness of STEC (colonies 1-9) from Hh4. The strains in profile 4 are closely related with 88.2-91.8 similarity level and 50% related to strains in profiles 5 and 6. In turn, strains in profile 6 are closely related with 88.9-100% similarity level and they are 67.3% related to strains in profile 5.

The profiles and corresponding dendrogram of each of the 10 colonies from Hh5 (Figure 3.4) indicate that they are closely related. This profile was designated 7 (Table 3.3).

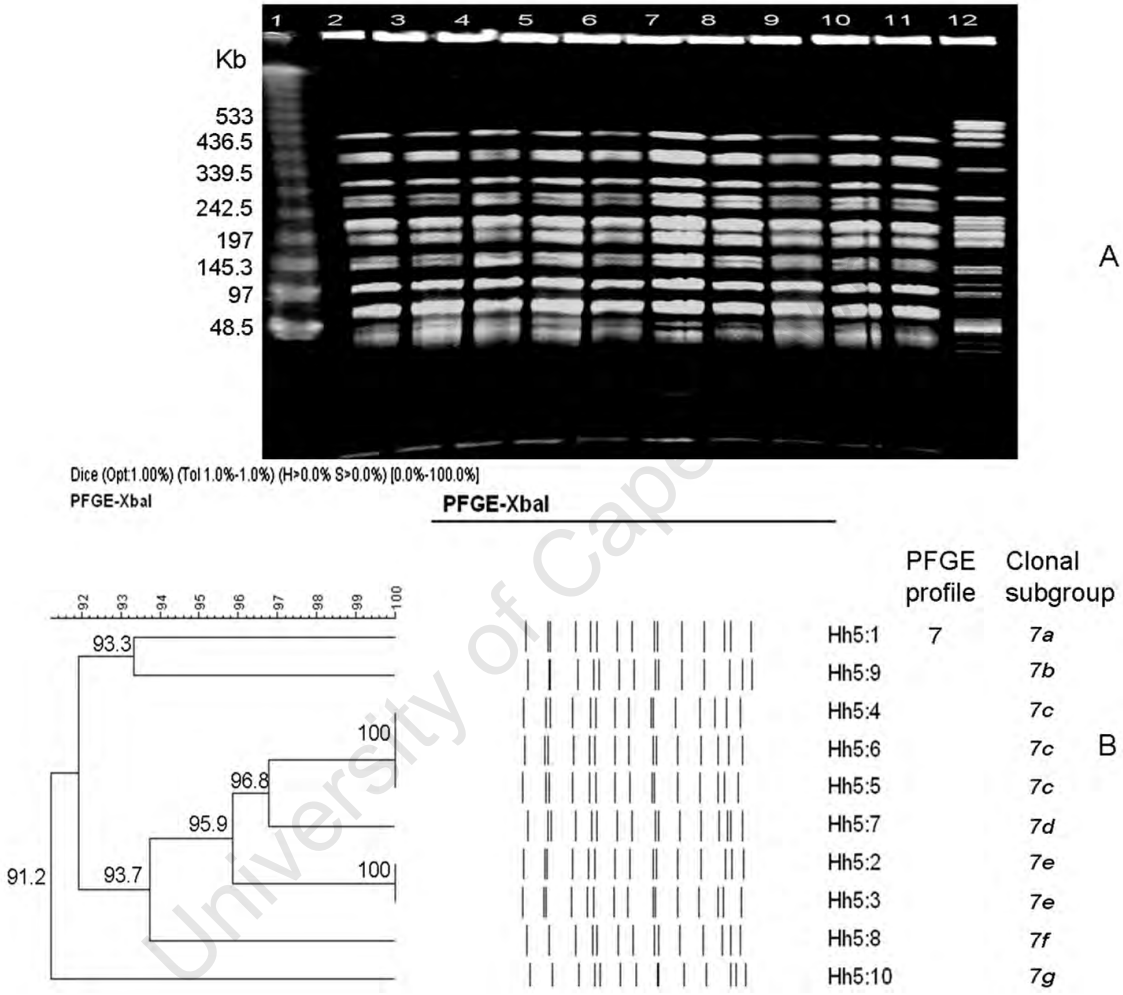

Figure 3.4: PFGE profiles and relatedness of 10 STEC colonies from Hh5.
A: PFGE of *Xba*I-digested genomic DNA. Lane 1, PFGE lambda ladder marker (New England Biolabs); lanes 2-11, colonies 1-10 from Hh5; lane 12, STEC O157:H7 that was included as a control.

B: Dendrogram showing the relatedness of STEC colonies from Hh5. The strains are closely related with 91.2-100% similarity level.

Similarly, the STEC from Hh6 (profile 8) or Hh3 (profile 3) were also related as shown in Table 3.3. Strains from Hh3 with the same PFGE profile but different *stx* gene content were designated Hh3/1 and Hh3/2. These strains showed a similarity level ranging from 90.7-97.7%. Similar assignation was applied to the bovine STEC.

3.3.2 Genetic fingerprints of individual clinical STEC colonies from phase 2

Analysis of PFGE profiles of 38 STEC from children in phase 2 was carried out (Table 3.4). Single colonies obtained from 4 children Hh8, Hh10, Hh11 and Hh12 were designated PFGE profiles 10, 12, 13 and 14, respectively (Table 3.4). PFGE profiles 11, 23 and 24 were assigned to colonies from Hh9, Hh17 and Hh18, respectively (Table 3.4). Each of 4 children Hh13, Hh14, Hh15 and Hh16 was co-infected with unrelated strains elaborating 2 profiles each (Table 3.4).

Table 3.4: PFGE profiles and subgroups of individual STEC colonies from each child in phase 2

Child	No. of colonies	PFGE profile	Clonal subgroup	Similarity coefficient (%)
Hh8	1	10		
Hh9	4	11	*11a, 11b, 11c, 11d*	84.9-86
Hh10	1	12		
Hh11	1	13		
Hh12	1	14		
Hh13i	7	15	*15a, 15b, 15c(2*), 15d(2*), 15e*	85-100
Hh13ii	1	16		
Hh14i	2	17	*17a(2*)*	100
Hh14ii	4	18	*18a, 18b, 18c, 18d*	82-100
Hh15i	5	19	*19a, 19b(3*), 19c*	84-100
Hh15ii	1	20		
Hh16i	1	21		
Hh16ii	4	22	*22a(2), 22b, 22c*	73.5-100
Hh17	2	23	*23a(2*)*	100
Hh18	3	24	*24*	100

*Number of identical colonies

3.3.3 Genetic relatedness of clinical STEC strains in phases 1 and 2

The PFGE profiles and dendrogram of the total clinical STEC strains (24) are shown in Figure 3.5. The strains are genetically diverse, forming 18 clusters with similarity levels ranging from 32.2-66.8% (Figure 3.5).

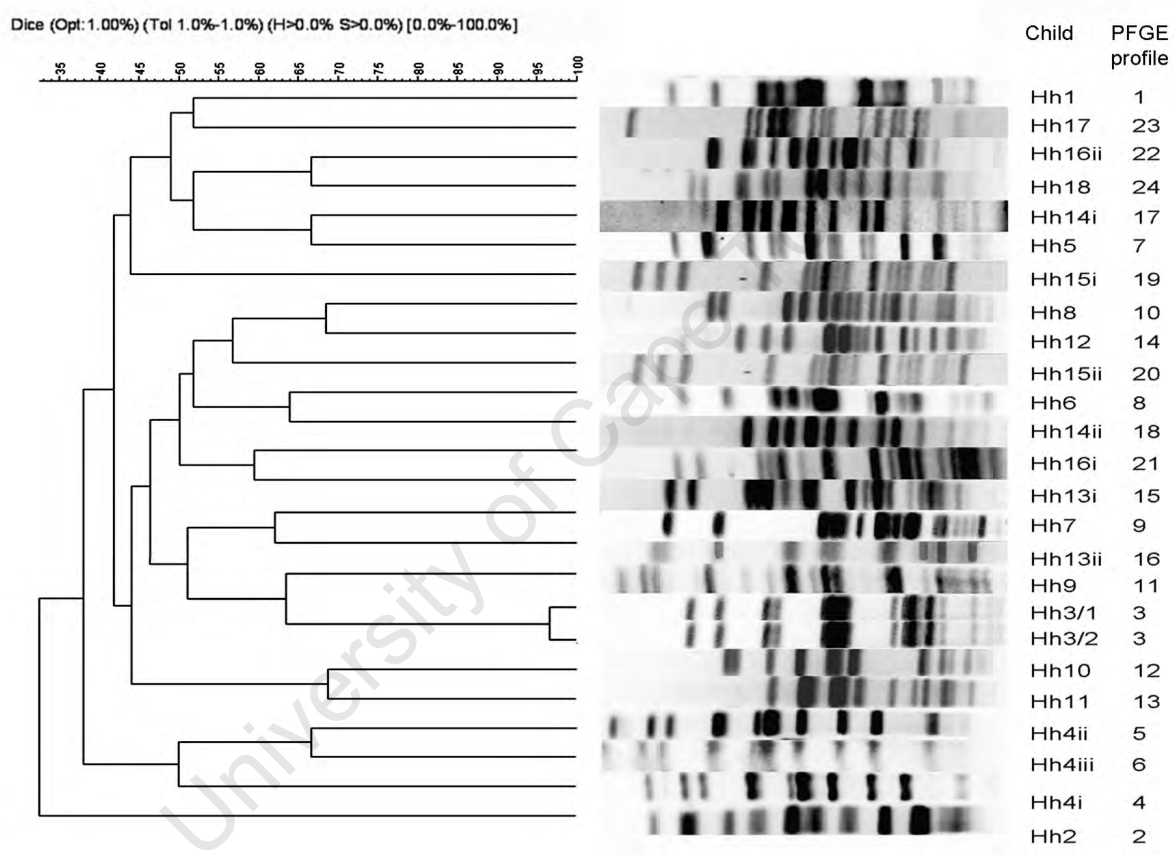

Figure 3.5: Dendrogram showing genetic relatedness of clinical STEC strains. PFGE profiles 1-9, phase 1 STEC; profiles 10-23, phase 2 STEC. 100%, identical strains; 70-99.9%, closely related; 65-69.9%, distally related; <65%, no genetic relationship.

As defined at 68.8% similarity level, the similarity between each of 2 pairs of profiles 10 and 14 (Hh8 and Hh12) and 12 and 13 (Hh10 and Hh11) suggests that the strains from each of the pairs of children may be distally related (Figure 3.5). Only 2 STEC from the 2 phases are possibly related: strains from Hh5 (profile 7) and Hh14i (profile 17) show 66% similarity (Figure 3.5).

3.3.4 Determination and analyses of individual bovine STEC colonies: relatedness to each other and to the clinical STEC

Eighteen (18) PFGE profiles with a similarity of 39-100% were obtained from 98 of 101 STEC colonies from the 15 bovines (Figure 3.6). Using a similarity level of 64% or more to define a cluster, no clustering was observed, indicating genetic diversity of the strains.

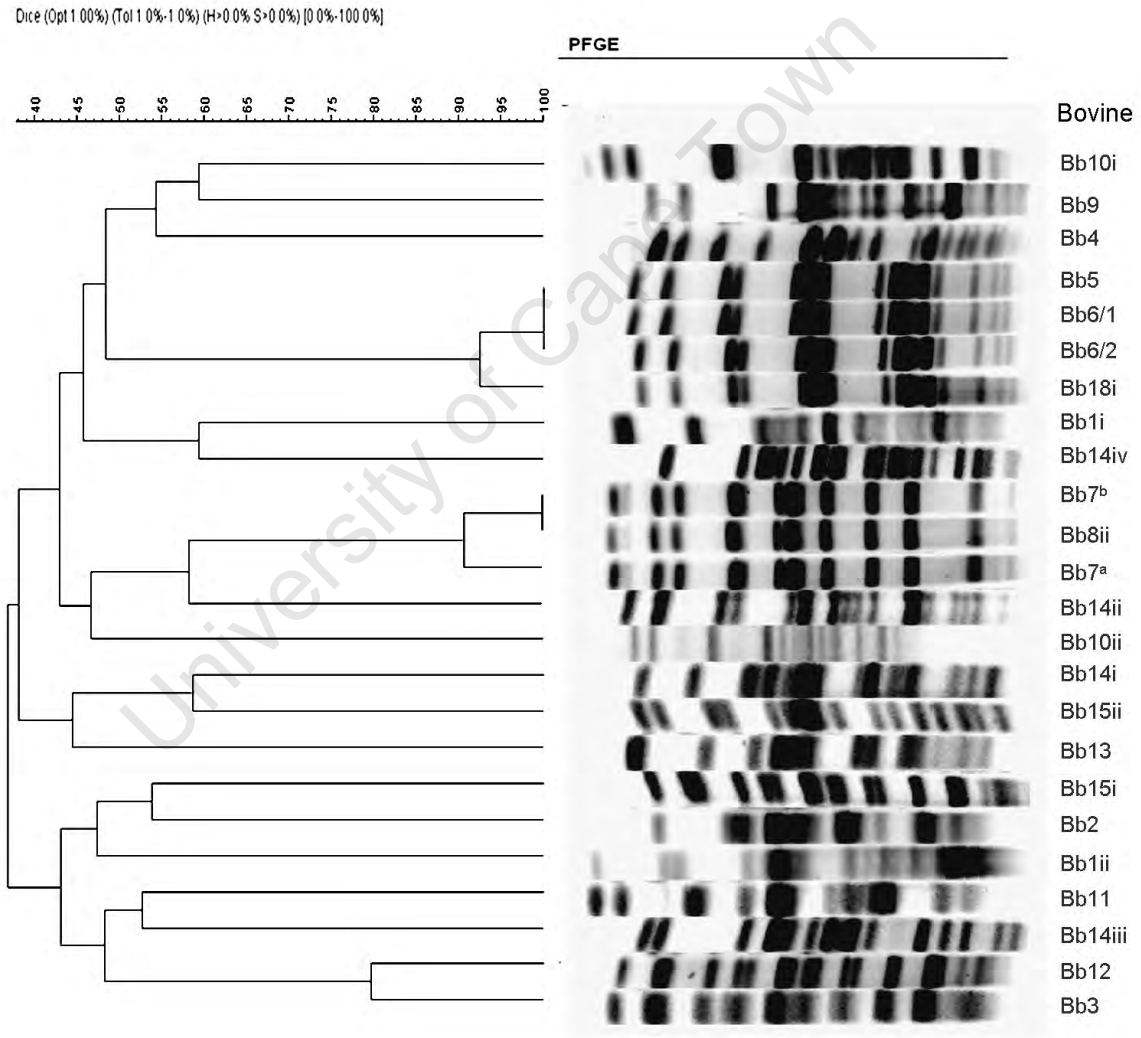

Figure 3.6: Dendrogram showing the relatedness of bovine STEC strains. 100%, identical strains; 70-99.9%, closely related; 65-69.9%, distally related; <65%, no genetic relationship. Cluster, 64% or more similarity level.

A comparison of these profiles with the corresponding profiles from the clinical isolates indicated that STEC from Bb7 (a and b) and Bb8ii are identical to profile 4 of STEC from child 4 (Figure 3.7; Table 3.5).

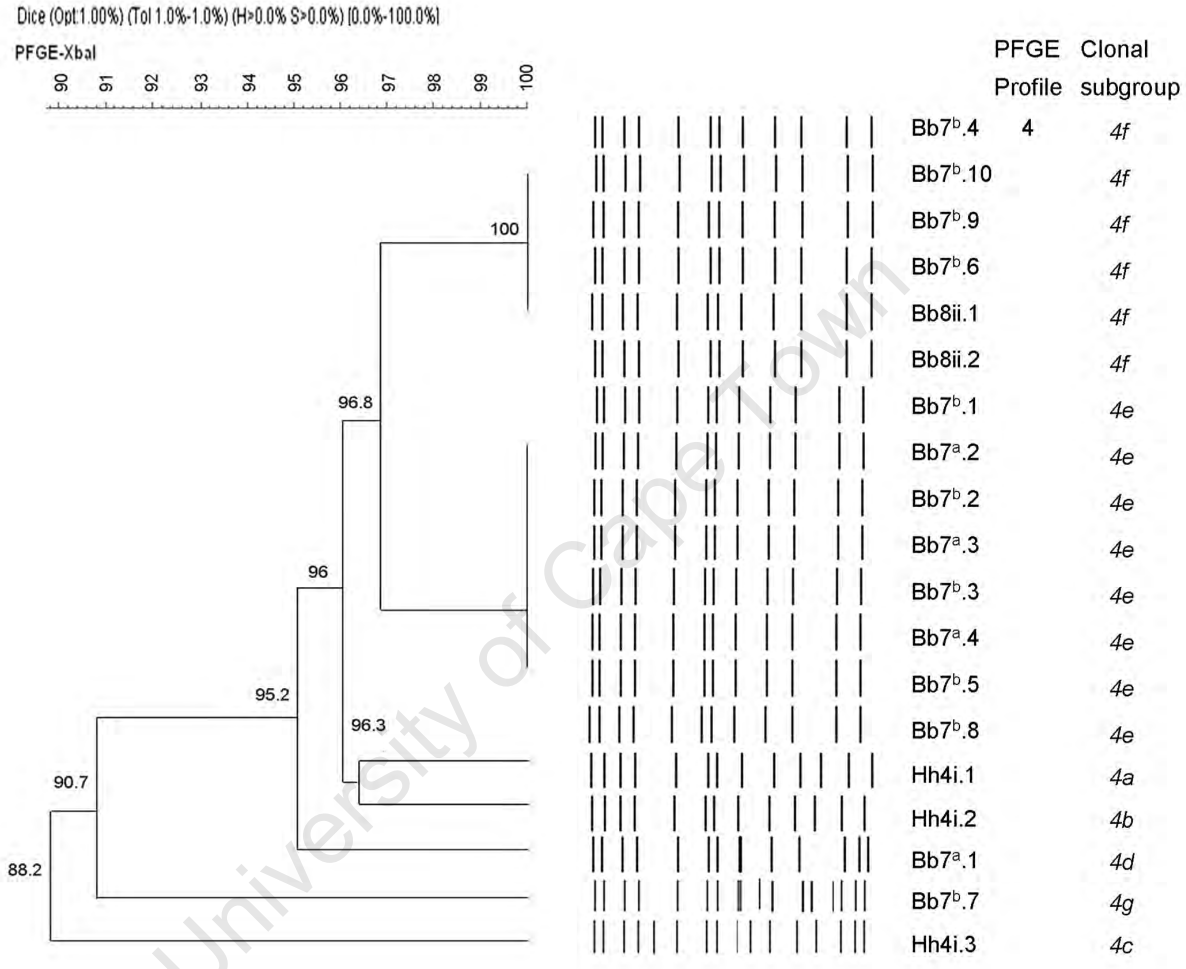

Figure 3.7: Dendrogram showing the relatedness of STEC from Bb7, Bb8 and Hh4. SF^- strains, $Bb7^a.1$-$Bb7^a.4$; SF^+ strains, $Bb7^b1$-$Bb7^b10$. Eight identical strains including 3 SF^- and 5 SF^+ (*4e*) have a similarity level of 96.8% with 6 identical strains in *4f*, which comprise 2 isolates from Bb8ii. In turn, these are 96% similar to 2 strains from Hh4i, and 90.7 and 88.2% similar to strains in *4g* and *4c*, respectively.

Additionally, the respective PFGE profiles of strains from Bb3 and Bb12 are 78.6-80 and 86% similar to profile 2 of STEC from Hh2 (Figure 3.8).

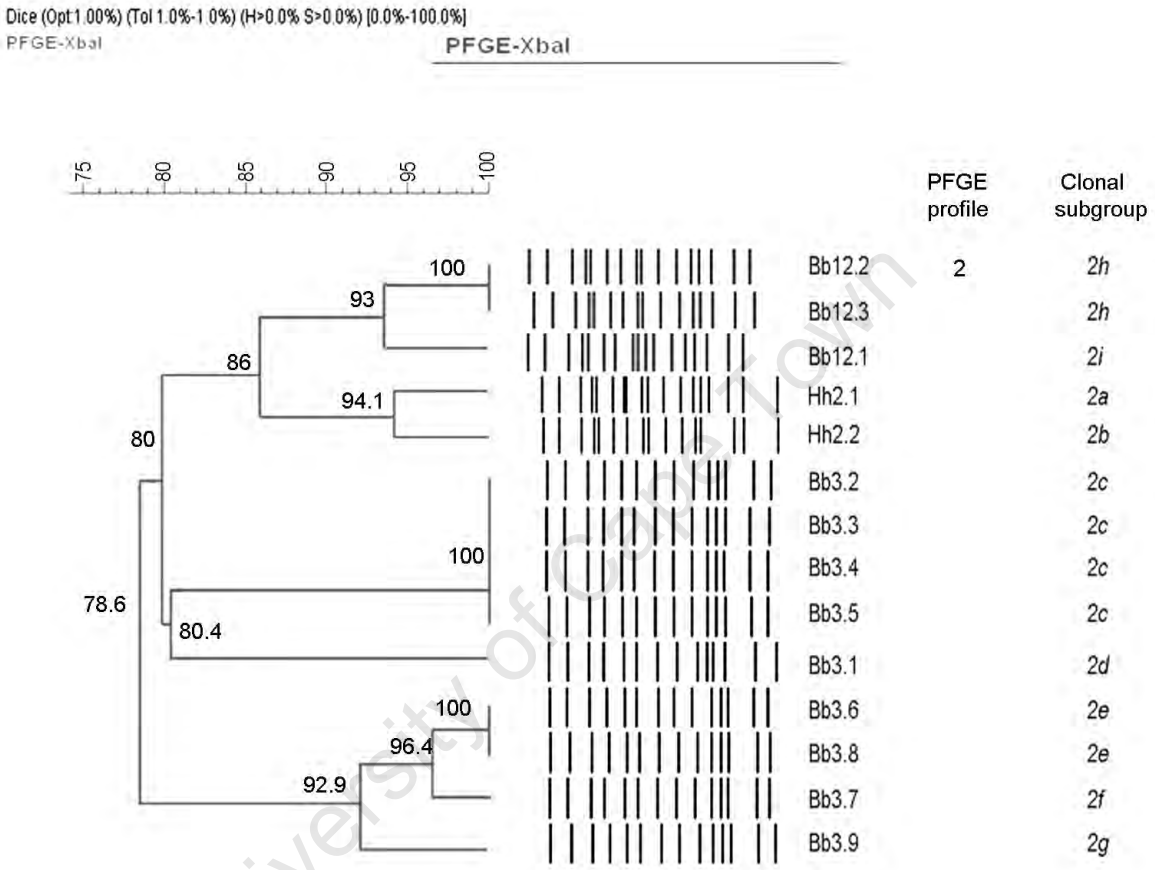

Figure 3.8: Dendrogram showing the relatedness of STEC colonies from Hh2, Bb3 and Bb12.
Strains from Bb3 are closely related (78.6-100%) and are similarly related (80%) to STEC from Hh2 and Bb12. Strains from Hh2 are 94.1%1 identical and 86% similar to strains from Bb12.

Table 3.5: PFGE profiles of multiple STEC colonies in individual bovines

Bovine No.	No. of colonies	PFGE profile	Clonal subgroup	Similarity coefficient (%)
Bb1i	10	25	*25a, 25b, 25c(3*), 25d, 25e, 25f*	80.9-100
Bb1ii		26	*26a, 26b*	97.6
Bb2	10	27	*27a(5*), 27b, 27c, 27d, 27e*	96.5-100
Bb3†	9	2	*2c(4*), 2d, 2e(2*), 2f, 2g*	78.6-100
Bb4	1	28	*28*	
Bb5†	6	29	*29a(6*)*	100
Bb6/1	8	29	*29a(2*), 29b(3*), 29c(2*), 29d, 29e*	86-100
Bb6/2	1	29	*29e*	
Bb7[a]	4	4	*4d, 4e(3*)*	
Bb7[b]	10	4	*4e(5*), 4f(4*) 4g*	91.7-100
Bb8i	8	29	*29a(3*), 29b, 29d(2*), 29f, 29g*	92.9-100
Bb8ii	2	4	*4f(2*)*	100
Bb9†	9	30	*30a, 30b, 30c, 30d, 30e, 30f, 30g, 30h, 30i*	83.2-97.1
Bb10i	2	31	*31*	100
Bb10ii	1	32	*32*	
Bb11	1	33	*33*	
Bb12	3	2	*2h(2*), 2i*	93
Bb13	1	34	*34*	
Bb14i	10	35	*35a, 35b*	95
Bb14ii		36	*36*	
Bb14iii		37	*37a, 37b*	90.5
Bb14iv		38	*38a, 38b, 38c, 38d, 38e*	80.4-91.9
Bb15i	2	39	*39*	
Bb15ii		40	*40*	

*Number of identical colonies

†One colony each was refractory to digestion

Hh6/1, stx_1 and stx_2; Hh6/2, stx_2-containing STEC

None of the other bovine STEC were related to the clinical isolates (Figure 3.9 and 3.10).

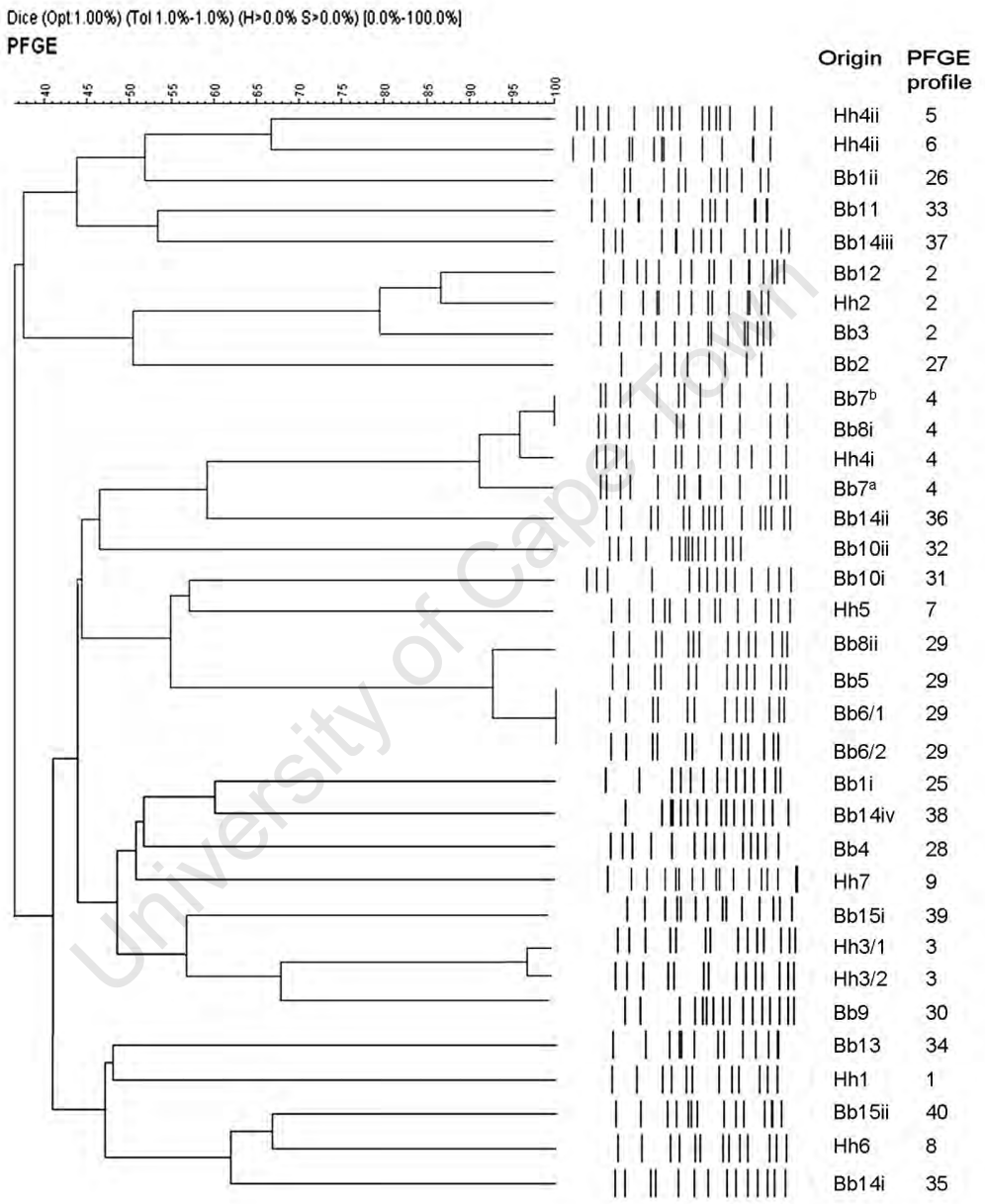

Figure 3.9: Dendrogram showing the relatedness between bovine and clinical STEC strains from phase 1.
100%, identical strains; 70-99.9%, closely related; 65-69.9%, distally related; <65%, no genetic relationship.

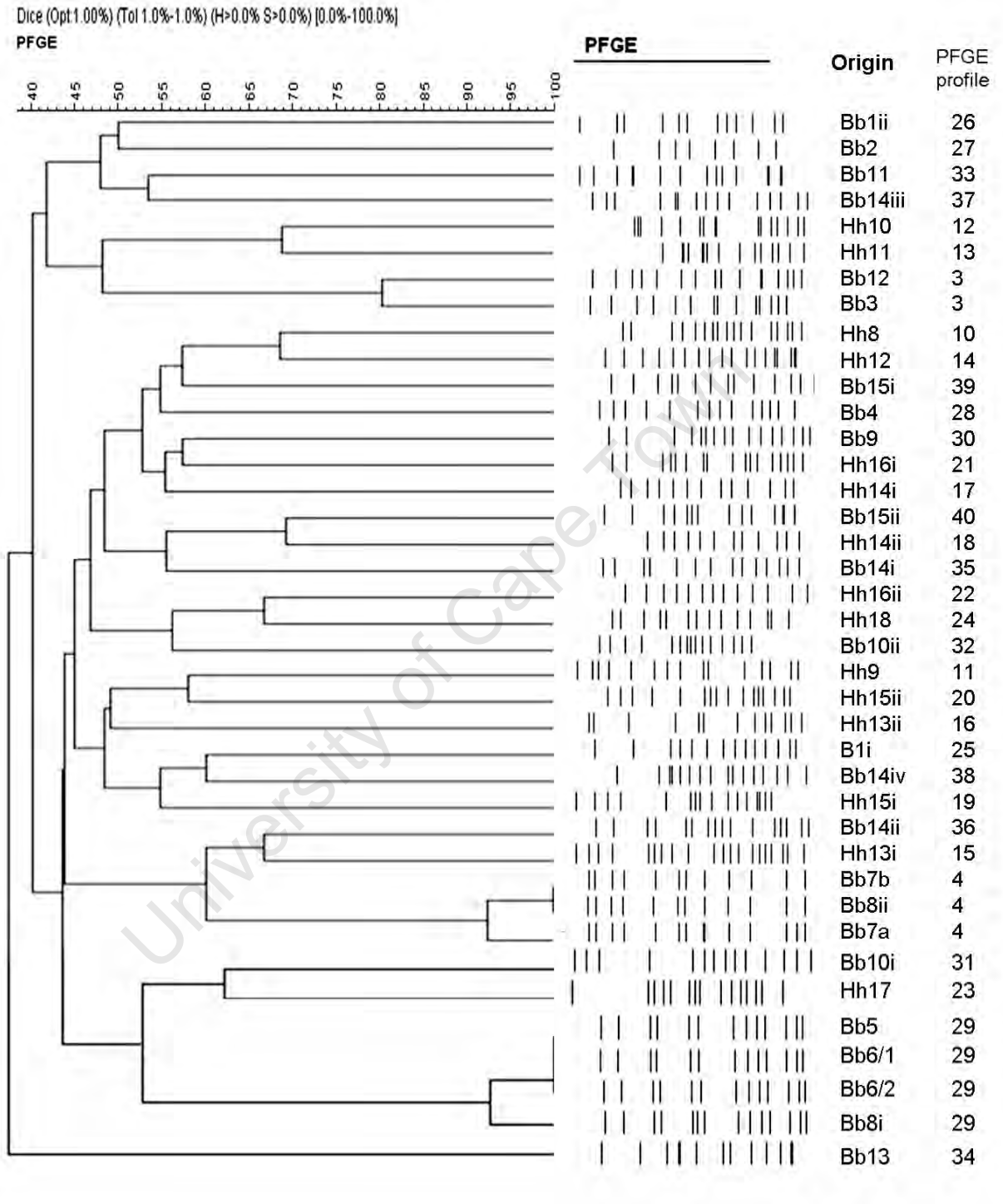

Figure 3.10: Dendrogram showing the relatedness between bovine and clinical STEC strains from phase 2.
100%, identical strains; 70-99.9%, closely related; 65-69.9%, distally related; <65%, no genetic relationship.

The remaining strains from 11 bovines were classified into 16 profiles (25-40) (Table 3.5). Of these, related strains (profile 29) were identified from Bb5, Bb6 and Bb8i (Figure 3.11).

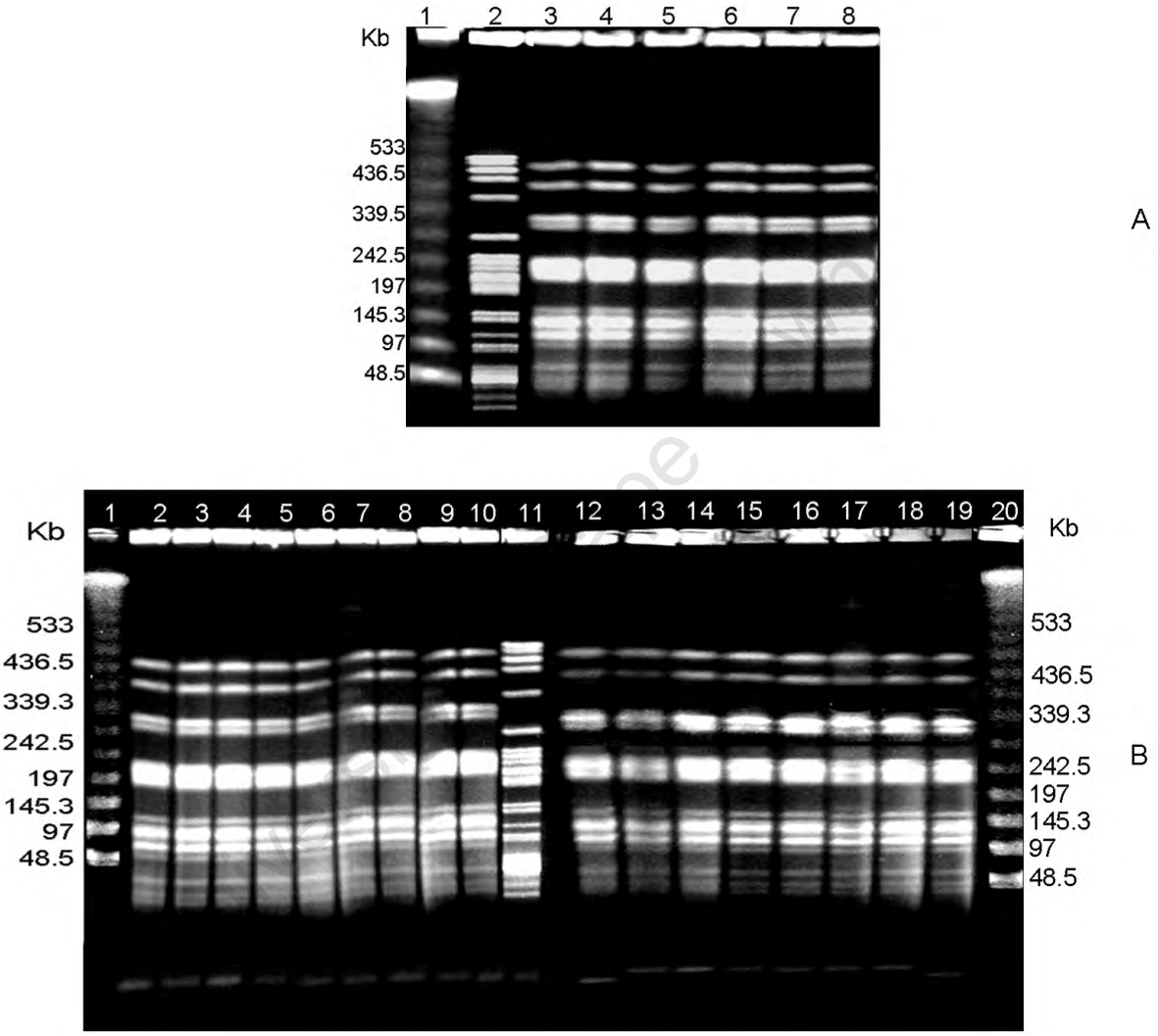

Figure 3.11: PFGE of *Xba*I-digested genomic DNA STEC strains from Bb5, Bb6 and Bb8.
A: Lane 1, PFGE lambda ladder marker (New England Biolabs); lane 2, O157:H7; lanes 3-8, colonies 1-7 from Bb5.
B: Lanes 1, 20, PFGE lambda ladder marker (New England Biolabs); lanes 2-10, Bb6 colonies 1-9; lane 11, O157:H7; lanes 12-19, Bb8 colonies 1-8 representing strain Bb8i. Strains in A and B are closely related.

One of the bovines (Bb14) was infected with 4 unrelated strains (profiles 35-39) as shown in Figure 3.12.

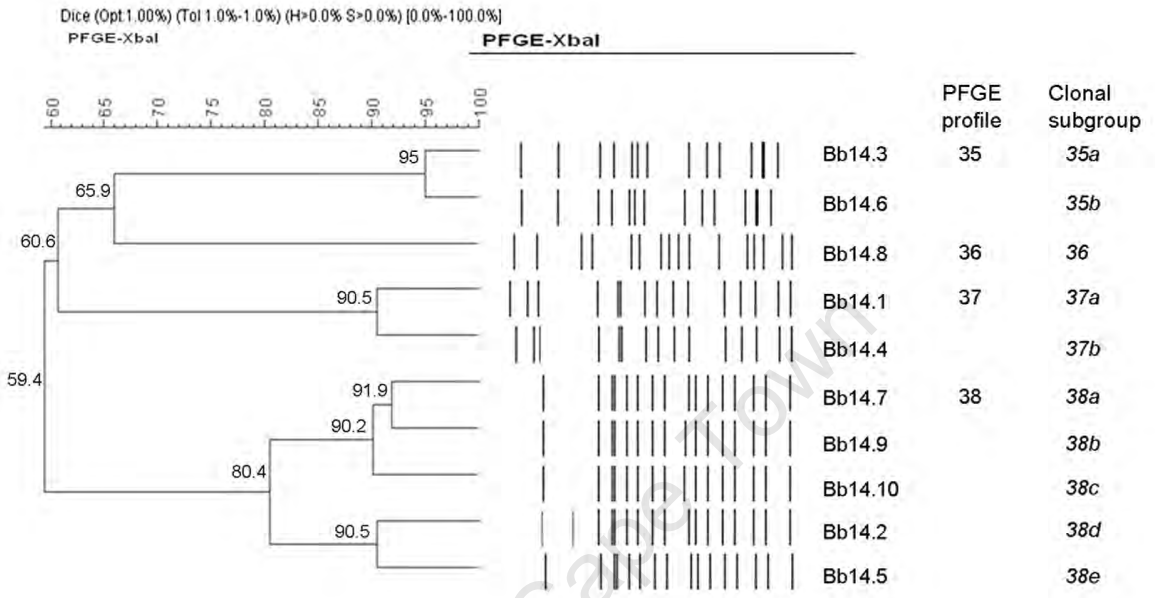

Figure 3.12: Dendrogram showing the genetic relatedness of STEC colonies from bovine Bb14.
Ten STEC colonies segregated into 4 profiles, 35-38, which have a similarity level of 59.4-65.9%.

3.3.5 Relatedness of STEC strains from water and their relatedness to clinical and bovine isolates

The profile of each STEC from water was unique (profiles 41-45); they are not closely related to each other, nor to any of the clinical or bovine isolates (Figures 3.13 and 3.14). Only STEC from Hh17 and Ww3, with 64% similarity segregated in the same cluster (Figure 3.13).

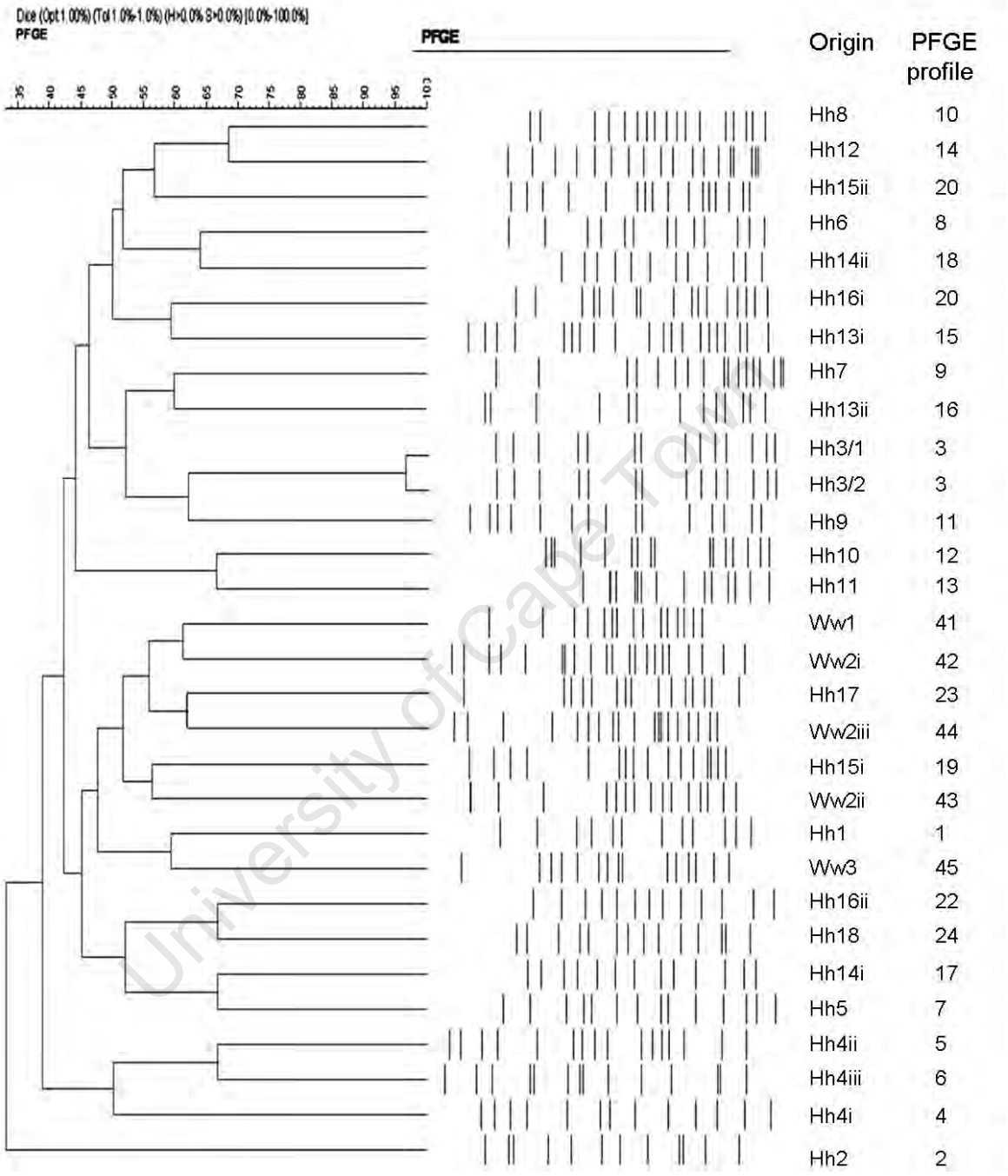

Figure 3.13: Dendrogram showing genetic relatedness between clinical and water STEC strains.
100%, identical strains; 70-99.9%, closely related; 65-69.9%, distally related; <65%, no genetic relationship.

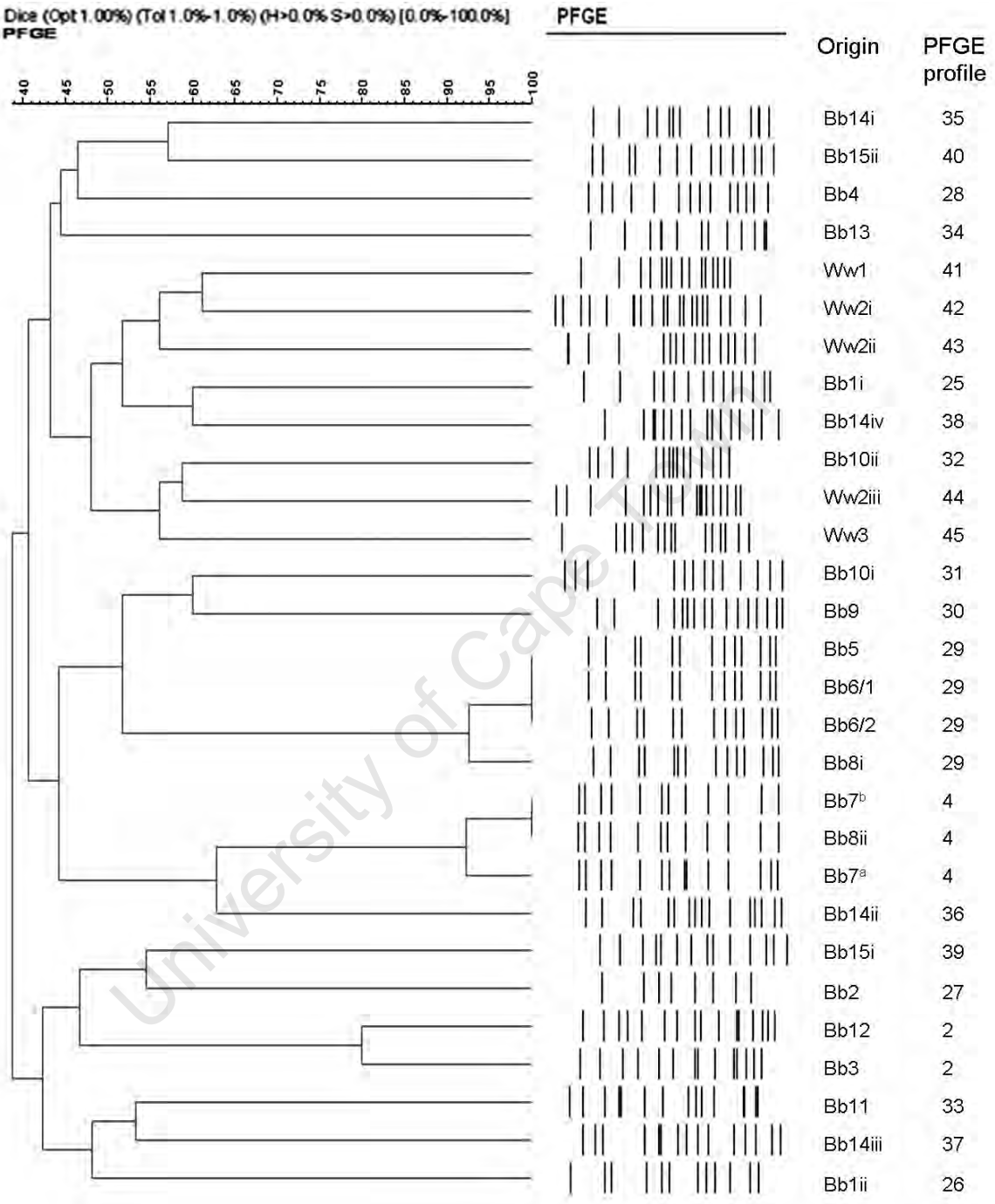

Figure 3.14: Dendrogram showing genetic relatedness between bovine and water STEC strains.
100%, identical strains; 70-99.9%, closely related; 65-69.9%, distally related; <65%, no genetic relationship.

3.4 Discussion

A majority of the bovine STEC (15 of 24) segregated into 15 unrelated PFGE profiles. That 14 of the 15 STEC were obtained from different herds of cattle suggests that these herds were infected with genetically distinct strains of STEC. This observation agrees with the findings from previous studies which have shown that different cattle populations are associated with distinct strains of STEC (Cobbold and Desmarchelier, 2001; Liebana *et al.* 2003). Although the bovines in my study were reared on range, animals within a herd commingle in the night enclosures (kraals), which could explain the infection of Bb5 and Bb6 with related STEC (profile 29). The 2 animals were from the same herd; cross-transmission of infection, or acquisition of infection from a common source, probably occurred within the kraal. This is not uncommon, close contact promoted STEC transmission within a herd of cattle has been described (Faith *et al.* 1996; Cobbold and Desmarchelier, 2001; LeJune *et al.* 2004). A different explanation must account for the relatedness of STEC from Bb5/6 and Bb8 or Bb3 and Bb12 which were not herd-related. Inter-farm clonal similarity of STEC has been previously reported between two unrelated farms over 121 km apart, which could be attributed to transmission by different vehicles including humans and birds, among others (Faith *et al.* 1996; Rice *et al.* 1999; Wetzel and LeJeune, 2006). Since the herds in Nyabushozi shared water sources, the bovines may have been infected through drinking contaminated water as suggested by the studies of Faith *et al.* (1996); Shere *et al.* (1998); LeJune *et al.* (2004). Alternatively, frequent movement of cattle between herds which occurs during traditional ceremonies such as marriage, could result in the transmission of STEC to animals in a different herd. Nevertheless, since the number of herds with STEC excreting cattle were few, it is not possible to deduce whether transmission of STEC among herds of cattle reared on range in Nyabushozi was common or not. A study involving a large number of herds of cattle would be required.

Bb7 was infected with SF^+ and SF^- closely related (90.7-100% similar) strains. A majority of the colonies characterised (8 of 14) segregated into clonal subgroup *4e* which is composed of both SF^+ and SF^- strains. This is not unusual, previous studies have reported genetic relatedness between fermenting and non-sorbitol-fermenting strains of EHEC O157:H7 and O157:NM which comprise a clonal complex (Whittam

et al. 1993; Feng *et al.* 1998). A stepwise evolutionary model has been proposed to explain the loss of sorbitol fermenting characteristics in EHEC O157 (Whittam *et al.* 1993; Feng *et al.* 1998; Donnenberg and Whittam, 2001; Wick *et al.* 2005). Likewise, the loss of sorbitol fermenting phenotype by STEC in my study could have occurred via a similar but parallel evolutionary pathway to STEC O157:H7 (Reid *et al.* 2000). Hence, both fermenting and non-fermenting strains have remained closely related.

Previous studies have characterised clonal subgroups of STEC in naturally infected individual cattle under range (Renter *et al.* 2003) or different production systems (Hancock *et al.* 1994; Shere *et al.* 1998; Rice *et al.* 1999) and in experimentally infected animals (Akiba *et al.* 2000). Clonal subgroups were observed in multiple STEC colonies from 11 of 12 bovines. Although the presence of multiple clonal subgroups in individual bovine STEC was consistent with previous findings obtained from range cattle (Renter *et al.* 2003), a larger proportion of animals in my study (11 of 13) than in a study (57 of the 93) by Renter *et al.* (2003) were infected with multiple clonal subgroups of STEC. In part, this might be a reflection of the difference in the number of colonies characterised in the two studies. The profiles of up to 10 colonies were obtained in my study, whereas only 3 colonies were analysed by Renter *et al.* (2003). Other factors such as different animal and STEC populations as well as environmental conditions may also be responsible for infection with multiple clonal subgroups of STEC. The exact mechanism of clonal diversification is unknown, nonetheless, there is consensus that it results from alterations in the genome through point mutations, insertions, deletions of DNA or internal re-arrangement events (Beutin *et al.* 1997; Akiba *et al.* 2000; Iguchi *et al.* 2003; Bielaszewska *et al.* 2006; Geue *et al.* 2006). This has been demonstrated in experimentally infected bovines where changes in the PFGE profiles of STEC were observed from day 1 to 50 days post-inoculation (Akiba *et al.* 2000). The presence of multiple clonal subgroups shed by individual bovines has demonstrated the heterogeneous nature of STEC and absence of a single predominant clonal subgroup. Thus, analysing PFGE profiles of multiple colonies provides a deeper understanding of the clonal characteristics of STEC within an individual host.

Several studies seem to suggest that closely related STEC from an individual host have similar *stx* gene content (Beutin *et al.* 1997; Shere *et al.* 1998; Sanderson

et al. 2006). Similarly, the *stx* gene content of closely related STEC from 7 of 8 bovines and 4 of 5 children was uniform. The loss of Stx2 phage is associated with alteration of the PFGE profiles of STEC (Murase *et al.* 1999; Bielaszewska *et al.* 2006), nonetheless, strains in Hh3 and Bb6 with stx_2 or both *stx* genes were closely related, suggesting that the absence of stx_1 did not alter their profiles. Although genetically related STEC from individual hosts carried uniform *stx* genes , equally, uniform *stx* genes were carried by unrelated STEC among different hosts. Therefore, the carriage of uniform *stx* genes in different STEC populations does not indicate genetic relatedness.

All five STEC strains from the water samples were genetically distinct, and showed no relatedness to the bovine STEC and all, except one clinical isolate. Isolates from Ww3 and Hh17 clustered together (64% similarity level), perhaps suggesting that they emerged from a distant ancestor (Kim *et al.* 2001; Yang *et al.* 2004). Perhaps due to temporal differences in sampling, cattle were not demonstrated as the source of STEC contaminating the water; nevertheless, they are the most likely culprits. In Nyabushozi STEC contaminated water may be a means of transmission or infection of these organisms to the bovines and children, alike. Studies have shown a genetic diversity of water STEC which might be associated with different sources of the organisms, such as other animals (domestic and wild) or birds (Hancock *et al.* 1994; Faith *et al.* 1996; Shere *et al.* 1998). Thus, although bovines were the main suspects, different animals or birds may be responsible for the STEC contaminated valley dams.

Transmission of STEC from bovines to humans in rural farming communities has been well documented (Crump *et al.* 2002; O'Brien *et al.* 2001; Grif *et al.* 2005). In my study PFGE indicated that STEC isolated from each of two children (Hh2 and Hh4) was acquired from bovines, or their environment. In this context, children could have been infected following consumption of raw milk or fermented milk (*es-habwe*), which is frequent in the community. Previous studies have linked human infection with ingestion of raw milk or cheese made from raw milk (Deschênes 1996; Hussein, 2005; Nielsen *et al.* 2005). Since children have direct contact with bovines, their faeces or their kraals, they could have contracted infection from one of these sources. These observations accord with previous reports on transmission STEC to humans (Wilson *et al.* 1996; Crampin *et al.* 1999; Bielaszewska *et al.* 2000; Crump

et al. 2002; Nielsen *et al.* 2005; Karch *et al.* 2005). Additionally, profiles of STEC from Hh13i and Hh14ii (phase 2), were distally related (66.7%) to bovine isolates Bb14ii and Bb15ii, respectively, despite the difference in sampling time. It may be that these strains have diverged from a recent common ancestor (Yang *et al.* 2004).

A majority of clinical isolates (16 of 25) were genetically distinct, implying that the isolates originated from different ancestors (Kim *et al.* 2001). Overall, this diversity of clinical STEC accords with findings from several studies which involve sporadic STEC infections in a number of countries (Rios *et al.* (1999; Khan *et al.* 2002a; Vaz *et al.* 2006). For instance, 21 of 23 clinical STEC from Chile were genetically diverse (Rios *et al.* (1999); similar diversity was observed in STEC associated with sporadic cases of diarrhoea in India (Khan *et al.* 2002a), or both diarrhoea and HUS in Brazil (Vaz *et al.* 2006).

STEC strains from 11 of the 13 children in my study exhibited multiple clonal subgroups and none of the clonal subgroups was dominant. This is not uncommon, STEC from individual patients showing multiple clonal groups have been reported in Germany (Liesegang *et al.* 2000). Probably, the diversity of the clinical isolates is linked to the diversity STEC in the bovines. From an epidemiological view point, it may be necessary to determine PFGE profiles of multiple clinical strains in a sample from the same patient. This is because multiple clones may be involved in an infection and may have different virulent profiles.

In conclusion, these data have shown high clonal heterogeneity among the bovine, clinical and water STEC isolates. Molecular typing strongly indicates transmission of STEC from bovines to 2 children.

Chapter 4

Serogroups and genetic heterogeneity of STEC

4.1 Introduction

Since its inception in 1947 (Kauffmann, 1947), serotyping has remained an important epidemiological and reference typing tool for *E.coli* (Orskov *et al.* 1977). Presently, more than 470 serogroups of STEC have been identified (http://www.lugo.usc.es/ecoli); however, only a small number of O antigenic groups including O26, O103, O111, O113, O145 and O157 have been associated with human illness (Nataro and Kaper, 1998; Thorpe, 2004; Caprioli *et al.* 2005). These strains, classified as enterohaemorrhagic, are frequently associated with more severe outcomes of infection, including HUS (Levine, 1987; Nataro and Kaper, 1998). Recently, additional serogroups, such as O8 and O76, which have emerged from among previously non or less pathogenic serogroups also play a role in human disease (Welinder-Olsson *et al.* 2002; Manning *et al.* 2007).

Thus, to obtain a more complete picture of the clinical STEC from Nyabushozi, the O antigenic serogroup of these organisms were determined. Additionally, to evaluate the potential health hazard of the bovines and water for the community, the serogroups of STEC from these sources were ascertained.

4.2 Experimental design

4.2.1 Bacterial strains

A single isolate of STEC representing each PFGE profile in association with *stx* gene content in the human, bovine and water, was investigated. In total 54 STEC strains were included: 5 water (Table 3.2), 25 human (Tables 3.3 and 3.4) and 24 bovine STEC strains (Table 3.5).

4.2.2 Serotyping

Serotyping for the O antigens was carried at the Enteric Diseases Reference Unit, National Institute of Communicable Diseases, National Health Laboratory Service, Sandringham, Johannesburg, South Africa. The tube agglutination test for serotyping based on an antigen/antibody reaction was used. Strains that did not form an agglutination with the O antisera were designated as nontypeable (NT). Typing for the H-antigens was not carried out. The O serogroups of 2 strains were not established because they were not viable, their serogroup have been recorded as not known (NK).

4.3 Results

4.3.1 O antigenic serogroups of STEC from the bovines

Twenty four bovine STEC were serotyped into 12 serogroups (Table 4.1). Four strains expressed non typeable O antigens. Strains expressing O157 were not isolated. Three serogroups accounted for 12 of the 20 typeable strains: O76 (5 strains), O8 (4 strains) and O113 (3 strains). The remaining 7 serogroups were distributed as follows; O142 (2 strains) and serogroups O20, O28ac, O107, O111, O158 and Poly9 contain 1 strain each (Table 4.1).

Table 4.1: Serogroups of bovine STEC

Bovine	Serogroup	Bovine	Serogroup	Bovine	Serogroup
Bb1i	O111	Bb7[a]	O113	Bb12	O76
Bb1ii	Poly9	Bb7[b]	O113	Bb13	NT
Bb2	NT	Bb8i	O8	Bb14i	NT
Bb3	O76	Bb8ii	O107	Bb14ii	O158
Bb4	O28ac	Bb9	O113	Bb14iii	O76
Bb5	O8	Bb10i	NT	Bb14iv	O142
Bb6/1	O8	Bb10ii	O20	Bb15i	O76
Bb6/2	O8	Bb11	O142	Bb15ii	O76

Bb7.1[a] (SF^+) and Bb7.2[b](SF^-) strains

4.3.2 Serogroups of STEC from the water

Three of the 5 strains from water (Ww1, Ww2i and Ww2ii) belonged to serogroup O166. Strain Ww3 expressed a non-typeable O antigen. The serogroup of the fifth strain (Ww2ii) was not established because the bacteria were not viable.

4.3.3 Serogroups of STEC from the children

Nineteen of the 25 strains expressed O antigens and segregated into 15 O serogroups (Table 4.2). For 5 strains, the O antigens were non-typeable (NT) (Table 4.2). The serogroup of an additional strain was not established because the cells were not viable.

The 10 strains in phase 1 were typed into 7 O serogroups, one additional strain expressed a nontypeable O antigen (Table 4.2). Serogroups O107 and O113 comprised 2 strains each (Table 4.2). The remaining 5 serogroups O8, O22, O74, O76 and O78 each comprised one strain (Table 4.2).

Table 4.2: Serogroups of STEC from children in phases 1 and 2

	Child	Serogroup	Child	Serogroup
Phase 1	Hh1	O74	Hh4ii	O107
	Hh2	O76	Hh4iii	O107
	Hh3/1	O22	Hh5	O78
	Hh3/2	O113	Hh6	NT
	Hh4i	O113	Hh7	O8
Phase 2	Hh8	O149	Hh14ii	NT
	Hh9	NT	Hh15i	O142
	Hh10	O29	Hh15ii	O142
	Hh11	O141	Hh16i	O176
	Hh12	O166	Hh16ii	NK
	Hh13i	NT	Hh17	O169
	Hh13ii	O111	Hh18	O142
	Hh14i	NT		

NK, serogroup not established

Of the 15 strains in phase 2, 10 were typed into 8 O serogroups, the O antigens of 4 strains were untypeable and one strain was not serotyped (Table 4.2). Of the 8 O serogroups, O142 comprised 3 strains. The remaining 7 serogroups: O29, O111, O141, O149, O166, O169 and O176 were each represented by a single strain (Table 4.2).

4.3.4 Correlation of *stx* gene content with serogroups containing multiple strains

Six serogroups were common to STEC from both children and the cattle, and one serogroup was present in STEC from water and a child (Table 4.3). Correlation of *stx*-genotype with serogroup showed that the *stx* content of clinical O antigen expressing STEC was not always the same as in the corresponding bovines or water related STEC (Table 4.3).

Table 4.3: *stx* content of STEC strains in various serogroups from different sources

	Origin and *stx* gene content of STEC			
Serogroup	Bovine/ water	*stx* content	Child	*stx* content
O8	Bb5	stx_1/stx_2	Hh7	stx_2
	Bb6/1	stx_1/stx_2		
	Bb6/2	stx_2		
	Bb8i	stx_1/stx_2		
O76	Bb3	stx_1/stx_2	Hh2	stx_1/stx_2
	Bb12	stx_1		
	Bb14iii	stx_1/stx_2		
	Bb15i	stx_1		
	Bb15ii	stx_1		
O107	Bb8ii	stx_2	Hh4ii	stx_1
			Hh4iii	stx_1
O111	Bb1i	stx_1/stx_2	Hh13ii	stx_1
O113	Bb7[a]	stx_1/stx_2	Hh3/2	stx_1/stx_2
	Bb7[b]	stx_1/stx_2	Hh4i	stx_1
O142	Bb11	stx_1	Hh15i	stx_2
	Bb14iv	stx_1	Hh15ii	stx_2
			Hh18	stx_1/stx_2
O166	Ww1	stx_2	Hh12	stx_1
O166	Ww2i	stx_2		
O166	Ww2ii	stx_2		

Bb7.1[a] (SF^+) and Bb7.2[b](SF^-) strains

4.3.5 Genetic heterogeneity of STEC clones within a serogroup

The clonal heterogeneity of STEC strains within a serogroup was investigated in 5 serogroups (O8, O76, O113, O142 and O166) containing 4 or more strains. The data was analysed as described [3.2.2.5] and results are presented in Figure 4.1).

Strains within each of the 5 serogroups belonged to 2 or more clonal subgroups. As in Figure 4.1A, 3 of the 5 isolates in serogroup O8, are identical (clonal subgroup

29a) and closely related to a strain in 29f.

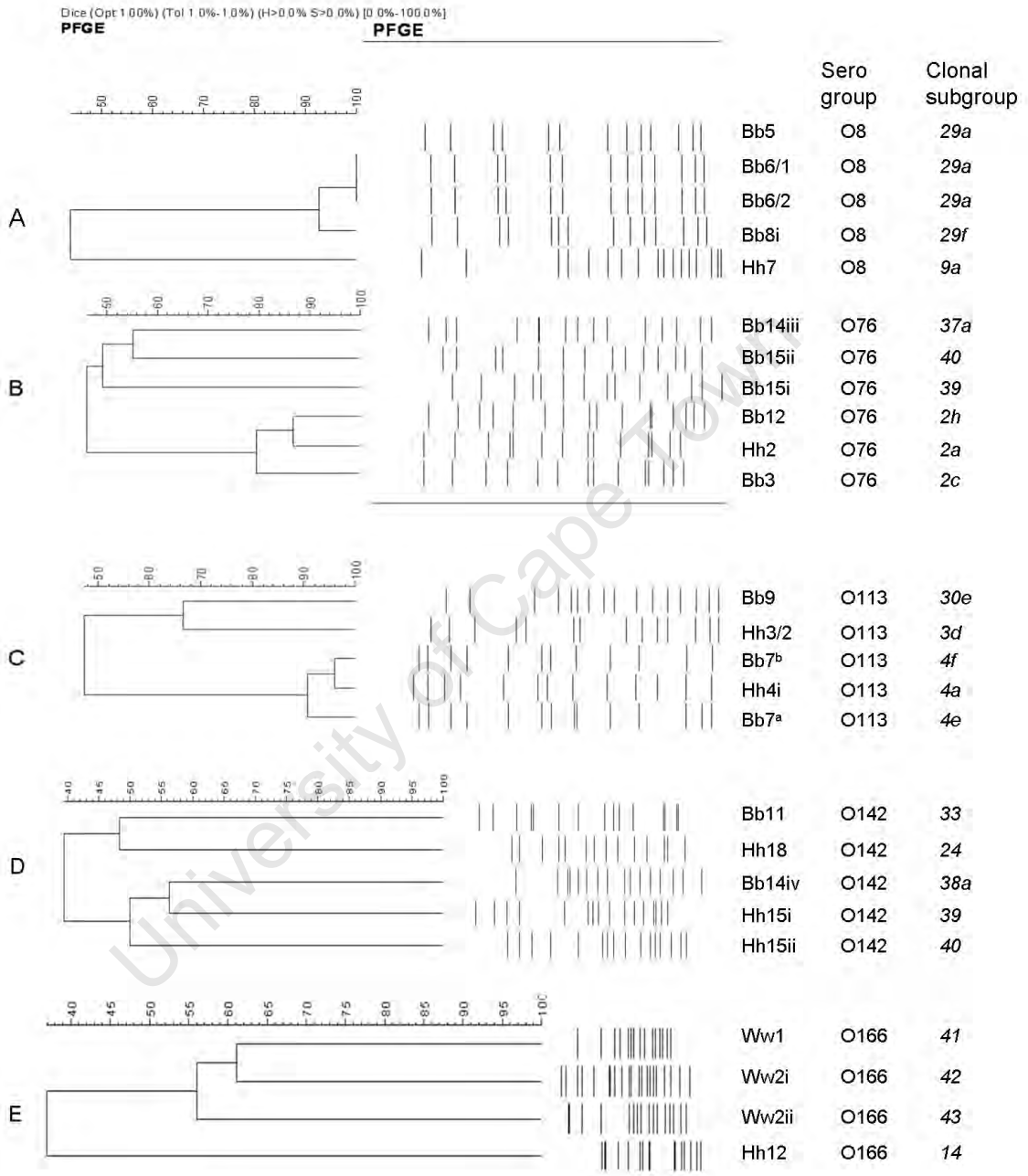

Figure 4.1: Dendrograms showing clonal heterogeneity of STEC within a serogroup. Macrorestriction digest of genomic DNA was carried out with *Xba*I. Similarity coefficient was generated by Dice coefficient using UPGMA with arithmetic averages at a position tolerance of 1%. The respective clonal subgroups are shown in Tables 3.3 and 3.5.

Strains (6) in serogroup O76 separated into 4 clonal complexes (Figure 4.1B) with

only clonal subgroups 2a, 2c and 2h being closely related. While 3 of the 5 strains in serogroup O113 are closely related (4a, 4e and 4f), they are distinct from strains in clonal subgroups 3d and 30e (Figure 4.1C). All strains in serogroups O166 and O142, separated into distinct unrelated clonal subgroups (Figure 4.1D and E).

4.4 Discussion

All the serogroups of STEC isolated from the bovines in Nyabushozi have been previously isolated from cattle in different countries, including Australia (Hornitzky *et al.* 2005; Brazil (Irino *et al.* 2005); Japan (Kobayashi et al. 2003, the USA (Cobbold *et al.* 2004) and a number of European countries (Beutin *et al.* 1997; Pradel *et al.* 2001; Pearce *et al.* 2006). Strains of serogroups O76 and O113 were reported in an earlier study by Kaddu-Mulindwa *et al.* (2001) in central Uganda, suggesting that these serogroups are prevalent in cattle from different parts of Uganda.

It is probably difficult to assess infection of bovines with O157:H7 from Uganda, and on the African continent in general, because of the few available studies. In one particular study, Kaddu-Mulindwa *et al.* (2001) investigated the occurrence of STEC in bovines from Uganda. These investigators did not isolate STEC O157:H7 from 43 STEC shedding bovines. Similarly, none of 15 STEC excreting bovines in my study shed STEC O157:H7. Corresponding studies from Australia (Hornitzky *et al.* 2002) and a number of countries in Europe suggest that cattle from these countries predominantly shed non-O157:H7 strains (Montenegro *et al.* 1990; Blanco *et al.* 2001). On the other hand, the available literature from North America, Brazil, United Kingdom and Japan (Izumiya *et al.* 1997; Slutsker *et al.* 1997; Michino *et al.* 1999; Thorpe, 2004; Vali *et al.* 2004; Irino *et al.* 2005) have shown that cattle predominantly shed O157:H7. From these observations, the shedding of O157:H7 or non-O157:H7 by bovines varies with the geographical location. Since only non-O157:H7 STEC were isolated from cattle in Nyabushozi, it can be suggested these strains are more associated with cattle in Uganda. However, these results cannot be generalised. Future studies involving large numbers of cattle and from different geographical and husbandry practices would need to be investigated.

It is known that cattle are frequently co-infected with multiple serogroups of STEC

(Beutin *et al.* 1997; Pradel *et al.* 2000; Pearce *et al.* 2004). Unsurprisingly, some bovines (Bb1, Bb8, Bb10 and Bb14) excreted two or more serotypes of STEC. Results from a study in Scotland showed that some cattle shed several serotypes (O26/O145 or O26/O103/O157) (Pearce *et al.* 2004), indicating that different serogroups of STEC can co-exist within the intestinal tract of bovines. Of course, co-existing serogroups may vary depending on the prevalent serogroups within an ecological niche.

That STEC serogroups O8, O20, O76, O111 and O113, known to cause bloody diarrhoea and HUS in humans (http://www.microbionet.com.au/vtectable.htm) were shed by cattle in the pastoralist community suggests that humans within the community are at risk of infection with STEC belonging to pathogenic serogroups.

Clinical isolates belonging to 15 serogroups were isolated indicating the existence of diverse STEC serogroups from children with diarrhoea in Nyabushozi. Interestingly, three of these: O29, O149 and O176 have not been previously described in humans. This shows the capability of diverse serogroups of STEC to colonise the human intestinal tract. Further, the isolation of novel STEC serogroups accords with the findings of Beutin *et al.* (2004), who observed that more human STEC strains remain to be identified. As the children infected with these serogroups were from phase 2 it is perhaps not surprising that their equivalents were not obtained from the bovines. In this context, STEC O176 has been isolated only from water buffaloes in Brazil (Oliveira *et al.* 2007); however, there are no water buffaloes in Nyabushozi county. Nonetheless, cattle remain the likely source of most of the clinical STEC, although this was not demonstrated.

Eight of the serogroups from children: O8, O74, O76, O78, O107, O111, O113 and O166 have been implicated as human pathogens (Beutin *et al.* 1998,2004; Keskimäki *et al.* 1998; Welinder-Olsson *et al.* 2002; Bettelheim, 2003; Blanco *et al.* 2003a; Ratchtrachenchai *et al.* 2004; Karch *et al.* 2005; Tarr *et al.* 2005; Manning *et al.* 2007). Of these, O76 from Hh2 is genetically related to the corresponding serotype from Bb3 and Bb12, while O113 from Hh4 is similarly related to O113 and O107 strains from Bb7 and Bb8ii, respectively (Figure 3.9 and Figure 4.1). One group, STEC O166, associated with human infection in Germany (Beutin *et al.* 2004), was also isolated from a child and water in Nyabushozi. Although STEC

O166 had not been isolated previously from water (http://www.lugo.usc.es/ecoli/; http://www.microbionet.com.au/vtectable.htm), the fact that these STEC can survive in an aquatic environment indicates the potential health hazard of STEC-contaminated water. It is noteworthy that, although the STEC strains from the different sources were not genetically related, water samples and faeces from the O166 infected child were obtained during the same sampling period. Serogroup O142 is believed to have emerged recently (Todd *et al.* 1999; Arthur *et al.* 2002). Interestingly, this serogroup was the most frequently identified group in the phase 2 children, suggesting that O142 STEC are able to thrive and proliferate within the intestinal tracts of children in Nyabushozi. In general, the diversity of serogroups displayed by the clinical STEC in Nyabushozi is comparable to diverse serotypes reported in sporadic infections in other countries (Beutin *et al.* 1998; Eklund *et al.* 2001; Blanco *et al.* 2004a). For example, in Spain, 126 isolates belonged to 41 O serogroups (Blanco *et al.* 2004a), 56 isolates from Thailand segregated into 20 O serogroups (Eklund *et al.* 2001) and 89 STEC from Germany belonged to 15 O serogroups (Beutin *et al.* 1998).

There is a clear distinction between serogroups of STEC collected 12-15 months apart, in phases 1 and 2. None of the serogroups isolated from phase 1 were identified in phase 2 and vice versa. Importantly, none of the children sampled in phase 2 were from the same homestead as both the children in phase 1 and the bovines, although they were from the same locality. These differences in the serogroups may be linked to the complex cattle-human-STEC dynamics in the ecosystem. This probably indicates that different populations of STEC were sampled in phase 1 and phase 2. Additionally, factors including high cattle population turn-over, changes in diet (Djordjevic *et al.* 2004; Pearce *et al.* 2004; Geue *et al.* 2006), the host immune response (Beutin *et al.* 1998) and seasonal human-cattle migration across the cattle corridor in Nyabushozi, which disrupt the stability of the ecosystem, would also alter the STEC population and the serogroups involved.

Simultaneous human infection with multiple serogroups of STEC has previously been reported in several countries including the USA (Gilmour *et al.* 2007b), Thailand (Leelaporn *et al.* 2003) and India (Khan *et al.* 2002b). Similarly, each of two children were co-infected with serogroups O107/O113 or O111/NT. Additionally, two other children were each co-infected with different strains of the same serogroup. Since

the serogroup of one of the strains from Hh16 is unknown, it is possible that this child was infected with strains belonging to more than one serogroup.

STEC within serogroups O8, O76 and O113 were clonally related (Figure 4.1), although within each serogroup one or more clones were distinct. While all clones within serogroups O142 or O166 were unrelated. These observations were unexpected, as previous studies have described unrelated and related clones of STEC within various serogroups (Geue *et al.*; Vaz *et al.* 2006). Recent studies have described the occurrence of genetically related STEC belonging to different O serogroups such as O22/O128 and O117/O128 (Urdahl *et al.* (2003). In agreement with these findings, related STEC of serogroups O22/O113 or O107/O113 were observed in my study, suggesting that these could have emerged from antigenic shift and switching of their O antigens. Previous studies suggested that antigenic shift resulted in the emergence of STEC O111 from STEC O26 (Feng *et al.* 1998; Whittam *et al.* 1993; Donnenberg and Whittam, 2001).

In conclusion, STEC from children and bovines in Nyabushozi belong to diverse serogroups. While a number of cattle excreted serogroups associated with severe human illnesses, children were infected by both pathogenic serogroups and previously unknown humans STEC serogroups. Both unrelated and closely related clones of STEC were isolated from various serogroups.

Chapter 5

Production of Stx, variants of *stx*$_2$ and *eae* genes of STEC strains

5.1 Introduction

To gain deeper understanding of the virulence of STEC strains described in Chapter 4, the presence of *stx*$_2$ variants and *eae* genes was examined. Clearly, the carriage of *stx* genes is fundamental to the pathogenesis of EHEC-associated human disease (Thorpe, 2004; Kaper *et al.* 2004). The two major bacteriophage-borne *stx*$_1$ and *stx*$_2$ genes (Karch *et al.* 2005; Beutin *et al.* 2004) may be present alone, or in combination (Karch *et al.* 2005). STEC that elaborate Stx2 only are more virulent than STEC that produce Stx1 alone or both toxins (Boerlin *et al.* 1999); however, strains elaborating only Stx1 or both Stx are equally virulent (Friedrich *et al.* 2002). While Stx1 is more conserved, greater amino acid variation in Stx2 is associated with proteins of different toxicity profiles in cultured cells or/and animals (Paton *et al.* 1995; Melton-Celsa *et al.* 1996,2002; Pierard *et al.* 1998). Clinical and epidemiological studies have implicated *stx*$_{2-EDL993}$ and *stx*$_{2c}$ in most cases of bloody diarrhoea and HUS, whereas the activatable *stx*$_{2d}$ genes (*stx*$_{2d-1}$ and *stx*$_{2d-2}$ are associated with mild to severe diarrhoea (Friedrich *et al.* 2002). The different subtypes of *stx*$_2$ were described in detail in Chapter 1.

Besides *stx*, EHEC contain a pathogenicity island LEE (Willshaw *et al.* 1985; Schmidt *et al.* 1994), which supports *eae*, the gene encoding adhesion to, and effacing of, the intestinal mucosal surface (Sherman *et al.* 1988; Jerse *et al.* 1990). While

the 3'-end of *eae* is conserved the 5'-end is heterogenous as a result of recombination events, involving foreign DNA, in this region (McGraw *et al.* 1999), also called Int280 (Adu-Bobie *et al.* 1998b; Frankel *et al.* 2001; Zhang *et al.* 2002b; Ramachandran *et al.* 2003). To date 18 distinct genes (α1, α2, β1, β2, ξ or β2B, $\delta(\kappa,\beta$2O), γ1, γ2(θ), ε1, ε2(νR), ζ, ι1, ι2(μR), λ, μB, νB, ξB and ρ) have been described (Adu-Bobie *et al.* 1998a; Oswald *et al.* 2000; Tarr and Whittam, 2002; Zhang 2002b; Jenkins *et al.* 2003a; Ramachandran *et al.* 2003; Blanco *et al.* 2004b; Mora *et al.* 2007; Cookson *et al.* 2007).

This chapter describes studies related to the production of Stx in STEC, and the identification of variants of *st*x_2 and *eae* genes carried by the strains.

5.2 Experimental design

5.2.1 Bacterial strains

A total of 54 STEC strains which are described in Chapter 4 were investigated. These included 25, 24 and 5 strains from children, bovines and water, respectively. STEC O157:H7 and *E. coli* K-12 were used as control strains, where necessary.

5.2.2 Shiga toxin production

Duopath Verotoxin detection kit (Merck KgaA, Darmstadt, Germany) was used according to the instruction manual to detect the production of Stx1 and Stx2. Minimal measurable quantities of Stx in amounts of 25ηg/ml and 62.5 ηg/ml for Stx1 and Stx2, respectively, were detected.

5.2.3 Detection of virulence genes by PCR amplification

5.2.3.1 PCR amplifications and detection of *st*x_2 variants

Primers designed to detect 8 *st*x_2 variants (Table 5.1) were used to amplify the *stx* genes according to the PCR amplification parameters presented in Table 5.1. PCR

conditions were identical to those for the previous amplification of *stx* [2.2.14.1], except for time and temperature (Table 5.1).

Table 5.1: Primers and PCR amplification conditions for the detection of variants of stx_2 gene

Primer	stx_2	Primer ($5' \rightarrow 3'$) sequence	Product size (bp)	PCR cycle conditions*	Reference
stv1F	stx_{2c},	TCGAATCCAGTACAACG	1379	94^{o}C, 60 s;	This
stv1R	stx_{2d-1},	ACATACCACGAATCAGG		57^{o}C, 60 s;	study
	stx_{2d-2},			72^{o}C, 90 s	
	stx_{2g},				
	stx_{2-O48},				
	stx_{2-OX3b},				
	$stx_{2-EDL993}$				
stv1F	stx_{2-vhc}	TCGAATCCAGTACAACG	1366	94^{o}C, 60 s;	This
stv3R		ATCCGGTTATGCCTCAG		55^{o}C, 60 s;	study
				72^{o}C, 90 s	

*Extension at 72 °C for 5 min completed the amplification process.

5.2.3.2 PCR amplifications and detection of *eae* and variants of *eae* genes

The presence of *eae* gene was detected using one of the 3 primers *UF*/*UR* (Mora *et al.* 2007), *lpF*/*lpR* (Cookson *et al.* 2007) or *orfU*/*escD* (Oswald *et al.* 2000) and conditions as shown in Table 5.2. While primers *UF*/*UR* (Mora *et al.* 2007) and *lpF*/*lpR* (Cookson *et al.* 2007) targeted the conserved 3'end of the *eae* gene, *orfU*/*escD* (Oswald *et al.* 2000) was used to amplify the entire structural gene. Six *eae* subtypes $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2\text{B}/\xi$, $\gamma 1$ and $\gamma 2/\theta$ were amplified using primers and conditions shown in Table 5.2. All *eae* amplification reactions included magnesium chloride at a concentration of 1.75mM rather than 1.5mM, used in other PCR reactions.

Table 5.2: Primers and PCR amplification conditions for the detection of *eae* and variants of *eae* genes

Primer	*eae* gene	Primer ($5' \rightarrow 3'$) sequence	Product size (bp)	PCR cycle conditions*	Reference
UF	*eae*[a]	GGAACGGCAGAGGTTAATCTGCAG	346	94^{o}C, 55s;	Mora
UR		GGCGCTCATCATAGTCTTTC		55^{o}C, 60s;	*et al.*
				72^{o}C, 60s.	(2007)
lpF	*eae*[a]	GATTCAAAACTGTTAACTCA	1848	94^{o}C, 60s;	Cookson
lpR		AGCCTTAATCTCAGTAATGCT		55^{o}C, 60s;	*et al.*
				72^{o}C, 120s.	(2007)
orfU	*eae*[b]	TATGATGATCTATGGCGTCTGT	3.7 Kb	94^{o}C, 30 s,	Oswald
escD		TATTTTCAAAAAGAATGATGTC		48 ^{o}C, 60s;	*et al.*
				72^{o}C, 120s.	2000
$\alpha 1F$	$\alpha 1$	AAAACCGCGGAGATGACTTC	820	94^{o}C, 60s;	Blanco
$\alpha 1R$		CACTCTTCGCATCTTGAGCT		60^{o}C, 60s;	*et al.*
				72^{o}C, 60s.	(2004c)
$\alpha 2F$	$\alpha 2$	AGACCTTAGGTACATTAAGTAAGC	517	94^{o}C, 60s;	Blanco
$\alpha 2R$		TCCTGAGAAGAGGGTAATC		60^{o}C, 60s;	*et al.*
				72^{o}C, 60 s	(2004c)
$\beta 1F$	$\beta 1$	ACTTCGCCACTTAATGCCAGC	730	94^{o}C, 60s;	Blanco
$\beta 1R$		TTGCAGCACCCCATGTTGAAT		65^{o}C, 60s;	*et al.*
				72^{o}C, 60s.	(2004c)
$\beta 2F$	β2B/	AAGGGGGGAACCCCTGTGTCA	604	94^{o}C, 60s;	Blanco
$\beta 2R$	ξ	ATTTATTCGCAGCCCCCCACG		62^{o}C, 60s;	*et al.*
				72^{o}C, 60s.	(2004c)
$\gamma 1F$	$\gamma 1$	AAAACCGCGGAGATGACTTC	804	94^{o}C, 60s;	Blanco
$\gamma 1R$		AGAACGCTGCTCACTAGATGTC		63^{o}C, 60s;	*et al.*
				72^{o}C, 60s.	(2004c)
$\gamma 2F$	$\gamma 2$/	AGAACGTTACTGGTGACTTA	414	94^{o}C, 60s;	Blanco
$\gamma 2R$	θ	CTGATATTTTATCAGCTTCA		50^{o}C, 60s;	*et al.*
				72^{o}C, 60s.	(2004c)

*Extension at 72 °C for 5 min completed the amplification process.

[a]Primers targeted a portion of the conserved 3'-end of the gene

[b]Primers targeted the structural gene

5.2.3.3 Optimisation and identification of *eae* gene variant for use as a control

Despite the authors's best efforts to obtain control strains, none were forthcoming. Therefore, the *eae* gene of STEC O157:H7, used in the diagnostic laboratory as a control for serotyping putative O157:H7 strains, was characterised for use as a control. PCR *eae*-related products were not obtained using primers for the detection of 6 *eae* subtypes $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2\text{B}/\xi$, $\gamma 1$ and $\gamma 2/\theta$ as shown in (Table 5.2). However, using PCR-RFLP the *eae* gene in this strain was identified as *eae*-$\gamma 1$ [5.3.4.1].

Since *eae* is frequently prevalent in EHEC O111, PCR amplifications were carried from O111 from Bb1i as template. Amplicons were obtained only with $\gamma 2F/\gamma 2R$ (Blanco *et al.* 2004c), indicating the presence of *eae*-$\gamma 2/\theta$. The amplicon was purified [2.2.14.3] and sequenced [2.2.14.4]. Analysis of the sequencing data confirmed the PCR product as a portion of *eae*-$\gamma 2/\theta$. Hereafter, serogroup O111 from Bb1i was used as positive control strain for the detection of *eae*-$\gamma 2/\theta$.

5.2.4 PCR-Restriction fragment length polymorphism (PCR-RFLP)

PCR amplification was carried out using primers *lpF*/*lpR* (Cookson *et al.* 2007) and amplification conditions shown in Table 5.2. The pair of primers delineated a fragment of 1848 bp and RFLP analysis of the fragments (Jenkins *et al.* 2003a; Cookson *et al.* 2007) was used to identify the *eae* types.

Between 200-400 ηg of the PCR product was digested with 5U of *Hae*III or *Hha*I in separate experiments in the recommended buffer (New England Biolabs, Cape Town South Africa), at 37^{o}C for 3 hours. Electrophoresis was carried out as previously described using 2.5% agarose gel [2.2.14.2]. Digital images were captured and stored [2.2.14.2]. The patterns generated were compared with the sequences of 18 intimin subtypes obtained from the Genbank (http://www.ncbi.nlm.nih.gov/) and aligned using DNAMAN (Lynnon Biosoft v4.0.0.1) sequence alignment editor. The *Hha*I and *Hae*III RFLP patterns for the different *eae* types were predicted from restriction fragments of the corresponding 1848bp nucleotide sequences (Jenkins *et al.* 2003; Cookson *et al.* 2007) generated by DNAMAN (Appendix E).

5.2.5 Southern hybridisation

Where necessary Southern hybridisation was carried out (Southern, 1975). All protocols, buffers, and reagents used were according to the instruction manual (Amersham International).

DNA probes were prepared by PCR and labeled DNA probes were labeled using the ECL® direct nucleic acid labeling and detection systems kit (Amersham International). Pre-hybridisation was carried out at 42^{o}C for one hour, followed by hybridisation at 42 °C with 50% formamide (14-16 hours) and subsequent washes at 55^{o}C, according to the instruction manual. Hybridisation was detected using alkalinephosphatase-labeled anti-digoxigenin monoclonal antibody with the ECL^{TM} direct nucleic acid labeling and detection systems (Amersham International).

5.3 Results

5.3.1 Production of Stx protein by clinical STEC strains

The production of Stx was investigated using Duopath Verotoxin detection kits (Merck KgaA, Darmstadt, Germany) [5.2.2]. The minimal detectable quantities for Stx1 and Stx2 of 25ηg/ml and 62.5ηg/ml, respectively were recorded. Expression of Stx1 and Stx2 was detected in O157:H7. As expected, no expression was detected from *E. coli* K-12.

Of the 25 STEC strains from children, Stx1 or Stx2 or both Stx were produced in 21 isolates (Table 5.3). In addition, expression of Stx1 was detected in 7 of 10 strains containing only stx_1 and in all of the STEC (6 strains) containing this gene in combination with stx_2 (Table 5.3). Stx2 expression was detected in 13 of 15 STEC strains carrying stx_2, including 7 of 8 strains containing this gene alone (Table 5.3).

Table 5.3: Stx production by clinical STEC strains

Child	Sero group	Detected *stx* 1	*stx* 2	Stx 1	Stx 2	Child	Sero group	Detected *stx* 1	*stx* 2	Stx 1	Stx 2
Hh1	O74	+	+	+	+	Hh11	O141	-	+	-	+
Hh2	O76	+	+	+	+	Hh12	O166	+	-	+	-
Hh3/1	O22	-	+	-	+	Hh13i	NT	+	-	+	-
Hh3/2	O113	+	+	+	+	Hh13ii	O111	+	-	+	-
Hh4i	O113	+	-	-	-	Hh14i	NT	+	-	+	-
Hh4ii	O107	+	-	-	-	Hh14ii	NT	+	-	+	-
Hh4iii	O107	+	-	-	-	Hh15i	O142	+	+	+	+
Hh5	O78	+	+	+	+	Hh15ii	O142	+	+	+	+
Hh6	NT	-	+		+	Hh16i	O176	-	+	-	-
Hh7	O8	-	+	-	+	Hh16ii	NK	-	+	-	+
Hh8	O149	+	-	+	-	Hh17	O169	-	+	-	+
Hh9	NT	+	-	+	-	Hh18	O142	+	+	+	-
Hh10	O29	-	+	-	+						

stx, Shiga toxin gene detected by PCR; NK, serogroup unknown
Stx, Shiga toxin detected using Duopath Verotoxin detection kit

5.3.2 Stx production by STEC strains from the bovines and water

A similar approach [5.3.1] was used to investigate Stx expression in STEC from the bovines and water. The results are presented in Table 5.4. Nineteen of 24 bovine STEC produced Stx1 or Stx2 or both toxins (Table 5.4). Expression of Stx1 and Stx2 was detected in 7 of 9 strains containing both *stx* genes (Table 5.4). While expression of the respective toxin was detected in 9 of 12 strains containing stx_1 and in two of the three strains with stx_2 (Table 5.4). Similarly, toxin expression was detected in 2 of the 5 STEC strains from water (Table 5.4).

Table 5.4: Stx production by STEC strains from the bovines and water

Bovine	Sero group	Detected *stx* 1	*stx* 2	Stx 1	Stx 2	Bovine	Sero group	Detected *stx* 1	*stx* 2	Stx 1	Stx 2
Bb1i	O111	+	+	+	+	Bb11	O142	+	-	+	-
Bb1ii	Poly9	-	+	-	+	Bb12	O76	+	-	+	-
Bb2	NT	+	-	+	-	Bb13	NT	+	-	+	-
Bb3	O76	+	+	+	+	Bb14i	NT	+	-	-	-
Bb4	O28ac	+	+	+	+	Bb14ii	O158	+	-	-	-
Bb5	O8	+	+	+	+	Bb14iii	O76	+	+	+	+
Bb6/1	O8	+	+	+	+	Bb14iv	O142	+	-	-	-
Bb6/2	O8	-	+	-	+	Bb15i	O76	+	-	+	-
Bb7^a	O113	+	+	+	-	Bb15ii	O76	+	-	+	-
Bb7^b	O113	+	+	+	+						
Bb8i	O8	+	+	-	-	Ww1	O166	-	+	-	+
Bb8ii	O107	-	+	-	-	Ww2i	O166	-	+	-	-
Bb9	O113	+	-	+	-	Ww2ii	O166	-	+	-	+
Bb10i	NT	+	-	+	-	Ww2iii	NK	+	-	-	-
Bb10ii	O20	+	-	+	-	Ww3	NT	+	-	-	-

stx, Shiga toxin gene detected by PCR; NK, serogroup unknown
Stx, Shiga toxin detected using Duopath Verotoxin detection kit
Bb, bovine strains; Ww, water strains; Bb7^a, SF$^-$; Bb7^b, SF$^+$ strains

5.3.3 Characterisation of *st*x_2 genes

Certain *stx*$_2$ variants are associated with severe sequelae following infection with STEC; thus, the identities of the *stx*$_2$ genes were more precisely determined. PCR assays were carried out with DNA released by CTAB method [2.2.13]. Amplification conditions and the primers used are shown in Table 5.1.

5.3.3.1 *st*x_2 variants in clinical STEC

An amplification product of 1379 bp with primers stv1F/stv1R was obtained from O157:H7 suggesting one of a number of variants in O157:H7 (Table 5.1). Using primers stv1F/stv3R a 1366 bp amplicon was obtained from STEC O111, suggestive of stx_{2-vhc} (Table 5.1). As expected, no PCR product was obtained with *E. coli* K-12.

Fifteen clinical strains contained either stx_2 alone or in combination with stx_1. Appropriately sized amplicons were obtained with primers stv1F/stv3R with 8 STEC from Hh1, Hh2, Hh3/1, Hh3/2, Hh5, Hh6, Hh7 and Hh11, indicating stx_{2-vhc} in these strains (Table 5.5). All the amplicons were sequenced and confirmed as stx_{2-vhc}.

Table 5.5: stx_1 and variants of stx_2 carried by clinical STEC strains

	Detected *stx* genes			Detected *stx* genes	
Child	stx_2 variant	stx_1	Child	stx_2 variant	stx_1
Hh1	stx_{2-vhc}	+	Hh11	stx_{2-vhc}	-
Hh2	stx_{2-vhc}, stx_{2d-2}	+	Hh12	-	+
Hh3/1	stx_{2-vhc}	-	Hh13i	-	+
Hh3/2	stx_{2-vhc}	+	Hh13ii	-	+
Hh4i	-	+	Hh14i	-	+
Hh4ii	-	+	Hh14ii	-	+
Hh4iii	-	+	Hh15i	$stx_{2-EDL993}$	+
Hh5	$stx_{2-EDL993}$, stx_{2-vhc}	-	Hh15ii	stx_{2c}	+
Hh6	stx_{2-vhc}	-	Hh16i	stx_{2d-2}	-
Hh7	$stx_{2-EDL993}$,stx_{2-vhc}	-	Hh16ii	stx_{2d-2}	-
Hh8	-	+	Hh17	stx_{2c}	-
Hh9	-	+	Hh18	stx_{2c}	+
Hh10	$stx_{2-EDL993}$	-			

+, gene detected; -, gene not detected

Products were obtained from 10 STEC using primers stv1F/stv1R. As these primers are not variant specific it was necessary to sequence the amplicons to ascertain their identities. Analysis of the sequencing data identified 4 amplicons as $stx_{2-EDL993}$ and 3 amplicons each as stx_{2c} and stx_{2-d2} (Table 5.5).

In summary, stx_{2-vhc} was the commonest variant identified (8 strains) and 3 STEC from Hh2, Hh5 and Hh7 contained 2 stx_2 variants (Table 5.5). Additionally, the STEC from 6 children (Hh1, Hh2, Hh3, Hh5, Hh15 and Hh18) carried stx_1 (Table 5.5).

5.3.3.2 *st*x_2 gene variants in STEC from bovines and water

Using a similar approach [5.3.3], variants of stx_2 were determined in the bovine and water STEC strains.

Twelve of the 24 bovine strains carried stx_2 alone or in combination with stx_1. Using primers stv1F/stv1R amplicons of the expected sizes were obtained from 10 bovine isolates. Sequencing of the amplicons identified them as stx_{2-d2} (7 amplicons) and $stx_{2-EDL993}$ (3 amplicons) as shown (Table 5.6).

Table 5.6: stx_1 and variants of stx_2 in STEC strains from the bovines and water

	Detected *stx* genes			Detected *stx* genes	
Bovine	stx_2 variant	stx_1	Bovine/water	stx_2 variant	stx_1
Bb1i	$stx_{2-EDL993}$, stx_{2-vhc}	+	Bb11	-	+
Bb1ii	stx_{2-vhc}	-	Bb12	-	+
Bb2	-	+	Bb13	-	+
Bb3	$stx_{2-EDL993}$, stx_{2-vhc}	+	Bb14i	-	+
Bb4	stx_{2d-2}, stx_{2-vhc}	+	Bb14ii	-	+
Bb5	stx_{2d-2}, stx_{2-vhc}	+	Bb14iii	stx_{2d-2}	+
Bb6/1	stx_{2d-2}, stx_{2-vhc}	+	Bb14iv	-	+
Bb6/2	$stx_{2-EDL993}$	-	Bb15i	-	+
Bb7^a	stx_{2d-2}	+	Bb15ii	-	+
Bb7^b	stx_{2d-2}, stx_{2-vhc}	+			
Bb8i	stx_{2d-2}	+	Ww1	stx_{2-vhc}	-
Bb8ii	stx_{2-vhc}	-	Ww2i	stx_{2-vhc}	-
Bb9	-	+	Ww2ii	stx_{2-vhc}	-
Bb10i	-	+	Ww2iii	stx_{2-vhc}	-
Bb10ii	-	+	Ww3		+

Bb, STEC from the bovines; Ww, STEC from water

Bb7^a, SF$^-$; Bb7^b, SF$^+$ strains; +, gene detected; -, gene not detected

When primers stv1F/stv3R were included in the reaction, amplicons were obtained from 8 STEC, indicating the presence of stx_{2-vhc} in these strains, which was confirmed by sequencing.

In summary, STEC containing stx_{2-vhc} alone (2 strains) or in combination with other variants, primarily stx_{2-d2}, were most common in the bovine STEC. Additionally, all except 3 of the strains, from Bb1ii, Bb6/2 and Bb8ii, contained stx_1 (Table 5.6).

With respect to the 4 water STEC isolates: amplicons were obtained only with primers stv1F/stv3R, indicating the presence of stx_{2-vhc} which was confirmed by sequencing (Table 5.6).

5.3.4 Detection and characterisation of *eae* genes

As a first step to understanding the *eae* gene content of the STEC, PCR assays [5.2.3.2] were carried out to detect this gene using universal primers UF/UR (Mora *et al.* 2007) (Table 5.2). Subsequently, *eae* types were determined using combinations of PCR and hybridization studies [5.2.5], and PCR-RFLP investigations [5.2.4].

5.3.4.1 *eae* genes in clinical STEC

Using universal primers UF/UR (Mora *et al.* 2007) (Table 5.2) PCR products of anticipated size (346 bp) were obtained with strains from 6 of 10 children representing phase 1, and 9 of 15 children from phase 2 (Table 5.7). An appropriately sized product was obtained with O157:H7, no PCR product was obtained with *E. coli* K-12.

Table 5.7: *eae*-containing clinical isolates from phases 1 and 2

Phase 1		Phase 2			
Child	*eae* gene	Child	*eae* gene	Child	*eae* gene
Hh1	-	Hh8	-	Hh15ii	+
Hh2	+	Hh9	+	Hh16i	-
Hh3/1	+	Hh10	-	Hh16ii	-
Hh3/2	+	Hh11	-	Hh17	-
Hh4i	+	Hh12	+	Hh18	+
Hh4ii	-	Hh13i	+		
Hh4iii	-	Hh13ii	+		
Hh5	+	Hh14i	+		
Hh6	+	Hh14ii	+		
Hh7	-	Hh15i	+		

+, *eae* gene detected; -, no *eae* gene detected

To further characterise the *eae* genes of the 6 STEC from phase 1, PCR assays were carried out using sets of primers for the detection of variants $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2\text{B}/\xi$, $\gamma 1$ and $\gamma 2/\theta$ (Table 5.2). Products were not obtained with *E. coli* K-12. As expected, using $\gamma 2F/\gamma 2R$ (Blanco *et al.* 2004c), for the detection of $\gamma 2/\theta$, products were obtained with O111 from Bb1 [5.2.3.2]. These primers ($\gamma 2F/\gamma 2R$) were extended in 4 of the 6 STEC, indicating *eae*-$\gamma 2/\theta$ in strains from Hh2, Hh3/1, Hh3/2 and Hh6 (Table 5.8).

Table 5.8: *eae* genes in clinical STEC from phase 1

Child	Serogroup	*eae* gene	Child	Serogroup	*eae* gene
Hh1	O74	-	Hh4ii	O107	-
Hh2	O76	$\gamma 2/\theta$	Hh4iii	O107	-
Hh3/1	O22	$\gamma 2/\theta$	Hh5	O78	*eae**
Hh3/2	O113	$\gamma 2/\theta$	Hh6	NT	$\gamma 2/\theta$
Hh4i	O113	*eae**	Hh7	O8	-

*eae**, unknown *eae* gene type; -, *eae* was not detected

To confirm the identification of the products from the clinical isolates hybridisation studies [5.2.5] were carried out using the PCR product from O111 (Bb1i), which was

identified as *eae*-$\gamma 2/\theta$ by DNA sequencing, as a probe [5.2.5]. Further, hybridisation studies were possible because there is only a 65% nucleotide similarity between *eae*-$\gamma 2/\theta$ and the corresponding regions of the other variants. The probe hybridised to each of the products from 4 STEC, confirming *eae*-$\gamma 2/\theta$ in these strains (Table 5.8 and Figure 5.1).

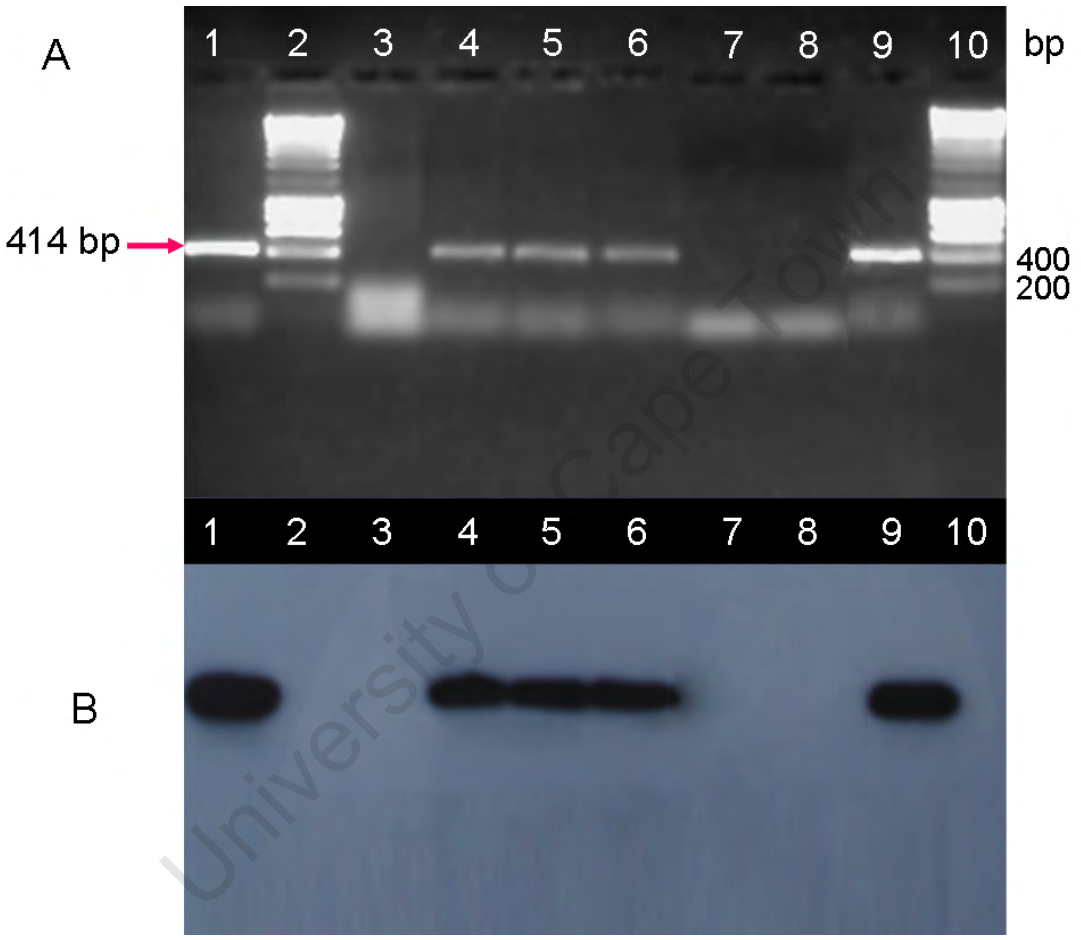

Figure 5.1: Identification of *eae*-$\gamma 2/\theta$ in clinical STEC (phase 1).
A: Agarose gel electrophoresis following PCR using primers for the detection of *eae*-$\gamma 2/\theta$. The presence of *eae*-$\gamma 2/\theta$ is indicated by the presence of 414bp fragments. STEC O111 was included as a positive control. Lane 1, O111; lanes 2 and 10, HyperLadder I (Bioline); lane 3, no DNA; lane 4, Hh2; lane 5, Hh3/1; lane 6, Hh3/2; lane 7, Hh4i; lane 8, Hh5; lane 9, Hh6.

B: Autoradiograph of DNA shown in A probed with a portion of *eae*-$\gamma 2/\theta$ prepared from O111. Lane 1, O111; lanes 2, 3, 10, no DNA; lane 4, Hh2; lane 5, Hh3/1; lane 6, Hh3/2; lane 7, Hh4i; lane 8, Hh5; lane 9, Hh6.

PCR-RFLP was used to characterise the *eae* genes of the 9 *eae*-positive clinical STEC (phase 2) and the corresponding genes from the 2 STEC (phase 1) which were not identified in the previous experiments. Using primers *lpF*/*lpR* (Cookson *et al.* 2007)(Table 5.2) products of the expected size (1848 bp) were obtained with STEC O157:H7.

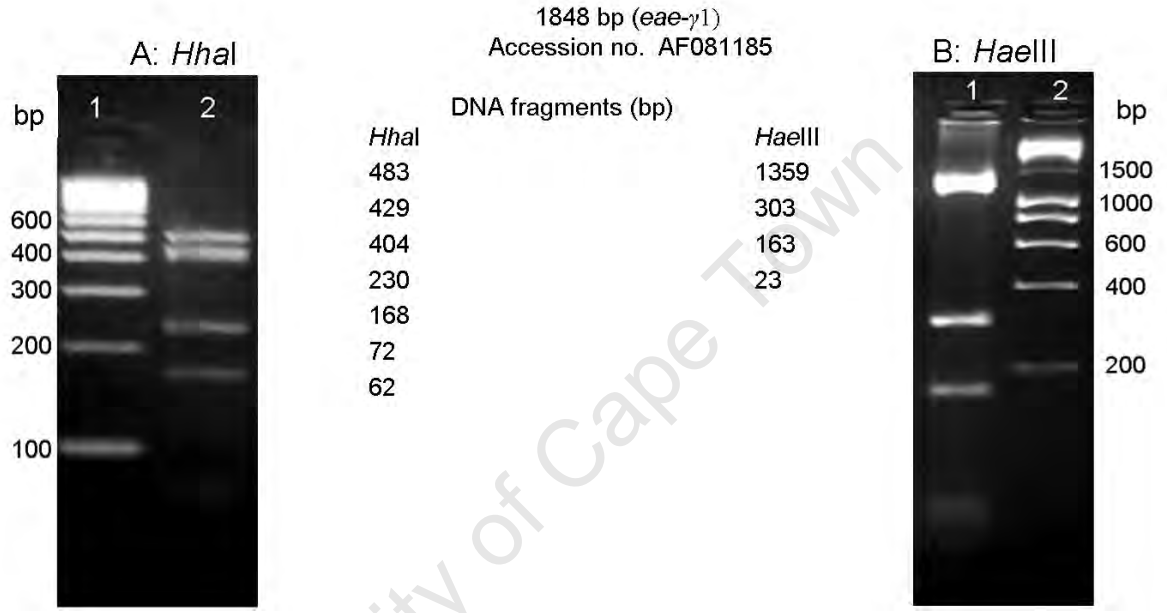

Figure 5.2: *Hha*I and *Hae*III restriction patterns of 1848 bp PCR product from O157:H7.
A: Restriction digest with *Hha*I. Lane 1, HyperLadder IV (Bioline); lane 2, 1848 bp fragment from O157:H7. The DNA fragments 72 168 230 408 429 483 bp are consistent with *eae*-γ1.

B: Restriction digest with *Hae*III. Lane 1, 1848 bp fragment from O157:H7; lane 2, HyperLadder I (Bioline). The DNA fragments 23 163 303 1359 bp are consistent with *eae*-γ1.

The amplicons were digested with *Hha*I or *Hae*III in separate experiments. The subsequent restriction patterns were compared with those of known *eae*-types (Appendix E). The *Hha*I and *Hae*III patterns of the amplicons from O157:H7 were consistent with those of *eae* γ1 (Figure 5.2, Table 5.9).

Similarly, the *Hha*I and *Hae*III patterns of the amplicons from STEC O111 (B1i) were consistent with those of *eae* $\gamma 2/\theta$ (Figure 5.3, Table 5.9).

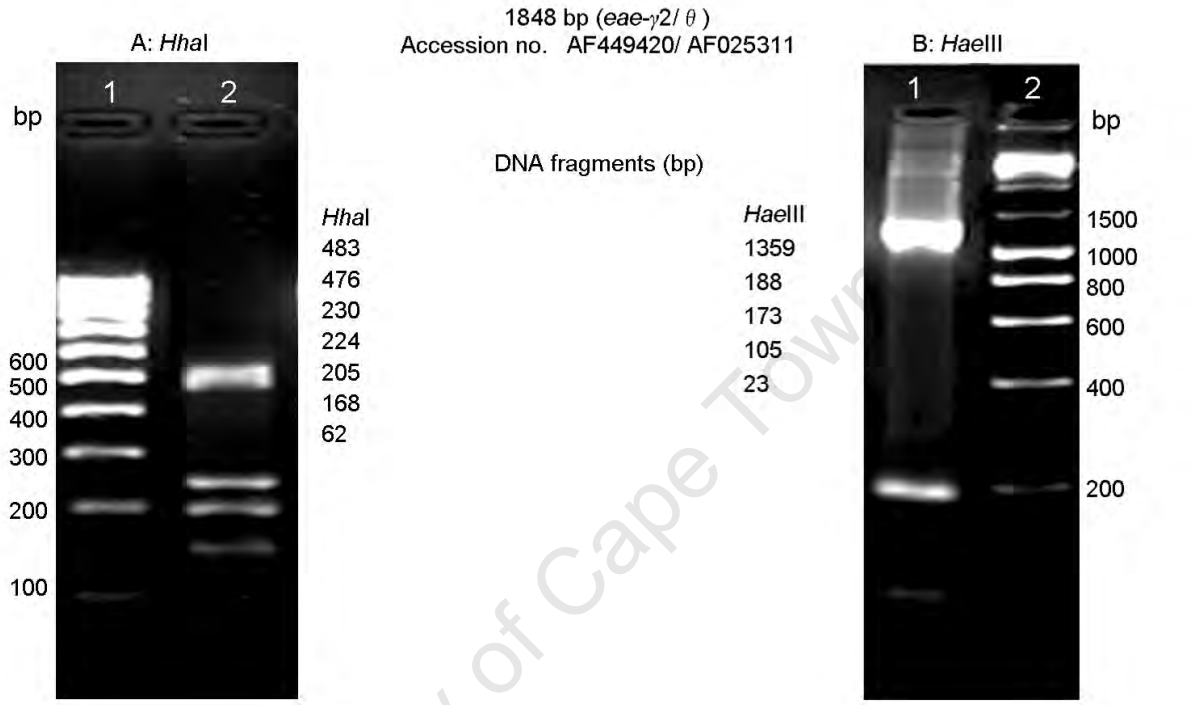

Figure 5.3: *Hha*I and *Hae*III restriction patterns of 1848 bp PCR product from STEC O111 (Bb1i).
A: Restriction digest with *Hha*I. Lane 1, HyperLadder IV (Bioline); lane 2, 1848 bp fragment from STEC O111. The DNA fragments 62 168 205 224 230 476 483 bp are consistent with *eae*-$\gamma 2/\theta$.

B: Restriction digest with *Hae*III. Lane 1, 1848 bp fragment from O111; lane 2, HyperLadder I (Bioline). The DNA fragments 23 105 173 188 1359 bp are consistent with *eae*-$\gamma 2/\theta$.

Products were obtained with each of the 9 clinical STEC from phase 2, and with the 2 STEC from phase 1. The restriction patterns of the 9 clinical STEC from phase 2 are presented in Figure 5.4, Table 5.9.

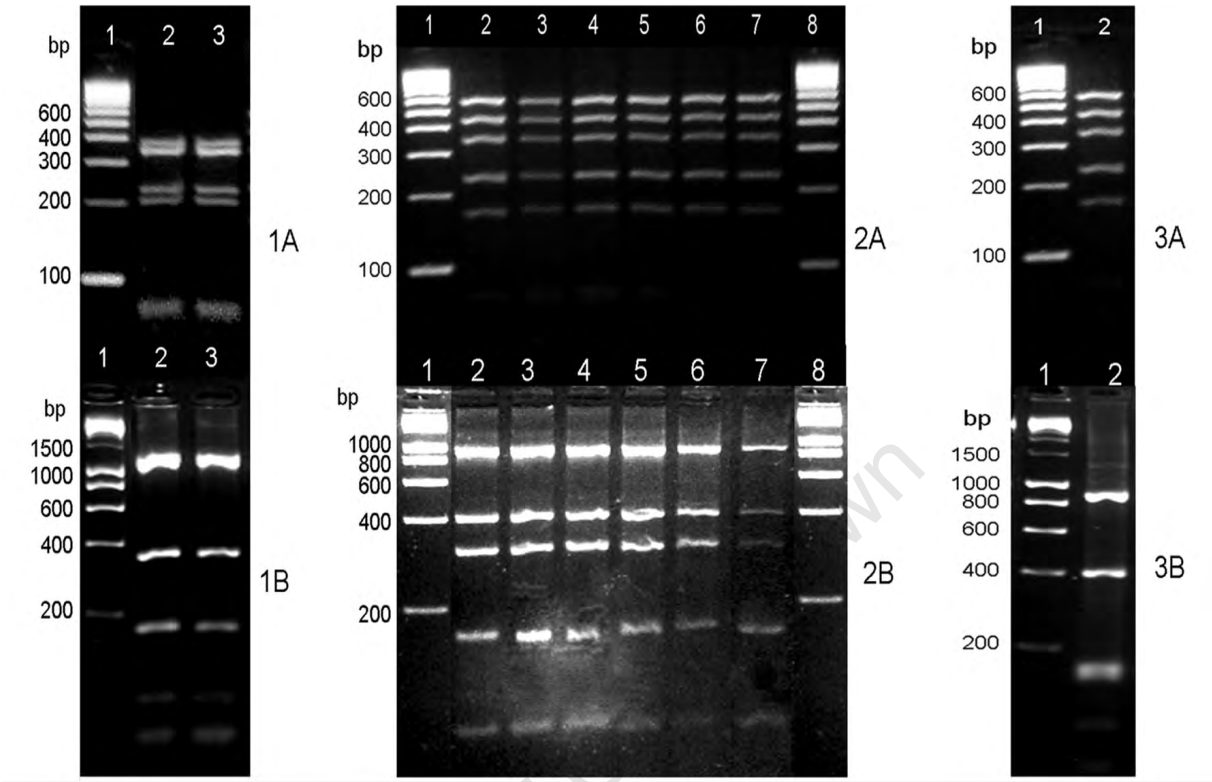

Figure 5.4: RFLP of *Hha*I and *Hae*III digests of PCR products from 9 STEC (phase 2).
1A: *Hha*I; 1B: *Hae*III. Lane 1, HyperLadder IV (Bioline) (1A) or HyperLadder I (Bioline) (1B); lane 2, Hh13i; lane 3, Hh13ii. RFLPs are consistent with *eae*-β1.

2A: *Hha*I; 2B: *Hae*III. Lanes 1, 8, HyperLadder IV (Bioline) (2A) or HyperLadder I (Bioline) (2B); lane 2, Hh12; lane 3, Hh14i; lane 4, Hh14ii; lane 5, Hh15i; lane 6, Hh15ii; lane 7, Hh18. RFLPs are consistent with *eae*-κ.

3A: *Hha*I; 3B: *Hae*III. Lane 1, HyperLadder IV (Bioline) (3A) or HyperLadder I (Bioline) (3B); lane 2, Hh9. RFLPs are consistent with eae-μ.

Table 5.9: *Hha*I and *Hae*III digests of 1848 bp portions of selected *eae* genes

eae	Accession no.	*Hha*I	*Hae*III
γ1	AF081185	62 72 168 230 404 429 483	23 163 303 1359
γ2/ θ	AF449420/ AF025311	62 168 205 224 230 476 483	23 105 173 188 1359
β1	AF453441	48 49 62 72 78 205 230 354 357 392	22 30 75 172 361 1187
κ/ δ	AJ308552/ AJ875027	63 72 168 230 341 429 545	23 83 163 303 399 877
μ	AJ705049	63 72 168 230 341 429 545	23 83 147 156 163 399 877

Additionally, the *HhaI* and *Hae*III patterns of the amplicons from 2 STEC (phase 1) identified them as *eae*-β1.

5.3.4.2 *eae* genes in STEC from water and bovines

Using the approach [5.3.4.1] to identify *eae* types in STEC from phase 2 children, PCR products were obtained in 3 of the 5 STEC from water, with primers *UF*/*UR* (Mora *et al.* 2007) and with 2 of these STEC using *lpF*/*lpR* (Cookson *et al.* 2007). The *Hha*I and *Hae*III RFLPs of the *lpF*/*lpR* (Cookson *et al.* 2007) generated 1848 bp product were consistent with those of eae-β1 (Table 5.10).

Using primers *orfU* and *escD* (Oswald *et al.* 2000) that anneal to sequences flanking *eae* (Table 5.2), no PCR product was obtained with the remaining *eae*-positive strain. The *eae* in this strain from Ww2iii was recorded as untypeable (Table 5.10).

Table 5.10: Detection of *eae* in bovine and water STEC strains

Bovine	*eae* gene	Bovine	*eae* gene	Bovine/water	*eae* gene
Bb1i	$\gamma2/\theta$	Bb8i	-	Bb14iii	$\gamma2/\theta$
Bb1ii	$\gamma2/\theta$	Bb8ii	-	Bb14iv	$\gamma2/\theta$
Bb2	$\gamma2/\theta$	Bb9	$\gamma2/\theta$	Bb15i	$\beta1$
Bb3	-	Bb10i	-	Bb15ii	$\gamma2/\theta$
Bb4	$\gamma2/\theta$	Bb10ii	-		
Bb5	$\gamma2/\theta$	Bb11	-	Ww1	$\beta1$
Bb6/1	*eae*	Bb12	$\gamma2/\theta$	Ww2i	$\beta1$
Bb6/2	-	Bb13	-	Ww2ii	-
Bb7[a]	-	Bb14i	-	Ww2iii	*eae*
Bb7[b]	$\gamma2/\theta$	Bb14ii	$\gamma2/\theta$	Ww3	-

Bb, bovine; Ww, water; *eae*, untypeable *eae* type; -, no *eae* detected
Bb7.1[a] (SF^+) and Bb7.2[b](SF^-) strains

Twenty four STEC were isolated from 15 bovines. Primers *UF*/*UR* (Table 5.2) detected *eae* in 14 strains. Using primers specific for variants $\alpha1$, $\alpha2$, $\beta1$, $\beta2B/\xi$, $\gamma1$ and $\gamma2/\theta$ products (414 bp) were obtained from 12 strains, suggesting the presence of $\gamma2/\theta$ in these strains. As in the previous study [5.3.4.1] the PCR products were hybridised to a $\gamma2/\theta$ probe prepared from O111 (Bb1i) [5.2.5].

A signal was obtained with each of the products, confirming the presence of $\gamma 2/\theta$ in the 12 strains (Table 5.10, Figure 5.5).

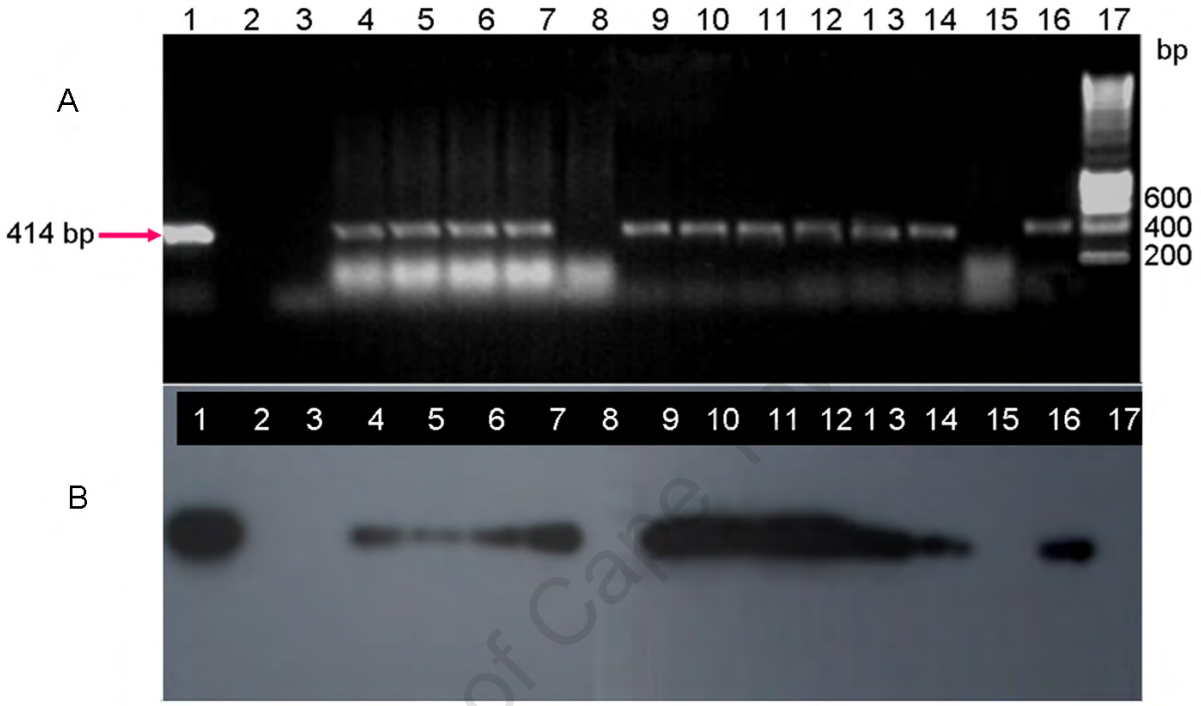

Figure 5.5: Identification of *eae*-$\gamma 2/\theta$ in bovine STEC.
A: Agarose gel electrophoresis following PCR using primers for detection of *eae*-$\gamma 2/\theta$. The presence of *eae*-$\gamma 2/\theta$ is indicated by the presence of 414bp fragments. STEC O111 was included as positive control strain. Lane 1, STEC O111; lane 2, blank; lane 3, no DNA; lane 4, Bb1ii; lane 5, Bb2; lane 6, Bb4; lane 7, Bb5; lane 8, Bb6/1; lane 9, $Bb7^b$; lane 10, Bb9; lane 11, Bb12; lane 12, Bb14ii; lane 13, Bb14iii; lane 14, Bb14iv; lane 15, Bb15i; lane 16, Bb15ii; lane 17, HyperLadder I (Bioline).

B: Autoradiograph of DNA shown in A probed with a portion of *eae*-$\gamma 2/\theta$ prepared from O111. Lane 1, Bb1i-O111; lanes 2, 3, 17, no DNA; lane 4, Bb1i; lane 5, Bb2; lane 6, Bb4; lane 7, Bb5; lane 8, Bb6/1; lane 9, $Bb7^b$; lane 10, Bb9; lane 11, Bb12; lane 12, Bb14ii; lane 13, 14.3; lane 14, Bb14iv; lane 15, Bb15i; lane 16, Bb15ii.

The *eae* genes of unknown type from the 2 bovine STEC (Bb6/1 and Bb15i) were characterised further using PCR-RFLP [5.3.4.1]. A PCR product was obtained with only one of the 2 STEC with primers *lpF*/*lpR* (Table 5.2). Restriction digest of the 1848 bp with *Hha*I and *Hae*III generated patterns consistent with *eae*-$\beta 1$ (Table 5.10).

Using primers *orfU* and *escD* (Table 5.2), no PCR product was obtained with the remaining *eae*-positive strain (Bb6/1), which was recorded as untypeable *eae* (Table 5.10).

5.3.5 Profiles of STEC

The data with respect to virulence markers, expression of Stx and serogroups of STEC was collated and their profiles are presented in Table 5.11 and Table 5.12.

Table 5.11: Presence and expression of *stx* genes and carriage of *eae* genes by clinical STEC

		Detected virulence marker				
Child	Serogroup	stx_2 type	Stx2	stx_1	Stx1	*eae*
Hh1	O74	stx_{2-vhc}	+	+	+	-
Hh2	O76	stx_{2-vhc}, stx_{2d-2}	+	+	+	$\gamma 2/\theta$
Hh3/1	O22	stx_{2-vhc}	+	-	-	$\gamma 2/\theta$
Hh3/2	O113	stx_{2-vhc}	+	+	+	$\gamma 2/\theta$
Hh4i	O113	-	-	+	-	$\beta 1$
Hh4ii	O107	-	-	+	-	-
Hh4iii	O107	-	-	+	-	-
Hh5	O78	$stx_{2-EDL993}$, stx_{2-vhc}	+	+	+	$\beta 1$
Hh6	NT	stx_{2-vhc}	+	-	-	$\gamma 2/\theta$
Hh7	O8	$stx_{2-EDL993}$,stx_{2-vhc}	+	-	-	-
Hh8	O149	-	-	+	+	-
Hh9	NT	-	-	+	+	μ
Hh10	O29	$stx_{2-EDL993}$	+	-	-	-
Hh11	O141	stx_{2-vhc}	+	-	-	-
Hh12	O166	-	-	+	+	κ/δ
Hh13i	NT	-	-	+	+	$\beta 1$
Hh13ii	O111	-	-	+	+	$\beta 1$
Hh14i	NT	-	-	+	+	κ/δ
Hh14ii	NT	-	-	+	+	κ/δ
Hh15i	O142	$stx_{2-EDL993}$	+	+	+	κ/δ
Hh15ii	O142	stx_{2c}	+	+	+	κ/δ
Hh16i	O176	stx_{2d-2}	-	-	-	-
Hh16ii	NK	stx_{2d-2}	+	-	-	-
Hh17	O169	stx_{2c}	+	-	-	-
Hh18	O142	stx_{2c}	-	+	+	κ/δ

NK, serogroup not established; Stx1, Shiga toxin 1; Stx2, Shiga toxin 2

Table 5.12: Presence and expression of *stx* genes and carriage of *eae* genes in bovine and water STEC

		Detected virulence marker				
Bovine/water	Serogroup	stx_2 type	Stx2	stx_1	Stx1	*eae*
Bb1i	O111	$stx_{2-EDL993}$, stx_{2-vhc}	+	+	+	$\gamma 2/\theta$
Bb1ii	Poly9	stx_{2-vhc}	+	-	-	$\gamma 2/\theta$
Bb2	NT		-	+	+	$\gamma 2/\theta$
Bb3	O76	$stx_{2-EDL993}$, stx_{2-vhc}	+	+	+	-
Bb4	O28ac	stx_{2d-2}, stx_{2-vhc}	+	+	+	$\gamma 2/\theta$
Bb5	O8	stx_{2d-2}, stx_{2-vhc}	+	+	+	$\gamma 2/\theta$
Bb6/1	O8	stx_{2d-2}, stx_{2-vhc}	+	+	+	*eae**
Bb6/2	O8	$stx_{2-EDL993}$	+	-	-	-
Bb7[a]	O113	stx_{2d-2}	-	+	+	-
Bb7[b]	O113	stx_{2d-2}, stx_{2-vhc}	+	+	+	$\gamma 2/\theta$
Bb8i	O8	stx_{2d-2}	-	+	-	-
Bb8ii	O107	stx_{2-vhc}	-	-	-	-
Bb9	O113	-	-	+	+	$\gamma 2/\theta$
Bb10i	NT	-	-	+	+	-
Bb10ii	O20	-	-	+	+	-
Bb11	O142	-	-	+	+	-
Bb12	O76	-	-	+	+	$\gamma 2/\theta$
Bb13	NT	-	-	+	+	-
Bb14i	NT	-	-	+	-	-
Bb14ii	O158	-	-	+	-	$\gamma 2/\theta$
Bb14iii	O76	stx_{2d-2}	+	+	+	$\gamma 2/\theta$
Bb14iv	O142	-	-	+	-	$\gamma 2/\theta$
Bb15i	O76	-	-	+	+	$\beta 1$
Bb15ii	O76	-	-	+	+	$\gamma 2/\theta$
Ww1	O166	stx_{2-vhc}	+	-	-	$\beta 1$
Ww2i	O166	stx_{2-vhc}	-	-	-	$\beta 1$
Ww2ii	O166	stx_{2-vhc}	+	-	-	-
Ww2iii	NK	stx_{2-vhc}	-	-	-	*eae**
Ww3	NT	-	-	+	-	-

Bb and Ww, STEC from bovines and water, respectively

Bb7[a], SF^-; Bb7[b], SF^+ strains; NK, serogroup not established

*eae**; untypeable eae gene; Stx1, Shiga toxin 1; Stx2, Shiga toxin 2

5.4 Discussion

Shiga toxins are the major virulence determinants of STEC associated with human illness, albeit in conjunction with the products of other genes such as *eae*. Essentially, Stx are the primary cause of the clinical signs associated with STEC infection. To induce diarrhoea, Stx may cause direct injury to the intestinal mucosa (O'Loughlin *et al.* 2001) or to the underlying intestinal capillaries (Sjogren *et al.* 1994). A majority of human and bovine STEC strains in this study expressed Stx, a result which was similar to findings reported in previous studies (Beutin *et al.* 2004; Zhang *et al.* 2005). However, expression was not detected in a few strains including members of serogroup O107. It is possible that in these strains the *stx* genes are located on non-inducible defective phages (Zhang *et al.* 2005). Alternatively, the genetic background of the Stx-negative strains does not enhance the expression of *stx* genes (Escobar-Paramo *et al.* 2004).

Systematic analysis of *stx* genes from the clinical STEC showed that 7 of the 10 strains which carried stx_1, expressed this gene (Table 5.11). Interestingly, among strains carrying stx_2, subtype stx_{2-vhc} was the most prevalent, carried by 8 of the 15 strains. This result contrasts the the findings of De Baets *et al.* (2004), who did not detect this variant from randomly selected clinical isolates in Belgium. Similarly, none of the clinical STEC from France contained stx_{2-vhc} (Pradel *et al.* 2001;2008). Thus the carriage of different stx_2 subtypes may be depend on the geographical location and STEC population.

STEC from serogroups O29, O149 and O176, not previously described in humans, contained $stx_{2-EDL993}$, stx_1 and stx_{2d-2}, respectively. While the pathogenicity of STEC with $stx_{2-EDL993}$ has been well described (Lindgren *et al.* 1993; Melton-Celsa *et al.* 1996; Friedrich *et al.* 2002), strains carrying stx_{2d-2} and possibly stx_{2-vhc} may be associated with moderate virulence (Friedrich *et al.* 2002) because both genes encode amino acid sequences which regulate mucous activation (De Baets *et al.* 2004). However, given the high disease burden, malnutrition and naive immunity among the rural poor children in Nyabushozi (Kikafunda *et al.* 1998; Mbonye, 2004), childhood infection with even the mildest form of STEC could present aggravated clinical signs. Therefore, strains carrying stx_{2-vhc} and stx_{2d-2} genes may be regarded

as moderately virulent and of public health importance in poor countries.

That the bovines contained more than a single copy of stx_2 was not surprising as a number of studies have identified more than one copy of this gene in bovine STEC (Schmitt *et al.* 1991; Bertin *et al.* 2001; Bielaszewska *et al.* 2006; Beutin *et al.* 2007). What was surprising, however, was that stx_{2-vhc}, rarely identified in cattle (De Baets *et al.* 2004), was the most frequent variant identified in the bovine STEC from a number of serogroups (Table 5.12). Of the variants identified in my study, $stx_{2-EDL993}$ was most prevalent in France (Bertin *et al.* 2001 and Australia (Brett *et al.* 2003a) and stx_{2d-2} was widespread among cattle from different locations (Bertin *et al.* 2001; Brett *et al.* 2003a; Beutin *et al.* 2007). Taken together these data probably indicate that different stx_2 genes are carried by STEC within specific ecological niches associated with distinct STEC populations.

A close association between stx_{2-vhc} and stx_{2d-2} observed among bovine STEC might indicate that the strains belong to a specific lineage of STEC (Girardeau *et al.* 2005). One study has shown that stx_{2d-2} genes are either not, or poorly expressed in both basal and induced conditions (de Sablet *et al.* 2008). Of the total STEC strains in my study, carrying this variant alone, Stx2 expression was not detected in 3 of the 5 strains. On the other hand, when stx_{2-vhc} was the sole variant, expression was detected in 8 of 11 strains. Expression of Stx2 was consistently detected in strains (5) containing both variants suggesting that the presence of stx_{2-vhc} may compensate for the poorly expressed stx_{2d-2} (Table 5.12).

The reasons for some STEC being more virulent than others are not fully understood; nevertheless, the link between the presence of *eae* in STEC and its ability to cause disease in humans has been well documented (Nataro and Kaper, 1998; Paton and Paton, 1998a; Donnenberg and Whittam, 2001; Karmali *et al.* 2003; Karch *et al.* 2005; Girardeau *et al.* 2005; Pradel *et al.* 2008). The observed high frequency of *eae*-positive isolates from the bovines (58.3%, 14 of 24) was unexpected, as previous studies have shown a much lower prevalence of *eae*-positive STEC in healthy cattle (Hornitzky *et al.* 2002; Blanco *et al.* 2004c; Caprioli *et al.* 2005). *eae*-$\gamma 2/\theta$ was the predominant subtype identified in the bovine STEC (10 of 14) comprising a variety of serogroups, indicating that it occurs in diverse serogroups. That this *eae* type is common among EHEC O111 strains (Beutin *et al.* 2004; Blanco *et al.* 2005)

suggests that it may confer a higher degree of virulence on serogroups containing this *eae* type.

Interestingly, 2 of the 3 members of serogroup O113 from bovines possessed $\gamma 2/\theta$. Strains belonging to this serogroup are prevalent in cattle from different regions (Kobayashi *et al.* 2003; Brett *et al.* 2003a), however, previously reported STEC O113 strains are typically *eae*-negative (Brett *et al.* 2003a; Kobayashi *et al.* 2003; Mellmann *et al.* 2005). Two of the O113 strains from Bb7^a and Bb7^b, which are SF$^-$ and SF$^+$ respectively, are closely related but possess different virulence genes. While, both shared stx_1 and stx_{2d-2}, the SF$^+$ STEC contained also stx_{2-vhc} and *eae*-$\gamma 2/\theta$. The close genetic relatedness of the 2 strains suggests that they diverged from a common ancestor and that *eae* was subsequently acquired by the SF$^+$ variant.

The detection of *eae* among STEC strains from the children revealed a high prevalence of *eae*-positive strains (60%; 15 of 25), and contrasts with results from other studies (Friedrich *et al.* 2002; Karch *et al.* 2005). It is noteworthy that strains of serogroups O28ac, O142 and O158 carried *eae*-$\gamma 2/\theta$. To the best of my knowledge this is the first report of *eae*-$\gamma 2/\theta$ in these serogroups. Previous studies have shown an association between severity of disease and the presence of *eae* in STEC (Boerlin *et al.* 1999; Eklund *et al.* 2002; Jenkins *et al.* 2003b), suggesting that non-EHEC strains containing this gene may be virulent.

A comparison of *eae*-subtypes carried by STEC in phase 1 and 2, showed distinct differences in *eae* types. Six of 9 phase 2 *eae*-positive strains possessed *eae*-κ/δ, 2 carried *eae*-$\beta 1$ and *eae*-μ was identified in one strain. None of 6 *eae*-positive strains in phase 1 carried *eae*-κ/δ, but *eae*-$\gamma 2/\theta$ and $\beta 1$ were present in 4 and 2 strains, respectively. The prevalence of *eae*-κ/δ in the clinical STEC (6 of the 15 strain) contrasts with the findings of a number of studies in which this gene was rarely isolated from clinical STEC in Europe (Oswald *et al.* 2000; Zhang *et al.* 2002b; Jenkins *et al.* 2003a). This may signify differences in populations and lineages of STEC. There is evidence that previously described *eae*-κ/δ genes have been associated with virulent O118 and O157:H7 strains (Zhang *et al.* 2002b), suggesting that this gene may be a marker of virulence. *eae*-$\beta 1$ genes are ubiquitous, having been identified in STEC from humans, animals and environmental sources (Oswald *et al.* 2000; Ramachandran *et al.* 2003). In this study STEC containing

this gene type were isolated from a bovine, children and water (Table 5.11 and Table 5.12). Several studies have suggested that *eae* type may indicate the phylogenetic origin of STEC (Adu-Bobie *et al.* 1998a; Mundy *et al.* 2007). The presence of *eae*-$\gamma 2/\theta$ among the majority of *eae*-positive STEC from phase 1 children and bovines might signal a phylogenetic relationship between the strains. Additionally, *eae*-$\gamma 2/\theta$ is closely associated with EHEC O111 (Oswald *et al.* 2000; Vaz *et al.* 2006) and has specific tissue tropism (Phillips *et al.* 2000; Fitzhenry *et al.* 2002), suggesting that STEC carrying this gene may have similar tissue tropism. Although previous studies have reported that *eae* subtypes show differential tissue tropism and host specificity (Phillips *et al.* 2000; Phillips and Frankel, 2000), no single *eae* type has been linked with HUS (Schmidt *et al.* 1999; Oswald *et al.* 2000).

Although *eae*-negative strains serogroup O113 are frequently associated with bloody diarrhoea and HUS in Australia and Germany (Elliott *et al.* 2001; Beutin *et al.* 2004), clinical strains carrying *eae*-$\gamma 2/\theta$ or *eae*-$\beta 1$ were isolated in my study. It may be that the *eae*-positive O113 strains have emerged from a distinct lineage of eae-carrying ancestor (McGraw *et al.* 1999; Oswald *et al.* 2000; Ramachandran *et al.* 2003). The *eae* gene had not been detected in strains of a different serogroup (O142) (Todd *et al.* 1999; Arthur *et al.* 2002); however, *eae*-positive O142 STEC were isolated from bovines and children in my study.

Collectively, the presence of *eae* genes in STEC strains belonging to serogroups known to cause mild disease was surprising. The spectrum of *eae*-carrying STEC serogroups, including O113, O142 and O166, isolated from humans in my study is wider than identified in other studies (Beutin *et al.* 1998; Elliott *et al.* 2001; Vaz *et al.* 2006; Pradel *et al.* 2008). The presence of *eae* containing STEC suggests a distinct population and lineage of these organisms which has evolved within the unique ecological niche in Nyabushozi.

Previous studies have shown that *eae*-positive strains more frequently carry $stx_{2-EDL993}$ (Boerlin *et al.* 1999; Beutin *et al.* 2004). A similar association was not apparent in my study: only 3 of all the STEC carried *eae* and $stx_{2-EDL993}$. The commonest variant identified in all strains was stx_{2-vhc}, either alone or in combination with stx_{2d-2}; a majority of these STEC contained *eae*-$\gamma 2/\theta$, suggesting an association between carriage of stx_{2-vhc} and *eae*-$\gamma 2/\theta$. That the majority of stx_{2d-2}

containing STEC were *eae*-negative was not unexpected and is consistent with the findings of others (Pradel *et al.* 2000; Beutin *et al.* 2004).

In conclusion, a significant proportion of STEC from bovines and water sources in Nyabushozi contained *eae* genes and *stx*$_2$-variants associated with pathogenicity. Therefore, these STEC may represent a potential public health risk to the community, especially children. A majority of STEC isolated from children carried *eae*, of which some contained *stx*$_2$ variants associated with severe infection.

Chapter 6

The phylogeny of STEC in association with virulence markers and seropathotypes

6.1 Introduction

In the previous chapter some of the virulence determinants of STEC were described in detail. Although the carriage of virulence genes is essential in the pathogenesis of disease, the importance of the genetic background in the acquisition and expression of the virulence genes is increasingly recognised (Johnson, 2002; Escobar-Páramo *et al.* 2004).

Substantial evidence indicates that combinations of horizontal transfer of exogenous DNA and mutation have resulted in the emergence of four major phylogenetic groups (PGs) of *E. coli*; A, B1, B2 and D (Clermont *et al.* 2000; Escobar-Páramo, *et al.* 2004). While extraintestinal *E.coli* predominantly belong to B2 and D (Donnenberg and Whittam, 2001; Johnson *et al.* 2001; Bingen-Bidois *et al.* 2002), STEC strains segregate mainly into PGs A, B1 and D (Clermont *et al.* 2000; Escobar-Páramo *et al.* 2004). Phylogenetic group (PG) D STEC frequently contain *eae* genes and have been associated with severe human illness (Girardeau *et al.* 2005), indicating the public health importance of this group. Some strains in PG B1 are associated with HUS and often carry *eae* (Pradel *et al.* 2008), while strains in PG A are commonly isolated from asymptomatic carriers and usually lack *eae* genes (Girardeau *et al.*

2005). In bovines, group A and B1 strains tend to predominate (Girardeau *et al.* 2005; Pradel *et al.* 2008).

The epidemiological and clinical data based on the incidence and association with HUS and diarrhoeal outbreaks has been used to classify STEC into five seropathotypes (SPTs), A to E (Karmali *et al.* 2003). Accordingly, EHEC O157:H7, the major cause of epidemic outbreaks of HUS, belongs to seropathotype (SPT) A. Serogroups O26, O103 and O111 which cause similar outbreaks of HUS but less frequently belong to SPT B. SPT C is comprised of serogroups such as O8, O113 which sporadically cause bloody diarrhoea and HUS (Girardeau *et al.* 2005). On the other hand, strains in SPT D are known to cause watery diarrhoea, while strains in SPT E have not been reported among human pathogens (Karmali *et al.* 2003; Girardeau *et al.* 2005). Intimin, a marker of virulence is more associated with strains in SPTs A, B and C than SPT D or E (Girardeau *et al.* 2005; Ziebell *et al.* 2008).

To gain deeper understanding of the evolutionary origin of STEC, the PGs of strains from different sources were determined in relation to their virulence markers. Additionally, using SPTs the potential human health risk associated with the clinical STEC was determined.

6.2 Experimental design

6.2.1 Bacterial strains used in the study

Fifty four STEC strains, 25 from children, 24 from cattle and 5 from the water (Chapter 4) were investigated.

6.2.2 Sorbitol fermenting phenotype

STEC cultures were inoculated on SMAC (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and incubated at 37^{o}C for 18-24 h as described [2.2.12]. SF^{+} or SF^{-} colonies were recorded.

6.2.3 Triplex PCR for phylogenetic grouping

The presence or absence of *chuA* and *yjaA* genes and an anonymous DNA fragment, *TspE4.C2* determine the PG (Clermont *et al.* 2000). Using DNA extracted by CTAB method [2.2.13], the triplex PCR was used to detect these genes and an anonymous DNA fragment. Amplification with primers shown in Table 6.1 proceeded with an initial DNA denaturation at 94^{o}C for 4 minutes, followed by 30 cycles of 5 s DNA denaturation at 94^{o}C, annealing temperature at 60^{o}C for 10 s and extension for 15 s at 72^{o}C. A final extension at 72^{o}C for 5 min completed the amplification.

Table 6.1: Primers used in triplex PCR for the amplification of target genes/ DNA fragment and the expected sizes of PCR products

Primer	Gene/ DNA fragment	Primer ($5' \rightarrow 3'$) sequence	Product size (bp)	Reference
ChuA.1	*chuA*	GACGAACCAACGGTCAGGAT	279	Clermont *et al.*
ChuA.2		TGCCGCCAGTACCAAAGACA		(2000)
YjaA.1	*yjaA*	TGAAGTGTCAGGAGACGCTG	211	Clermont *et al.*
YjaA.2		ATGGAGAATGCGTTCCTCAAC		(2000)
Tsp1	*TspE4.C2*	GAGTAATGTCGGGGCATTCA	152	Clermont *et al.*
Tsp2		CGCGCCAACAAAGTATTACG		(2000)

6.3 Results

6.3.1 Phylogenetic distribution of STEC from children, bovines and water

A PCR product (279 bp) indicative of *chuA* and consistent with PG D STEC was obtained with O157:H7 (Figure 6.1 and Table 6.2). Similarly, an appropriately sized amplicon (152 bp) consistent with the presence of *TspE4.C2* was obtained with STEC O111, and indicative of group B1. A 211 bp *yjaA*-related amplicon was obtained with *E. coli* K-12 and no PCR product was obtained from a non-STEC clinical *E. coli* strain isolated from the faeces of a child. Both of these are consistent

with PG A strains (Figure 6.1 and Table 6.2). Thereafter, these were used as control strains in further phylogenetic studies.

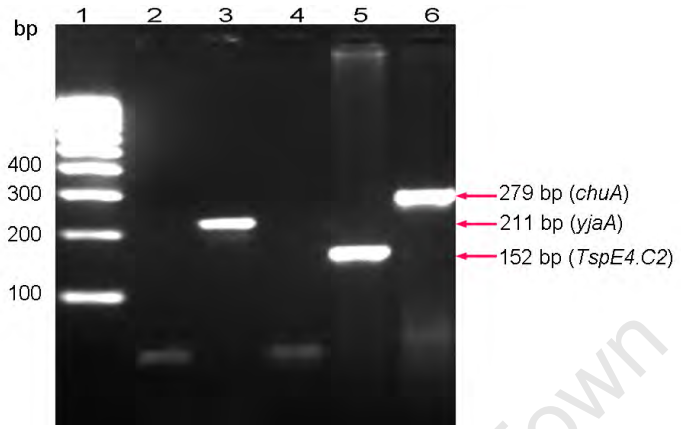

Figure 6.1: PCR for the identification of the PGs of the control STEC strains. Agarose gel electrophoresis following triplex PCR for phylogenetic classification of STEC. The phylogenetic classification is based on the presence/absence of one or more fragments (297, 211 or 154 bp). Lane 1, HyperLadder IV (Bioline, UK); lane 2, no DNA; lane 3, *E. coli* K-12; lane 4, non-STEC clinical *E. coli*; lane 5, STEC O111 (Bb1i); lane 6, O157:H7

Table 6.2: Phylogenetic grouping of *E. coli* using PCR amplification of the *chuA* and *yjaA* genes and DNA fragment *TspE4.C2*

	Phylogenetic groups						
Target	A		B1	B2		D	
chuA	-	-	-	+	+	+	+
yjaA	-	+	-	+	+	-	-
TspE4.C2	-	-	+	-	+	-	+

+, obtained PCR product; -, no PCR product obtained
Adopted from Clermont *et al.* (2000).

Of the 25 clinical STEC, an equal number of STEC, 10 (40%) segregated into PGs A or D, and the remaining 5 (20%) strains belonged to B1 group (Figure 6.2). All strains lacking a PCR product were repeated three times, and consistently none yielded an amplicon.

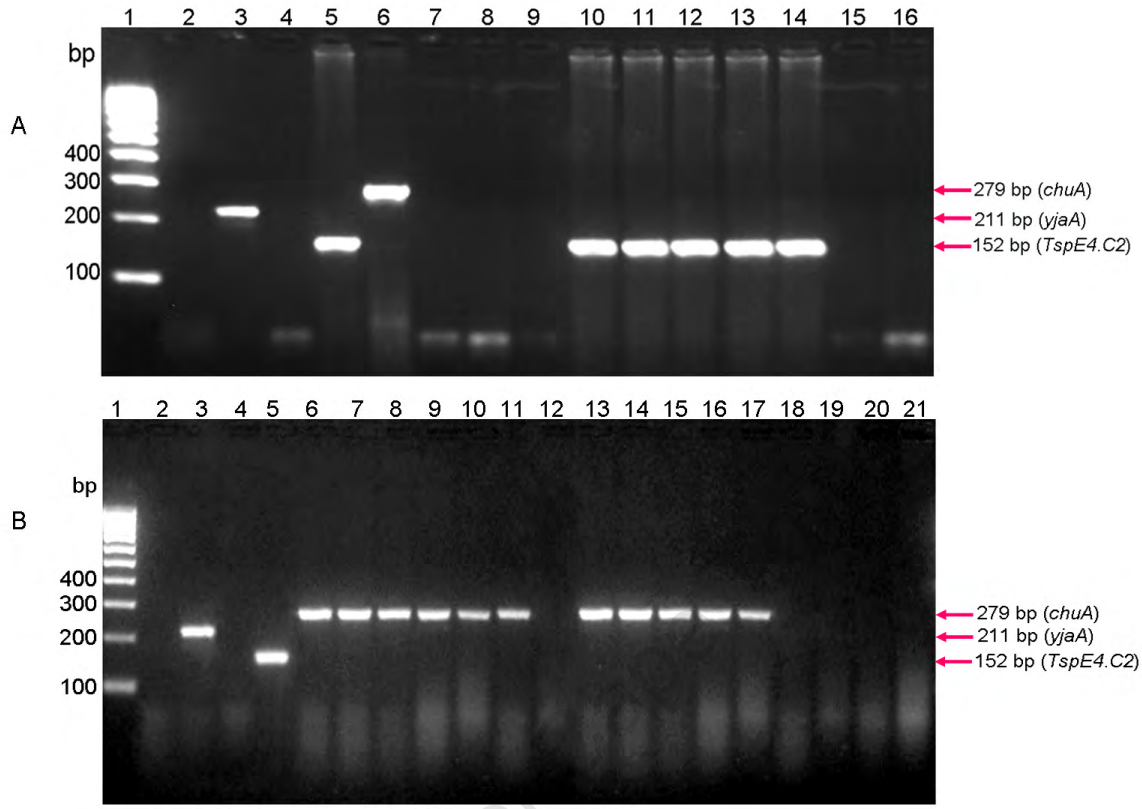

Figure 6.2: PCR for the identification of the PGs of clinical STEC.
A: Agarose gel electrophoresis following triplex PCR for phylogenetic classification of STEC. The phylogenetic classification is based on the presence/absence of one or more fragments (297, 211 or 154 bp). Lane 1, HyperLadder IV (Bioline); lane 2, no DNA; lane 3, *E. coli* K-12; lane 4, non-STEC clinical *E. coli*; lane 5, STEC O111 (Bb1i); lane 6, O157:H7; lane 7, Hh1; lane 8, Hh2; lane 9, Hh3/1; lane 10, Hh3/2; lane 11, Hh4i; lane 12, Hh4ii; lane 13, Hh4iii; lane 14, Hh5; lane 15, Hh6; lane 16, Hh7.

B: Lanes 1, HyperLadder IV (Bioline); lane 2, no DNA; lane 3, *E. coli* K-12; lane 4, non-STEC clinical *E. coli*; lane 5, STEC O111 (Bb1i); lane 6, O157:H7; lane 7, Hh8; lane 8, Hh9; lane 9, Hh10; lane 10, Hh11; lane 11, Hh12; lane 12, Hh13i; lane 13, Hh13ii; lane 14, Hh14i; lane 15, Hh14ii; lane 16, Hh15i; lane 17, Hh15ii; lane 18, Hh16i; lane 19, Hh16ii; lane 20, Hh17; lane 21, Hh18.

Similarly, of the 24 bovines STEC, most strains belonged to B1 group (13 strains, 54.2%), 9 strains (37.5%) belonged to A group and 1 strain to group D. An additional strain which could not be typed was designated non typeable (NT). The five water STEC belonged to PG A.

6.3.2 Phenotypic and genotypic attributes of PG A STEC from children, bovines and water

Twenty four (24) STEC in PG A comprised 10 clinical, 9 bovine and 5 water STEC. The strains belonged to 9 serogroups including O176 not previously described in humans (Table 6.3).

Table 6.3: Phenotypic and genotypic attributes of PG A STEC from children, bovines and water

	Sero		Detected virulence marker				
PG	group	Source	stx_2 type	Stx2	stx_1	Stx1	*eae*	SF
A	O8	Hh7	$stx_{2-EDL993}$, stx_{2-vhc}	+	-	-	-	+
A	O8	Bb5	stx_{2d-2}, stx_{2-vhc}	+	+	+	$\gamma 2/\theta$	-
A	O74	Hh1	stx_{2-vhc}	+	+	+	-	+
A	O76	Hh2	stx_{2-vhc}, stx_{2d-2}	+	+	+	$\gamma 2/\theta$	+
A	O76	Bb3	$stx_{2-EDL993}$, stx_{2-vhc}	+	+	+	-	+
A	O76	Bb15i	-	-	+	+	$\beta 1$	+
A	O76	Bb15ii	-	-	+	+	$\gamma 2/\theta$	+
A	O113	Hh3/2	stx_{2-vhc}	+	+	+	$\gamma 2/\theta$	+
A	O113	Bb7[b]	stx_{2d-2}, stx_{2-vhc}	+	+	+	$\gamma 2/\theta$	+
A	O142	Hh18	stx_{2c}	-	+	+	κ/δ	+
A	O158	Bb14ii	-	-	+	-	$\gamma 2/\theta$	+
A	O166	Ww1	stx_{2-vhc}	+	-	-	$\beta 1$	+
A	O166	Ww2i	stx_{2-vhc}	-	-	-	$\beta 1$	+
A	O166	Ww2ii	stx_{2-vhc}	+	-	-	-	+
A	O169	Hh17	stx_{2c}	+	-	-	-	+
A	O176	Hh16i	stx_{2d-2}	-	-	-	-	+
A	NT	Hh6	stx_{2-vhc}	+	-	-	$\gamma 2/\theta$	+
A	NT	Hh13i	-	-	+	+	$\beta 1$	-
A	NK	Hh16ii	stx_{2d-2}	+	-	-	-	+
A	NT	Bb2	-	-	+	+	$\gamma 2/\theta$	+
A	NT	Bb13	-	-	+	+	-	+
A	NT	Bb14i	-	-	+	-	-	+
A	NK	Ww2iii	stx_{2-vhc}	-	-	-	*eae**	+
A	NT	Ww3	-	-	+	-	-	+

PG, phylogenetic group; Hh, child; Bb, bovine; Ww; water; *eae**; untypeable *eae* gene
NK, serogroup not established; Stx1, Shiga toxin 1; Stx2, Shiga toxin 2
SF, ability (+)/inability (-) to ferment sorbitol

Three of the serogroups O8, O76 and O166 had multiple strains (Table 6.3). Isolates of serogroups O74, O142 and O169 were from clinical STEC, while O8, O76 and O113 were shared between clinical and bovine isolates. STEC O158 and O166 were from the bovine and water, respectively (Table 6.3).

Eight of the strains in group A contained stx_1 as the only *stx* gene, while 16 strains contained stx_2 or both *stx* genes (Table 6.3). Twelve of the 16 strains carrying stx_2, contained stx_{2-vhc} gene alone, or in combination with other genes (Table 6.3). Variant stx_{2d-2} was carried alone, or in combination with stx_{2-vhc} in 3 clinical and 2 bovine strains. Variants stx_{2c} was present in 2 clinical strains while $stx_{2-EDL993}$ was present in a clinical and bovine isolate (Table 6.3).

A majority of group A strains, 14 of 24 (58.3%) contained *eae* gene. Most strains (8 of 13) harboured *eae*-$\gamma 2/\theta$, 4 strains carried *eae*-$\beta 1$, while one strain each carried *eae*-κ/δ or a nontypeable intimin gene (Table 6.3). The ability to ferment sorbitol was assessed, and 22 of 24 (91.7%) group A STEC were SF^+ strains (Table 6.3).

6.3.3 Phenotypic and genotypic attributes of PG B1 STEC from children and bovines

Of the 18 STEC in PG B1, a majority (13) were bovines STEC and the remainder (5) were clinical strains (Table 6.4). Nine of the strains belonged to 3 serogroups O8, O107 and O113. The remaining strains belonged to different O serogroups. Serogroups O107 and O113 were shared between clinical and bovine STEC while O8, O76 and O111 were from the bovines (Table 6.4).

Ten strains contained one or two variants of stx_2 alone or in association with stx_1 (Table 6.4). stx_{2-vhc} was carried by most strains (7), while stx_{2d-2} and $stx_{2-EDL993}$ were carried by 4 and 3 strains, respectively (Table 6.4), showing that different stx_2 genes are carriage by STEC in group B1.

Ten of 18 (55.6%) group B1 STEC carried *eae* gene including *eae*-κ/δ (6 strains of different serogroups), *eae*-$\beta 1$ in 2 strains and one strain each possessed *eae*-κ/δ or a non-typeable *eae* gene (Table 6.4). In addition, 11 of 18 (61.1%) group B1 strains fermented sorbitol.

Table 6.4: Phenotypic and genotypic attributes of PG B1 STEC from children and bovines

PG	Sero group	Source	Detected virulence marker					SF
			stx_2 type	Stx2	stx_1	Stx1	*eae*	
B1	O8	Bb6/1	stx_{2d-2}, stx_{2-vhc}	+	+	+	*eae**	-
B1	O8	Bb6/2	$stx_{2-EDL993}$	+	-	-	-	-
B1	O8	Bb8i	stx_{2d-2}	-	+	-	-	-
B1	O20	Bb10ii	-	-	+	+	-	+
B1	O22	Hh3/1	stx_{2-vhc}	+	-	-	κ/δ	+
B1	O28ac	Bb4	stx_{2d-2}, stx_{2-vhc}	+	+	+	$\gamma 2/\theta$	+
B1	O76	Bb12	-	-	+	+	$\gamma 2/\theta$	+
B1	O78	Hh5	$stx_{2-EDL993}$, stx_{2-vhc}	+	+	+	$\beta 1$	-
B1	O107	Hh4ii	-	-	+	-	-	+
B1	O107	Hh4iii	-	-	+	-	-	+
B1	O107	Bb8ii	stx_{2-vhc}	+	-	-	-	-
B1	O111	Bb1i	$stx_{2-EDL993}$, stx_{2-vhc}	+	+	+	$\gamma 2/\theta$	+
B1	O113	Hh4i	-	-	+	-	$\beta 1$	+
B1	O113	Bb7[a]	stx_{2d-2}	-	+	+	-	-
B1	O113	Bb9	-	-	+	+	$\gamma 2/\theta$	-
B1	O142	Bb14iv	-	-	+	-	$\gamma 2/\theta$	+
B1	Poly9	Bb1ii	stx_{2-vhc}	+	-	-	$\gamma 2/\theta$	+
B1	NT	Bb10i	-	-	+	+	-	+

PG, phylogenetic group; Hh, child; Bb, bovine; *eae**; untypeable intimin gene
Stx1, Shiga toxin 1; Stx2, Shiga toxin 2
SF, ability (+)/inability (-) to ferment sorbitol

6.3.4 Phenotypic and genotypic attributes of PG D STEC from children and bovines

Similarly, analyses of STEC in PG D revealed that 8 of the 11 STEC belonged to 7 serogroups including O111. Eight strains are *eae*-positive and *eae*-κ/δ was detected in most strains (5) while one strain each possessed *eae*-$\beta 1$, $\gamma 2/\theta$ or μ (Table 6.5). Variants of stx_2 carried alone or in combination with stx_1 include $stx_{2-EDL993}$ (2 strains) and one strain each possessed stx_{2c}, stx_{2-vhc} or stx_{2d-2} (Table 6.5). Nine 9

of 11 (81.8%) strains were SF^+.

Table 6.5: Phenotypic and genotypic attributes of PG D STEC strains from children and bovines

PG	Sero group	Source	Detected virulence marker					SF
			stx_2 type	Stx2	stx_1	Stx1	*eae*	
D	O29	Hh10	$stx_{2-EDL993}$	+	-	-	-	+
D	O76	Bb14iii	stx_{2d-2}	+	+	+	$\gamma 2/\theta$	+
D	O111	Hh13ii	-	-	+	+	$\beta 1$	-
D	O141	Hh11	stx_{2-vhc}	+	-	-	-	-
D	O142	Hh15i	$stx_{2-EDL993}$	+	+	+	κ/δ	-
D	O142	Hh15ii	stx_{2c}	+	+	+	κ/δ	-
D	O149	Hh8	-	+	+	+	-	-
D	O166	Hh12	-	+	+	+	κ/δ	-
D	NT	Hh9	-	+	+	+	μ	-
D	NT	Hh14i	-	+	+	+	κ/δ	-
D	NT	Hh14ii	-	+	+	+	κ/δ	-

Hh, child; Bb, bovine; SF, ability (+)/inability (-) to ferment sorbitol
Stx1, Shiga toxin 1; Stx2, Shiga toxin 2

The PG of a sorbitol fermenting bovine STEC Bb11 (O142) could not be established and was recorded as untypeable (NT). The strain was *eae*-negative and carried stx_1.

6.3.5 Distribution of clinical STEC in different SPTs

The classification of clinical STEC strains into the various SPTs (A to D) was adopted from Karmali *et al.* (2003). Each O serogroup was assigned to an appropriate SPT based on the available published data (Karmali *et al.* 2003; Girardeau *et al.* 2005; de Sablet *et al.* 2008; Ziebell *et al.* 2008) and internet databases (http://www.microbionet.com.au/vtec2u.htm, http://www.who.int/emcdocuments/zoonoses/docs/whocsraph988.html, and http://www.lugo.usc.es/ecoli).

Of the 25 clinical STEC, a majority (20, 80%) separated into SPT D, 4 strains (15%) into SPT C and 1 strain (5%) into SPT B (Table 6.6).

Table 6.6: Phenotypic and genotypic attributes of clinical STEC in different SPTs

	Sero		Detected virulence marker							
SPT	group	Child	stx_2 type	Stx2	stx_1	Stx1	*eae*	Diar	SF	PG
B	O111	Hh13ii	-	-	+	+	$\beta 1$	WD	-	D
C	O8	Hh7	$stx_{2-EDL993}$, stx_{2-vhc}	+	-	-	-	WD	+	A
C	O76	Hh2	stx_{2-vhc}, stx_{2d-2}	+	+	+	$\gamma 2/\theta$	WD	+	A
C	O113	Hh3/2	stx_{2-vhc}	+	+	+	$\gamma 2/\theta$	WD	+	A
C	O113	Hh4i	-	-	+	-	$\beta 1$	WD	+	B1
D	O22	Hh3/1	stx_{2-vhc}	+	-	-	$\gamma 2/\theta$	WD	+	B1
D	O29	Hh10	$stx_{2-EDL993}$	+	-	-	-	WD	+	D
D	O74	Hh1	stx_{2-vhc}	+	+	+	-	WD	+	A
D	O78	Hh5	$stx_{2-EDL993}$, stx_{2-vhc}	+	+	+	$\beta 1$	BD	-	B1
D	O107	Hh4ii	-	-	+	-	-	WD	+	B1
D	O107	Hh4iii	-	-	+	-	-	WD	+	B1
D	O141	Hh11	stx_{2-vhc}	+	-	-	-	BD	-	D
D	O142	Hh15i	$stx_{2-EDL993}$	+	+	+	κ/δ	WD	-	D
D	O142	Hh15ii	stx_{2c}	+	+	+	κ/δ	WD	-	D
D	O142	Hh18	stx_{2c}	-	+	+	κ/δ	BD	+	A
D	O149	Hh8	-	-	+	+	-	BD	-	D
D	O166	Hh12	-		+	+	κ/δ	WD	-	D
D	O169	Hh17	stx_{2c}	+	-	-	-	BD	+	A
D	O176	Hh16i	stx_{2d-2}	-	-	-	-	WD	+	A
D	NT	Hh6	stx_{2-vhc}	+	-	-	$\gamma 2/\theta$	BD	+	A
D	NT	Hh9		-	+	+	μ	BD	-	D
D	NT	Hh13i	-	-	+	+	$\beta 1$	WD	-	A
D	NT	Hh14i	-	-	+	+	κ/δ	WD	-	D
D	NT	Hh14ii	-	-	+	+	κ/δ	WD	-	D
D	NK	Hh16ii	stx_{2d-2}	+	-	-	-	WD	+	A

SPT, seropathotype; NK, serogroup not established; Diar, type of diarrhoea
PG, phylogenetic group; SF, ability (+)/inability (-) to ferment sorbitol
Stx1, Shiga toxin 1; Stx2, Shiga toxin 2

The *eae*-$\beta 1$ containing STEC O111 belonged to SPT B, while serogroups O8, O76 and O113 belonged to SPT C. Three of the *eae*-positive STEC in SPT C contained

$\gamma 2/\theta$ (O76, O113) or *eae*-$\beta 1$ in O113 (Table 6.6).

Twenty STEC in SPT D belong to 11 O serogroups (Table 6.6). Slightly over half of the strains (11 of 20) carried *eae*, mostly eae-κ/δ (6 strains), $\beta 1$ (2 strains) and one strain each carried $\gamma 2/\theta$ or μ (Table 6.6). In addition, the strains contained different *stx* genes (Table 6.6). There was an equal split, 10 strains possessed SF^+ or SF^- phenotype.

6.4 Discussion

Several phylogenetic studies of STEC have been carried out using the triplex PCR method (Girardeau *et al.* 2005; Ishii *et al.* 2007; Pradel *et al.* 2008), because it is quick and may not require reference collection of strains (Clermont *et al.* 2000). An equal proportion of clinical strains (10 strains, 40%) separated into PGs A or D; a result which contrasts with findings from studies in France (Girardeau *et al.* 2005; Pradel *et al.* 2008) and Canada (Ziebell *et al.* 2008), where a majority of clinical STEC belonged to PG B1. This may be attributed to the differences in the STEC populations or their clonal descent. In addition, inherent host factors and climatic conditions may influence the distribution of the various PGs of STEC (Duriez *et al.* (2001). Although PG D STEC are frequently associated with HUS (Girardeau *et al.* 2005), available evidence suggests that PG B1 STEC could potentially cause a spectrum of disease from mild diarrhoea to HUS (Girardeau *et al.* 2005; Ziebell *et al.* 2008). That none of PG B2 STEC was identified, was expected because group B2 *E. coli* rarely cause diarrhoeal disease (Escobar-Paramo *et al.* 2004; Girardeau *et al.* 2005).

More than half of bovine STEC (13 strains, 54.2%) in this study, belonged to PG B1, a finding which accords with results of other studies (Girardeau *et al.* (2005; Ishii *et al.* 2007). An additional 9 (37.5%) STEC segregated into PG A while one strain belonged to PG D. Probably this indicates that STEC in PGs A and B1 are more adapted to the gastrointestinal tracts of bovines in Nyabushozi than PG D STEC. PG A STEC from water and bovines represent strains that are probably associated with mild illness (watery diarrhoea) in humans (Girardeau *et al.* 2005).

In the majority of STEC the expression of Stx1, Stx2 or both toxins was detected among the different PGs. This observation suggests that the expression of Stx in these strains is not entirely dependent on the genetic background. However, the genetic background may play a significant role in the differential basal expression of Stx among STEC strains (de Sablet *et al.* 2008). This was not verified in the present study because the Duopath Verotoxin assays determined the qualitative production of Stx. That certain strains of STEC in PGs A and B1 contained multiple variants of stx_2 was expected, as this has been reported in a previous study (Pradel *et al.* 2008). In contrast to Pradel *et al.* (2008), none of the STEC in PG D in this study possessed more than one variant of stx_2, possibly because of the differences in STEC populations.

A majority of PG A STEC (14 of 24) in this study were *eae*-positive. This finding accords with results from a recent study in Canada (Ziebell *et al.* 2008), suggesting that PG A *eae*-positive STEC occur in different geographical locations and/or STEC populations. However, *eae* gene types carried by PG A STEC described by Ziebell *et al.* (2008) remain unknown because the *eae* gene types were not characterised. Nonetheless, STEC in PG A from this study carried various *eae* gene types; a majority of strains contained *eae*-$\gamma 2/\theta$ (8 of 14) and 4 strains contained *eae*-$\beta 1$, while *eae*-κ/δ and an untypeable *eae* were each carried by one strain (Table 6.3).

The carriage of *eae* in PG B1 STEC (10 of 18) was not unusual as previous studies have reported *eae*-positive STEC in the PG (Pradel *et al.* 2008; Ziebell *et al.* 2008). What was unusual, however, was the fact that most of the *eae*-positive STEC contained *eae*-$\gamma 2/\theta$. This result differs from findings of Pradel *et al.* (2008), who reported *eae*-β and ε among the clinical STEC, instead of *eae*-$\gamma 2/\theta$. This may be attributed to the difference in STEC populations and/or their geographical location.

Few phylogenetic studies have characterised *eae* gene types in STEC (Ishii *et al.* 2007; Pradel *et al.* 2008), and to the best of my knowledge this is the first description of *eae*-$\gamma 2/\theta$ in STEC of different genetic backgrounds. The carriage of *eae*-$\gamma 2/\theta$ gene in a majority of *eae*-positive STEC in group B1 as well as group A in this study, probably indicates that *eae*-$\gamma 2/\theta$ is associated with STEC of different genetic backgrounds (A and B1). It can be suggested that during evolution, genomes with

A or B1 genetic background preferentially acquired and retained *eae*-$\gamma 2/\theta$. This is supported by previous studies that have shown a close association between the clonal lineage EHEC-1 such as EHEC O157:H7 and *eae*-$\gamma 1$ (Wieler *et al.* 1997; McGraw *et al.* 1999; Donnenberg and Whittam, 2001). On the other hand, the presence of *eae* may indicate that *eae*-$\gamma 2/\theta$ is widely distributed among clinical and bovine PG A and B1 STEC in Nyabushozi. The carriage of *eae*-$\beta 1$ by STEC in PGs A and B1 was not surprising as this gene is known to be ubiquitous among STEC (McGraw *et al.* 1999; Oswald *et al.* 2000), and most probably of different genetic backgrounds.

It is noteworthy that *eae*-positive STEC O113 and specifically carrying *eae*-$\gamma 2/\theta$ in PGs A and B1 are being reported for the first time. Previous studies have reported *eae*-negative STEC O113 with genetic background A or B1 (Girardeau *et al.* 2005; Pradel *et al.* 2008; Ziebell *et al.* 2008). Therefore, it is reasonable to suggest that STEC O113 from Nyabushozi emerged from distinct clones of *eae*-carrying progenitors within the ecological niche.

Nine of the 11 STEC in PG D (with the exception of O76 and O111) were collected in phase 2, suggesting that they might have evolved from a common distant ancestor. This also suggests that their ancestral descent is different from phase 1 clinical or the bovine STEC. As expected, a majority of strains in PG D (8 of 11) carried *eae* genes. This accords with previous studies which have shown a strong correlation between the D genetic background and the presence of *eae* (Girardeau *et al.* 2005; Ziebell *et al.* 2008). The carriage of *eae* in STEC with genetic background D in this study might indicate virulence, since the virulent strains od EHEC O157:H7 are predominantly *eae*-positive and belong to PG D (Bidet *et al.* 2005; Girardeau *et al.* 2005; Ziebell *et al.* 2008).

Besides EHEC O111, the *eae*-positive STEC in PG D, including STEC O29 and O149, are non-EHEC strains. This probably indicates that non-O157:H7 STEC with the D genetic background are wide spread among the clinical STEC in Nyabushozi. Five of the 7 of these STEC carried *eae*-κ/δ. To the best of my knowledge, the carriage of *eae*-κ/δ by strains in PG D has not been previously reported.

The carriage of stx_{2d-2} has been previously associated with absence of *eae* in the STEC genome (de Sablet *et al.* 2008); unsurprisingly 4 of 5 STEC containing this gene as the only stx_2 gene in PG A and B1 were *eae*-negative. However, a strain in

group D contained stx_{2d-2} and *eae*-$\gamma 2/\theta$, suggesting that the D genetic background played a role in the retention of both *eae* and stx_{2d-2} genes within the genome (Escobar-Paramo *et al.* 2004).

SPTs provide a basis for molecular risk assessment in order to predict the virulence potential of STEC (Coombes *et al.* 2008). A majority of clinical strains (20 of 25) belonged to SPT D, indicating that they could potentially cause mild illness (watery diarrhoea). The presence of PG A strains in SPT D (7 of 20) was expected because genetic background A is associated of low pathogenicity (Girardeau *et al.* 2005; Ziebell *et al.* (2008). On the other hand, 4 and 9 strains in SPT D belonged to PG B1 and D, respectively. This was probably because triplex PCR was unable to discriminate sufficiently among the various serotypes of STEC (Ziebell *et al.* 2008). In general, SPT was able to segregate clinical STEC in different PGs into SPTs B, C and D, showing its usefulness as a molecular analysis tool. This observation is in agreement with the previous study , which suggests that SPTs was more precise than triplex PCR in predicting the virulence potential of STEC (Coombes *et al.* 2008).

Since both SF^+ and SF^- STEC were detected among SPT D strains, it indicates that this phenotype is a weak marker of virulence. This is supported by previous studies which have shown that most EHEC strains implicated in severe human illness ferment sorbitol (March and Ratnam *et al.* 1986; Bielaszewska and Karch, 2000; Tarr *et al.* 2005). It can therefore be suggested that SF^+ or SF^- phenotype within the different genetic backgrounds of STEC is an indicator of clonal descent.

In conclusion, STEC of genetic background A are widely distributed in the different sources (humans, bovine and water). Genetic backgrounds B1 and D were closely associated with bovine and clinical STEC, respectively. Of note *eae*-κ was predominantly carried by genetic background D STEC whereas $\gamma 2/\theta$ was associated with background A or B1. Although a majority of clinical STEC were in SPT D, the isolation of strains in SPTs B and C suggests that clinical STEC from Nyabushozi are potentially pathogenic; capable of causing human illness ranging from mild to severe diarrhoea and HUS.

Chapter 7

Conclusions and general discussion

Cattle play a significant role in the livelihood of the rural pastoral communities in Nyabushozi; a county which supports one of the highest populations of cattle in Uganda. Undeniably, a large cattle population, coupled with the close human-cattle contact are risk factors for STEC infection in the community, especially to the children.

Using biochemical and molecular methods, STEC was isolated from 18 of 222 (8.1%) *E. coli* carrying diarrhoeal children. To the best of my knowledge this is the first report describing clinical STEC in children from Nyabushozi county and Uganda in general. This probably suggests that previous STEC infections were misdiagnosed, or not detected during laboratory testing, since screening for STEC is not mandatory in public health centres in Uganda. In addition, the lack of technical and human resource impedes the detection of STEC in most laboratories. The prevalence of STEC (8.1%) in my study accords with findings from a similar study in a rural community of Nigeria (Okeke *et al.* 2000, whereas similar studies in peri-urban communities from Uganda (Nasinyama, 1996; Kaddu-Mulindwa *et al.* 2001), Tanzania (Gascon *et al.* 2000) and Mozambique (Rappelli *et al.* 2005) did not identify STEC. This suggests that children from rural farming communities are more at risk from STEC infections than in peri-urban communities, perhaps because of the close contact with cattle or the farm environment.

The numerous PFGE profiles shown by clinical STEC indicated genetically diverse STEC. This observation was similar to results of previous studies (Rios *et al.* 1999;

Khan *et al.* 2002a). The genetic diversity accords with previous studies which reported that non-O157:H7 STEC is a heterogenous group of pathogens from diverse ancestral clones (Whittam *et al.* 1993; Feng *et al.* 1998). Further, the clonal subgroups of STEC within each host exhibited genetic heterogeneity, which probably indicates the dynamic nature of STEC genome. The host immune system or the prevailing external factors may have induced the genomic alteration (Akiba *et al.* 2000).

The clinical STEC belonged to 15 serogroups including O8, O74, O76, O78, O107, O111, O113 and O166, previously associated with severe human infections. In addition, O29, O149 and O176 which have not been previously described in humans were isolated, suggesting the occurrence of additional unidentified human STEC. O142 was the most frequent serogroup from the children, suggesting that strains of STEC O142 are able to thrive and proliferate within the intestinal tracts of children in Nyabushozi.

There was a clear distinction between clinical serogroups of STEC collected in phases 1 and 2, possibly because phase 2 children were from homesteads different from children and bovines in phase 1. This suggests that there are diverse STEC populations within the ecological niche of Nyabushozi. Probably the high cattle population turn-over, changes in the bovine diet (Geue *et al.* 2006) or seasonal human-cattle migration might have disrupted the ecosystem and altered the population of STEC.

Fifteen of 25 clinical strains contained either stx_2 alone or in combination with stx_1. Variant stx_{2-vhc} was the commonest variant, carried alone or in combination with $stx_{2-EDL993}$ or stx_{2d-2c}. The predominance of stx_{2-vhc} in clinical STEC from Nyabushozi contrasts the findings from other studies in Europe (Stephen and Hoelzle, 2000; Pradel *et al.* 2008). This strongly suggests that stx_{2-vhc} is associated with clinical STEC in Nyabushozi.

Most clinical STEC contained *eae* gene (15 of 25, 60%); and since STEC carrying this gene have been associated with severe human illnesses (Boerlin *et al.* 1999; Eklund *et al.* 2002), this probably indicates that *eae*-positive STEC are potentially virulent.

A majority of clinical strains in phase 2 contained *eae*-κ/δ, while phase 1 STEC

carried either *eae*-$\gamma 2/\theta$ or *eae*-$\beta 1$, which might be attributed to the different STEC populations between the phases. The few serotypes of clinical STEC previously found to harbour *eae*-κ/δ are known for their virulence (Zhang *et al.* 2002b), thus this gene could be a marker of virulence among the clinical STEC from Nyabushozi.

Phylogenetic analysis classified clinical STEC predominantly into A and D groups. In the majority of STEC in the various PGs the expression of Stx1, Stx2 or both toxins was detected. However, further studies are needed to establish the relationship between PG and the differential basal expression of Stx.

The exclusive carriage of *eae*-κ/δ in PG D STEC from phase 2, suggests that these STEC belonged to a distinct lineage and possibly STEC population. Although the triplex PCR ndoes not sufficiently predict the virulence potential of STEC, strains carrying LEE are probably more virulent than *eae*-negative clinical strains.

Rectal faeces containing *E. coli* from 15 of 216 (7.85%) bovines under range management in Nyabushozi were positive for STEC. This was the same prevalence as in bovines from the USA under similar husbandry practice (Renter *et al.* 2003. However, this prevalence was much lower than 28.3% for cattle from a farm in central Uganda (Kaddu-Mulindwa *et al.* 2001), indicating that shedding of STEC varies with husbandry practices and the locality.

The analysed PFGE profiles of a majority of bovine STEC revealed that they were genetically diverse, suggesting that they were derived from different STEC populations. This is probable because STEC were isolated from different herds, which are thought to harbour distinct STEC strains. This accords with Cobbold and Desmarchelier (2001), who identified strains specific to individual farms in Australia.

The intra-herd clonal similarity suggests that transmission of STEC among cattle within the herd must have occurred, possibly promoted by commingling of cattle within the night enclosures (kraals). On the other hand, the inter-farm clonal similarity of STEC, suggests that the transmission of STEC among the different herds may have occurred, possibly via drinking water at the communal valley dams, through cattle movement between herds during traditional ceremonies or transmission by humans, birds and other vehicles within the environment in Nyabushozi. However, the possibility of transmitting STEC from contaminated pastures to other

cattle was probably minimal because the chances of sharing the same contaminated pastures are remote.

The genetic relatedness between some bovine and clinical STEC indicates a possible transmission of STEC from the cattle to children in the community. Infection could have been acquired through direct contact with cattle, the farm environment or via ingestion of raw or fermented milk (*eshabwe*). Contact with cattle or the farming environment are major risk factors for STEC transmission from bovines to humans in farming communities (O'Brien *et al.* 2001; Crump *et al.* 2002; Grif *et al.* 2005), such as the pastoralists in Nyabushozi.

The bovine STEC belonged to previously described serogroups, of which O8, O76, O111 and O113 were also shed by the children, suggesting that bovines in Nyabushozi harboured STEC pathogenic for humans. In addition, bovines harboured *eae*-positive STEC of serogroups O28ac, O113, O142 and O158. Previously described STEC in these serogroups lacked *eae*, suggesting that these strains belong to distinct clonal descent and STEC populations within the ecological niches of Nyabushozi.

Half of the 24 bovine STEC carried stx_2 alone or in combination with stx_1. While STEC containing stx_{2-vhc} alone or in combination with stx_{2-d2} were most common. Since stx_{2d-2} is poorly expressed (de Sablet *et al.* 2008), STEC carrying this gene perhaps compensates for the poor expression by carrying stx_{2-vhc} as well. However, this would need further investigation. Stx2d-2 and Stx-vhc share amino acid sequences associated with mucous activation, and this would suggest that both proteins have similar virulence profiles and may be associated with mild to moderate human illness.

The *eae* gene was prevalent in the bovine STEC, carried by 14 of the 24 strains. Specifically *eae*-$\gamma 2/\theta$ was carried in 12 of these strains, suggesting that *eae*-$\gamma 2/\theta$ was a prevalent *eae* type among the bovine STEC. The carriage of untypeable *eae* indicates the presence of additional unknown *eae* types.

Phylogenetic studies of the bovine STEC classified most of the strains in groups A and B1. The carriage of *eae*-$\gamma 2/\theta$ gene in a majority of *eae*-positive STEC in group B1 as well as group A, probably indicates that *eae*-$\gamma 2/\theta$ is associated with bovine STEC of different genetic backgrounds in Nyabushozi.

The valley dams extensively used by humans and their bovines were contaminated by STEC which belonged to PG A. Most of the strains carried stx_{2-vhc} and *eae* gene. STEC O166 was simultaneous isolated from water and a child, indicating the health risk associated with drinking of the water. Although STEC from the water was genetically diverse, the valley dams remain an important source of infection to humans and the bovines.

Since the H-antigen of STEC was not typed, the risk assessment based on the SPT of the bovine STEC could not be established. Nonetheless, the bovine STEC should be regarded as potential human pathogens which require further investigation. Clinical STEC in SPT B and C potentially cause severe illness since they have previously been associated with epidemic and sporadic outbreaks of HUS (Girardeau *et al.* 2005). A majority of clinical STEC segregated into SPT D, suggesting that they could potentially cause watery diarrhoea. However, given the level of childhood malnutrition (Kikafunda *et al.* 1998) and the disease burden among the rural children in Uganda, even the mild STEC could cause severe illness. In general, clinical STEC should be regarded as potentially pathogenic and capable of causing severe illness in children with naive immunity.

Certain STEC were classified in SPT C basing on their O antigen, however, appropriate classification would required typing of their H-antigens. In order to predict more precisely the virulence potential of *eae*-positive STEC with the D genetic background, analysis of the non-LEE pathogenic islands would be required in future studies.

In conclusion, the genetically diverse STEC from the pastoral system of Nyabushozi, armed with *stx* and *eae* genes within their respective genetic backgrounds have the potential to cause childhood illnesses ranging from watery to bloody diarrhoea and HUS.

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Appendices

Appendix A

Research approval

Uganda National Council For Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

UNCST

Your Ref:.....

Our Ref: HS 74 *Date:* 10-Oct-05

Dr. Majalija Samuel
C/o Faculty of Veterinary Medicine
Makerere University
KAMPALA

Dear Dr. Majalija,

RE: RESEARCH PROJECT, "HUMAN AND ENVIRONMENTAL HEALTH IMPACT OF FARM WASTE ON THE SPREAD OF RESISTANT BACTERIA IN BAHIMA PASTORAL SYSTEMS"

This is to inform you that the Uganda National Council for Science and Technology (UNCST) approved the above research proposal on **August 17, 2005**. The approval will expire on **August 17, 2006.** If it is necessary to continue with the research beyond the expiry date, a request for continuation should be made in writing to the Executive Secretary, UNCST.

Any problems of a serious nature related to the execution of your research project should be brought to the attention of the UNCST, and any changes to the research protocol should not be implemented without UNCST's approval except when necessary to eliminate apparent immediate hazards to the research participant(s).

This letter also serves as proof of UNCST approval and as a reminder for you to submit to UNCST timely progress reports and a final report on completion of the research project.

Yours sincerely,

P. Julius Ecuru
for: Executive Secretary
UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

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Appendix B

Informed consent form

Dear Parent/ Guardian

I am Dr Samuel Majalija, of the Faculty of Veterinary Medicine, Makerere University. I am investigating the human and environmental health impact of farm waste on the spread of resistant bacteria in Bahima pastoral systems

I will explain to you about my research work: The excess use of animal drugs (antibiotics) are associated with antibiotic resistance among some indicator bacteria, which also transmitted to humans. The purpose of this research is to identify antibiotic resistant bacteria in children suffering from diarrhoea and cattle from the same homesteads.

I am inviting you to participate because it is important to test whether your child is infected by antibiotic resistant bacteria.

If you are willing, I will ask you a few questions and also the nurse will assist you to collect a stool sample from your child. In addition, I will visit your home within the next 3 days in order to collect faecal samples from the cattle.

Informed consent
I .....(parent /guardian/ cattle owner),
I have understood the objectives of this research and I agree to participate in the study on: *The human and environmental health impact of farm waste on the spread of resistant bacteria in Bahima pastoral systems.*

I understand that participation involves:
Answering questions regarding the study.
Providing a stool sample from my child

Allowing the investigator to visit my home where faecal samples will taken from the cattle.

I understand that participation is completely voluntary and has no financial bearing whatsoever.

Confidentiality
I understand that my name, the child's name or a specific cattle identification will not appear with the results of this research.
I understand that my decision to participate or not to participate in this study will not alter the health care of my child/ or the cattle.
I understand the results may be communicated to the relevant authorities for future action.
I understand that in the use of the information generated from this study, my identity or the identities of my child/ animals will remain anonymous.
I also understand that I am free to withdraw consent at any time during the study.

I understand that for any questions or explanation about the tests or results, I may contact Dr Samuel Majalija (Tel. Number +256 77 404710).

I understand that by signing in the space below, I indicate that I have understood the purpose of this study to which I agree to participate.

Signature.....Parent/Guardian
Age..... Date: .....
Name of investigator: .....
Signature of investigator:.....
Date.....
Name and signature of witness.....

Appendix C

Questionnaire

Patient biodata

1: Clinical number of patient:
2: Patient identity code:
3: Patient age.....
4: Patient sex.....
5: Name of household head
6: Patient contact address: Local village council (LC1).....
7: Chairperson of LC1:.....
8: Name of nearest primary school/ church/ Mosque.....
9: Duration of stay in the village.....
10: Number of cattle at home.....
11: Source of drinking water.....
12: How often do you drink raw milk or fermented milk (eshabwe) in the home?.....
13: Did this child drink raw milk or fermented milk (eshabwe) in the last 2 weeks?.....

Clinical data

14: Time and date of presentation for care.....
15: Maximum number of loose or liquid motions in any 24-hour period since the diarrhoea began.....
16: Duration of diarrhoea.....
17: Type of diarrhoea.....
18: Whether treatment given before reporting to clinic.....
19: Types of antibiotic given at the time of sample collection.....

Appendix D

Media and buffers

D.1. Media

Caye Broth containing Caye broth supplement
Caye broth 7.95g
Distilled water 200 ml
Autoclave
Cool to 45^{o}C
Add Caye supplement

mTEC Agar (Difco)
Protease Peptone 35.0g
Yeast Extract 3.0g
Lactose 10.0 g

Stuart's transport medium (BBL^{TM})
14.1 g of Stuart's transport medium powder
Dissolve in 1000ml of high grade water.
Dispense in small screw-capped bottles
Autoclave at 121^{o}C for 10 minutes.

2X yeast-Tryptone (YT) broth
Tryptone 16g
Yeast extract 10g
Nacl 5g
Add to 1000ml high grade water and autoclave at 121^{o}C for 10 minutes.

D.2. Buffers

Cell Lysis Buffer (50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl)
25 ml of 1 M Tris, pH 8.0
50 ml of 0.5 M EDTA, pH 8.0 50 ml of 10 % Sarcosyl (N-Lauroylsarcosine, Sodium salt)
Dilute to 500 ml with sterile Ultrapure (Reagent Grade Type 1) water

Cell Suspension Buffer (100 mM Tris:100 mM EDTA, pH 8.0)
10 ml of 1 M Tris, pH 8.0
20 ml of 0.5 M EDTA, pH 8.0
Dilute to 100 ml with sterile Ultrapure (Reagent Grade Type 1) water

50X TAE Buffer for agarose
Tris (base) 2M (242g)
EDTA (disodium salt)
5M (18.6g)
Adjust to pH 8 with 57ml glacial acetic acid
Make up to 1 litre with distilled water.
Autoclave. For preparing agarose gel and running use 1x TAE, i.e 1ml TAE:49ml water

0.5X TBE buffer for PFGE
Tris 8.1g
Boric Acid 1.38g
EDTA 0.47g
Dilute to 1000 ml with sterile Ultrapure (Reagent Grade Type 1)
Autoclave

TE Buffer
10 ml of 1 M Tris, pH 8.0
2 ml of 0.5 M EDTA, pH 8.0
Dilute to 1000 ml with sterile Ultrapure (Reagent Grade Type 1) water

Appendix E

Restriction fragments

E.1. Restriction fragments generated by *Hha*I digestion of 1848 nucleotide sequences of 18 intimin types from the Genbank

Intimin type	Accession number	DNA fragment generated by restriction digest with *Hae*III
Alpha (α) 1	AF022236	63, 72, 230, 341, 545, 597
Alpha (α) 2	AJ579368	63, 72, 168, 230, 341, 429, 545
Beta (β) 1	AF453441/ AJ277443	48, 49, 62, 72, 78, 205, 230, 354, 357, 392
Beta (β) 2/B	AJ715407	62, 63, 167, 230, 413, 429, 483
Gamma (γ) 1	AF081185	62, 72, 168, 230, 404, 429, 483
Gamma (γ)2 theta (θ)	AF449420 / AF025311	62, 168, 205, 224, 230, 476, 483
Epsilon (ϵ) 1	AF116899	62, 72, 78, 205, 230, 392, 404, 405
Epsilon (ϵ) 2	AF530554/ DQ523614	62, 72, 168, 205, 224, 230, 404, 483
Zeta (ζ)	AF449417	72, 168, 230, 404, 429, 545
Zeta (ζ)	AJ298279	72, 168, 205, 224, 230, 404, 545
Eta (η)	DQ523604/ AJ308550	62, 63, 78, 168, 230, 341, 429, 477
Iota (ι)	DQ523601/ AJ308551	72, 168, 230, 404, 429, 545
Iota (ι) 2	AF530553	23, 47, 62, 72, 78, 183, 220, 354, 404, 405

Continued...

Table E.1 – Continued

Intimin type	Accession number	DNA fragment generated by restriction digest
Kappa (κ/)	AJ308552	63, 72, 168, 230, 341, 429, 545
delta (δ)/	AJ875027	
(β2O)	U66102	
Lambda (λ)	AF530557	72, 168, 230, 404, 429, 545
Mu (μ)	AJ705049	63, 72, 168, 230, 341, 429, 545
Nu (μ)	AJ705050	23, 47, 62, 72, 78, 183, 220, 354, 404, 405
xi (ξ)	AJ705051	62, 72, 78, 205, 230, 392, 403, 405
Omicron (o)	AJ876648	23, 47, 62, 72, 78, 183, 220, 354, 404, 405
rho (ρ)	AJ748084	47, 62, 72, 78, 183, 243, 354, 404, 405

E.2. Restriction fragments generated by*Hae*III digestion of 1848 nucleotide sequences of 18 intimin types from the Genbank

Intimin type	Accession number	DNA fragment generated by restriction digest with *Hae*III
Alpha (α) 1	AF022236	23, 83, 163, 303, 399, 877
Alpha (α) 2	AJ579368	106, 163, 303, 1276
Beta (β) 1	AF453441/ AJ277443	22, 30, 75, 172, 361, 1187
Beta (β) 2/B	AJ715407	23, 83, 162, 303, 399, 877
Gamma (γ) 1	AF081185	23, 163, 303, 1359
Gamma (γ)2	AF449420 /	23, 105, 173, 188, 1359
theta (θ)	AF025311	
Epsilon (ϵ) 1	AF116899	23, 30, 75, 168, 172, 193, 1187
Epsilon (ϵ) 2	AF530554/ DQ523614	23, 163, 303, 482, 877
Zeta (ζ)	AF449417	23, 147, 156, 163, 482, 877
Zeta (ζ)	AJ298279	23, 482, 1343
Eta (η)	DQ523604/ AJ308550	23, 83, 147, 156, 163, 222, 225, 829
Iota (ι)	DQ523601/ AJ308551	23, 163, 303, 482, 877
Iota (ι) 2	AF530553	30, 436, 1382
Kappa (κ/)	AJ308552	23, 83, 163, 303, 399, 877
delta (δ)/	AJ875027	
(β2O)	U66102	
Lambda (λ)	AF530557	23, 163, 303, 1359
Mu (μ)	AJ705049	23, 83, 147, 156, 163, 399, 877
Nu (μ)	AJ705050	23, 30, 83, 436, 447, 829
xi (ξ)	AJ705051	22, 30, 75, 172, 361, 1187
Omicron (o)	AJ876648	12, 18, 106, 436, 447, 829
rho (ρ)	AJ748084	12, 18, 23, 436, 1359