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Detection and characterization of *E. coli* O157:H7 and induced shiga toxin-2 coding bacteriophages

by

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"Ek verklaar dat die verhandeling wat ek hiermee aan die Universiteit van Pretoria vir die M.Sc. (Geneeskundige Virologie)- graad voorlê, my eie werk is en nie vantevore deur my aan enige ander tersiêre inrigting vir enige graad voorgelê is nie."

"I certify that the thesis hereby submitted to the University of Pretoria for the degree of M.Sc. (Medical Virology) is my own work and has not previously been submitted by me in respect of a degree at any other tertiary institution."

Signature: ______________________

Date: 27 May 2002

This thesis is dedicated to my parents

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I found it!) but 'That's funny…'

Isaac Asimov (1920-1992)

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LIST OF ABBREVIATIONS

μl	Microlitre
μm	Micrometre
A	Absorbancy
AE	Attaching and effacing
AFLP	Amplified Fragment Length Polymorphism
AMP	Adenylate Monophosphate
BFP	Bundle forming pilus
bp	Base pairs
CDC	Centers for Disease Control and Prevention
cfu	Colony forming units
CT	Cefixime-tellurite
DAEC	Diffusely adherent *Escherichia coli*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	diNucleotide Triphosphate
E. coli	*Escherichia coli*
*Eae*A	*E. coli* attaching and effacing
EAEC	Enteroaggregative *E. coli*
EAF	EPEC adherence factor
EAST	Heat stabile enterotoxin
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
EPEC	Enteropathogenic *E. coli*
ERIC	Enterobacterial repetitive intergenic consensus
Esp	Extracellular serine protease
ETEC	Enterotoxigenic *E. coli*
GB3	Globotriaosylceramide
GB4	Globotetraaosylceramide
GC	Guanylate cyclase
GMP	Guanylate monophosphate
HAS	Human serum albumin
HC	Haemorrhagic colitis
HeLa	Human cervical cancer cells
Hly	Enterohaemolysin
HUS	Haemolytic uraemic syndrome
IL	Interleuken
IMS	Immunomagnetic sepration
ICTV	Internation Committee on Virus Taxonomy
IP	Inositol phosphate
kb	Kilo Bases
kDa	Kilo Dalton
LB	Luria Bertani
LEE	Locus for enterocyte effacement
LPS	Lipopolysaccaride

LT	Heat-labile toxin
Mda	Mega dalton
mg	Milligram
$MgCl_2$	Magnesium Chloride
ml	Millilitre
MM	Millimolar
MPC	Magnetic Particle Concentrator
MSA	Modified Scholtens Agar
MSB	Modified Scholtens Broth
NaCl	Sodium Chloride
NaN_3	Sodium Azide
ng	Nanogram
OD	Optic Density
OMP	Outer Membrane Protien
P	Plasmid
PAGE	Polyacrylamide Gel Electrophoreses
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulse-field Gel Electrophoreses
pfu	Plaque Forming Unit
Phage	Bacteriophage
PKC	Phosphokinase C
PTA	Phosphotungstic Acid
RAPD	Random Amplified Polymorphic DNA
RE	Restriction Enzyme
REP's	Repetitive Extragenic Polendromic Elements
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RPM	Revolutions per Minute
RSA	Repetitive Sequence Analysis
SMAC	Sorbitol MacConkey Agar
ST	Heat Stable Toxin
STEC	Shigatoxigenic *E. coli*
Stx	Shiga Toxin
Tir	Translocator Intimin Receptor
TNF	Tumor Necrosis Factor
TTP	Thrombotic Thrombocytopaenic Purpura
UHQ	Ultra High Quality
UV	Ultra Violet light
VCC	Vancomycin-Cefixime-Cefsulodin
Vero	Monkey Kidney Cells
VT	Verocytotoxin
VTEC	Verocytotoxigenic *E. coli*
WHO	World Health Organization
λ	Lambda

LIST OF PUBLISHED AND SUBMITTED PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

PUBLICATIONS:

1. Müller, E.E., Ehlers, M.M. and Grabow, W.O.K. (2001) The Occurrence of *E. coli* O157:H7 in South African Water Sources Intended for Direct and Indirect Human Consumption. *Water Research.* **35,** 3085-3088

2. Müller, E.E., Grabow W.O.K., and Ehlers, M.M. (2001) Application of the Immunomagnetic Separation Method for the Detection and Isolation of *Escherichia coli* O157:H7 from Grounded Beef, Milk, Sewage and Environmental Waters. Submitted for publication in: *Journal of Medical Microbiology*

3. Müller, E.E., Taylor, M.B., Grabow, W.O.K. and Ehlers, M.M. (2001) Isolation and Characterization of *Escherichia coli* O157:H7 and Shiga Toxin - converting Bacteriophages from Strains of Human, Bovine and Porcine Origin. Submitted for publication in: *Water, Science and Technology.*

4. Müller E.E., Grabow, W.O.K., and Ehlers, M.M. (2001) Host range susceptibility of toxin-converting bacteriophages infecting *Escherichia coli* O157:H7. Submitted for publication in: *Journal of Medical Microbiology.*

5. Grabow, W.O.K., Müller, E.E., Ehlers, M.M., de Wet, C.M.E., Uys, M. and Clay, C.G. (2002) Occurrence of *E coli* O157:H7 and other pathogenic *E coli* strains in water sources intended for direct and indirect human consumption. Water Research Commission. WRC Report No 1068/1/01.

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1. Müller, E.E., Clay, C.G. and Grabow, W.O.K. (2000) Detection and isolation of *Escherichia coli* O157:H7 from sewage and environmental waters using immunomagnetic separation. Water Institute of Southern Africa. (WISA 2000 Conference). Sun City, South Africa 31 May-2 June 2000. Poster presentation.

2. Müller, E.E., Clay, C.G. and Grabow, W.O.K. (2000) Detection and isolation of *Escherichia coli* O157:H7 from sewage and environmental waters using immunomagnetic separation. Faculty Day, Faculty of Health Sciences. Poster presentation.

3. Müller, E.E., Taylor, M.B., Grabow, W.O.K. and Ehlers, M.M. (2001) Isolation and Characterization of *Escherichia coli* O157:H7 and Shiga Toxin - converting Bacteriophages from Strains of Human, Bovine and Porcine Origin. Faculty Day, Faculty of Health Sciences. Poster presentation.

4. Müller, E.E., Ehlers, M.M. and Grabow, W.O.K. (2002) The Occurrence of *E. coli* O157:H7 in South African Water Sources Intended for Direct and Indirect Human Consumption. South African Society for Microbiology Conference 2002 2-4 April 2002. Oral presentation.

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1. Müller, E.E., Clay, C.G. and Grabow, W.O.K. (2000) Improvement of the immunomagnetic separation method to detect *Escherichia coli* O157:H7 in sewage and environmental waters. The 1st World Congress of the International Water Association (IWA). Conference Preprints nr 7 (HRMP-A40). Health-Related Water Microbiology. Paris, France 3-7 July 2000. Poster presentation.

2. Müller, E.E., Taylor, M.B., Grabow, W.O.K. and Ehlers, M.M. (2001) Isolation and Characterization of *Escherichia coli* O157:H7 and Shiga Toxin - converting Bacteriophages from Strains of Human, Bovine and Porcine Origin. The 2nd World Congress of the International Water Association (IWA). (B0308). Health-Related Water Microbiology. Berlin, Germany 15-19 October 2001. Oral presentation

3. Müller, E.E., Grabow, W.O.K. and Ehlers, M.M. (2002) Characterisation of *Escherichia coli* O157:H7 isolated from water sources and animal reservoirs. Waterborne Pathogen Symosium, Lisbon, Portugal 22-25 September 2002. Accepted for poster presentation.

4. Müller, E.E., Grabow, W.O.K. and Ehlers, M.M. (2002) Repetitive sequence analysis of *Escherichia coli* O157:H7 isolated from water sources and animal reservoirs. Waterborne Pathogen Symosium, Lisbon, Portugal 22-25 September 2002. Accepted for oral presentation.

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DETECTION AND CHARACTERIZATION OF *E. COLI* O157:H7 AND INDUCED SHIGA TOXIN-2 CODING BACTERIOPHAGES

by

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SUMMARY

Escherichia coli O157:H7 is classified as a member of the enterohaemorrhagic *E. coli* (EHEC) family. These organisms are responsible for a variety of clinical manifestations ranging from non-bloody diarrhoea to gross bloody diarrhoea with complications that include haemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopaenic purpura (TTP). Infection occurs by the ingestion of faecally contaminated food products, water sources and through person-to-person contact. Outbreaks of *E. coli* O157:H7 have been reported worldwide although most outbreaks seem to be from countries in the Northern hemisphere. Very little information is available on the prevalence of *E. coli* O157:H7 in South Africa. The only data available on *E. coli* O157:H7 were from a 1992 outbreak in Swaziland with some cases spreading to the adjacent provinces of South Africa.

Selective methods were assessed and optimised to identify and isolate *E. coli* O157:H7 from food, water and faeces. These methods included culture techniques, immunomagnetic separation, immunoassays and molecular confirmation techniques. The methods optimised

and assessed in this study proved to be suitable for the detection and isolation of *E. coli* O157:H7 from environmental water, food and faecal samples. In addition to the isolation of *E. coli* O157:H7 from these sources, methods were also optimised for the characterisation of *E. coli* O157:H7 using repetitive sequence analysis and induction of shiga toxin (Stx)-converting phages which carry the genes coding for Stx from strains of *E. coli* O157:H7.

The prevalence of *E. coli* O157:H7 in human-, bovine- and porcine faeces, sewage and recreational waters was investigated in a selected region of South Africa. Data suggested a low prevalence in sewage (0.76%), recreational waters (0%) and human faecal (0%) samples with a higher prevalence among carriers such as cattle (12.5%) and pigs (14.29%).

UV-induced Stx-converting phages were examined and found to have different phage morphologies to the previously described lambdoid structure. In order to establish the host range susceptibility of these phages, all induced phages were subjected to conditions favourable for infecting *E. coli* O157:H7, non-O157 *E. coli* and other members of the enterobacteriaceae family including *Salmonella*, *Shigella*, *Enterobacter*, *Klebsiella*, and *Proteus*. These results have shown that Stx-phages were able to infect *Salmonella cholerasuis* and produce infectious progeny from these strains. Stx-converting phages propagated in *Salmonella cholerasuis* were able to re-infect strains of *E. coli* O157:H7.

This study has shown that IMS in combination with molecular techniques was a sensitive tool for the isolation, identification and characterisation of *E. coli* O157:H7 from different sources. Results indicated that Stx-phages induced from *E. coli* O157:H7 demonstrated lambdoid structure as well as phages with long hexagonal heads and long non-contractile tails.

OPSPORING EN KARAKTERISERING VAN *E. COLI* O157:H7 EN GE-INDUSEERDE SHIGA TOKSIEN-2 KODERENDE BAKTERIOFAGE

deur

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OPSOMMING

Escherichia coli O157:H7 kan as 'n lid van die enterohemorragiese *E. coli* familie geklassifiseer word. Hierdie organismes is verantwoordelik vir 'n verskeidenheid van kliniese manifestasies wat strek van nie-bloederige diaree tot erge bloederige diaree met komplikasies wat hemoragiese kolitis (HK), hemolitiese uremiese sindroom (HUS) en trombotiese trombositopeniese purpura (TTP) insluit. Infeksie geskied deur die inname van fekaal gekontamineerde voedselprodukte, waterbronne en deur persoon-tot-persoon kontak. Uitbrake van *E. coli* O157:H7 kom wereldwyd voor alhoewel die meeste uitbrake in lande in the Noordelike halfronde plaasvind. Baie min inligting is beskikbaar oor die voorkoms van *E. coli* O157:H7 in Suid-Afrika. Die enigste data beskikbaar oor *E. coli* O157:H7 is afkomstig van 'n uitbraak in 1992 in Swaziland met 'n paar gevalle wat versprei het na die aangrensende provinsies van Suid-Afrika.

Geselekteerde metodes was gevalueer en geoptimiseer vir die isolasie van *E. coli* O157:H7 uit voedsel, water en stoelgang. Hierdie metodes sluit in kultuur tegnieke, immunomagnetiese skeiding, immunotoetse en molekulêre tegnieke. Hierdie geoptimiseerde en ge-evalueerde metodes blyk toepaslik te wees vir die opsporing en isolasie van *E. coli* O157:H7 uit omgewingswaterbronne, voedsel en fekale monsters. Addisioneel is metodes geoptimiseer vir die karaktisering van *E. coli* O157:H7 deur gebruik te maak van herhaalbare nukleotied-volgorde analise van shiga toksien (Stx)-fage wat die gene bevat wat kodeer vir Stx vanuit *E. coli* O157:H7 stamme.

Daar is gekyk na die voorkoms van *E. coli* O157:H7 in 'n geselekteerde gebied van Suid-Afrika in menslike stoelgange, bees en vark mis, riool en ontspannings waterbronne. Data dui 'n lae voorkoms van *E. coli* O157:H7 aan in riool (0.76%), ontspannings water bronne (0%) en menslike stoelgange (0%) met 'n hoër voorkoms in draers soos beeste (12.5%) en varke (14.29%).

UV-geinduseerde Stx-fage is bestudeer en daar is bevind dat dié fage morfologies verskillend was van die voorheen beskryfde lambdoiëd struktuur. Om die gasheer vatbaarheid te bepaal is alle geinduseerde fage bloodgestel aan kondisies gunstig vir hierde fage om *E. coli* O157:H7, nie-O157 *E. coli* en ander lede van die enterobacteriaceae familie te infekteer. Dit sluit in *Salmonella*, *Shigella*, *Enterobacter*, *Klebsiella* en *Proteus*. Resultate dui aan dat Stx-fage instaat was om *Salmonella cholerasuis* te infekteer en om 'n infektiewe nageslag te produseer. Stx-fage wat in *Salmonella cholerasuis* gegroei het was instaat om *E. coli* O157:H7 te her-infekteer.

Hierdie studie het aangetoon dat IMS in kombinasie met molekulêre tegnieke, sensitiewe metodes was vir die isolasie, identifikasie en karakterisasie van *E. coli* O157:H7 vanuit verskillende bronne. Resultate het bewys dat Stx-fage geinduseerd uit *E. coli* O157:H7, lambdoiëde struktuur so wel as fage met lang heksagonale koppe en lang nie-saamtrekbare sterte bevat.

CHAPTER 1

INTRODUCTION

In 1982, investigation of two outbreaks of a bloody diarrheal syndrome led to the identification of a new bacterial pathogen, *Escherichia coli* 0157:H7. This pathogen, which belongs to the group of enterohaemorrhagic *E. coli* (EHEC) has emerged as an important cause of bloody diarrhea or haemorrhagic colitis (HC), which is characterized by severe crampy abdominal pain, watery diarrhea followed by grossly bloody diarrhea and little or no fever (Strockbine *et al.*, 1986). This organism is responsible for the serious microangiopathic disorder of haemolytic uremic syndrome (HUS) which can be defined by a triad of acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia (Nataro and Kaper, 1998).

The Shiga toxin family (Stx1 and Stx2) has been extensively reviewed (O'Brien *et al*, 1987, Okrend *et al.*, 1990, Tesh and O'Brien, 1991; O'Brien *et al.*, 1992; O'Brien and Holmes, 1996) and decided as the major virulence factor that leads to death and many other symptoms in patients infected with EHEC. It is also important to mention that EHEC possess other virulence factors which together with Stx are necessary for pathogenesis for example the *eae*A gene (*eae*, for *E. coli* attaching and effacing) and the enterohemolysin plasmid (Nataro and Kaper, 1998). Genes for both toxins are phage-encoded, which suggests that *E. coli* 0157:H7 may have acquired these toxin genes through phage-mediated transfer (Strockbine *et al.*, 1986). It is hypothesized that at some point a bacterial virus (phage) acquired an Stx gene during infection of a *Shigella* bacterium and subsequently transferred the gene to an enteropathogenic *E. coli* (Nataro and Kaper, 1998). This theory illustrates how acquisition of whole genes, sometimes from different bacterial species can markedly affect a microbe's pathogenic or epidemic potential (Wachsmuth *et al.*, 1997).

It was reported by O'Brien *et al.* (1984) that *E. coli* 0157:H7 strain 933, harbors two different toxin converting phages designated 933J and 933W and suggested that a family

of shiga-like toxin converting phages exist in nature. Phage 933J is a lambdoid phage that codes for Stx1 while phage 933W codes for Stx2 (O'Brien *et al.*, 1984). Lysogenization of *E. coli* K-12 with a toxin converting phage resulted in a dramatic increase in the amount of shiga-like toxin produced (O'Brien *et al.*, 1984). Therefore, converting phages could contain either the toxin structural genes or regulatory elements that act on structural genes already present in the host bacterium.

Waterborne transmission of *E. coli* O157:H7 has been reported from both recreational waters and contaminated drinking water (Swerdlow *et al.*, 1992; Keene *et al.*, 1994; Promed, 2000). A recent outbreak of *E. coli* O157:H7 in the small farming community of Walkerton in Ontario, Canada, attracted world-wide attention. More than 2000 people fell ill and six died when the borehole (raw water source of the water supply system) got contaminated by stormwater run-off containing cattle manure (ProMed, 2000). The treatment and chlorination processes for the water supply failed to control the number of *E. coli* O157:H7 in the final water (ProMed, 2000). This incident was seen as an example of new attitudes towards responsibility and accountability with major financial implications for water utilities and authorities concerned. The only documented waterborne outbreak of *E. coli* O157:H7 in Southern Africa was the 1992 outbreak in Swaziland (Effler *et al.*, 2001) with some cases spreading to the adjacent provinces of South Africa. This outbreak was linked to contaminated surface water supplies.

The high number of enterohaemorrhagic *E. coli* organisms isolated from the faeces of patients, has led to the concern that these organisms, especially *E. coli* O157:H7, could pose a significant health risk to people utilising contaminated water supplies (Griffin, 1995). The number of *E. coli* O157:H7 in sewage environments might give us an indication of the prevalence of these organisms in the human population. Foodborne transmission of *E. coli* O157:H7 is another important source of infection in humans (Griffin, 1995). The most common vehicle of transmission is through the ingestion of faecally contaminated meat products (Griffin, 1995). Cattle is the main reservoir of *E. coli* O157:H7, although it has been isolated from other animals such as chickens, pigs and sheep (Griffin and Tauxe, 1991; Griffin, 1995). A variety of food sources other than

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meat products have been implicated in the transmission of *E. coli* O157:H7: raw cow's milk and cheese, pasteurised milk, mayonnaise, apple cider, contaminated fruit and vegetables (Besser *et al.*, 1993; Griffin, 1995; McCarthy, 1996; Nataro and Kaper, 1998). In addition *E. coli* O157:H7 has the potential for person-to-person transmission once it is introduced into the community through a food or water vehicle (Paton and Paton, 1998). Person-to-person transmission occurs in day-care centres, nursing homes or where there is close contact between individuals (Karmali, 1989; Griffin, 1995). The modes of transmission for sporadic *E. coli* O157:H7 infections appear to be similar to those for outbreaks (Griffin, 1995).

Advances in the development of laboratory techniques to detect these organisms in human faeces have been crucial in the reliable diagnosis of *E. coli* O157:H7 infection. Immunomagnetic separation, culture techniques, cytotoxic activity, immunoassays, ELISA methods, DNA probes and PCR for the detection of genes coding for Stx, *eae*A, and pO157/ haemolysin are among the most common *E. coli* O157:H7 detection methods currently used (Nataro and Kaper, 1998). Serological and strain subtyping techniques proved valuable in *E. coli* O157:H7 diagnostical and epidemiological studies (Nataro and Kaper, 1998). Most of these methods were not optimized to detect *E. coli* O157:H7 in water but exclusively for diagnostic purposes. All waterborne transmission studies used direct culture techniques for *E. coli* O157:H7 isolation (Swerdlow *et al.*, 1992; Keene *et al.*, 1994; Ackman *et al.*, 1997).

It can be concluded that *E. coli* O157:H7 is globally recognized as an important cause of both epidemic and sporadic disease. More extensive studies are needed to assess the detection, isolation and prevalence of *E. coli* O157:H7 in water environments. According to Effler *et al.* (2001) more research is needed to determine the prevalence of *E. coli* O157:H7 in Africa. It is also necessary to determine the carriage rates among livestock in South Africa. This new information will be helpful to assess the potential for future outbreaks of *E. coli* O157:H7, specifically in South Africa.

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The objectives of this study were:

1. To assess techniques for the isolation of *E. coli* O157:H7 from sewage, river water, animal reservoirs and food products such as milk and beef.
2. To assess the prevalence of *E. coli* O157:H7 in selected sewage and environmental water samples in South Africa.
3. To assess the prevalence of *E. coli* O157:H7 among livestock (bovine and porcine) in South Africa.
4. To characterise *E. coli* O157:H7 isolates and induced Stx-converting phages using molecular techniques and electron microscopy.
5. To investigate the host range and Stx-phage susceptibility among non-pathogenic *E. coli* and other members of the enterobacteriaceae family.

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CHAPTER 2

Literature review

2.1 Introduction

Escherichia coli (*E. coli*) is the most investigated bacterium and the predominant facultative anaerobe of the human colonic flora (Drasar and Hill, 1974). The organism colonizes the infant gastrointestinal tract at birth or immediately after birth and remains confined to the intestinal lumen without harmful interference (Drasar and Hill, 1974). Previously *E. coli* was not considered to be a pathogen; however, it has become evident that *E. coli* can cause a variety of diseases in humans such as diarrhoea, dysentery, renal infections, septicaemia, pneumonia and meningitis (Drasar and Hill, 1974).

E. coli is rod-shaped and about 2 µm long and 0.7 µm in diameter (Smith-Keary, 1988). Three types of appendage may be found attached to the outer membrane (Smith-Keary, 1988):

(i) **Flagellae:** These are responsible for motility of the cell, each about 20 µm long and 20 nm in diameter (Smith-Keary, 1988).

(ii) **Common pili or fimbriae**: These are smaller than the flagellae (1 µm x 10 nm) and can be numerous. The presence of surface adherence fimbriae is a property of virtually all *E. coli* strains (Levine *et al.*, 1984; Vial *et al.*, 1988). Pathogenic *E. coli* strains possess specific fimbrial antigens that enhance their intestinal colonizing ability and adherence to host cells situated in the small bowel mucosa (Levine *et al.*, 1984; Vial *et al.*, 1988).

(iii) **The F- or sex-pilus:** A single sex-pilus may be present on cells that harbour an F-factor or other conjugate plasmid. These pili are plasmid encoded. They have an unusual role of providing the specific receptor sites for certain bacteriophages (Smith-Keary, 1988).

There are more than 160 known serotypes of *E. coli* (Salyers and Whitt, 1994). Classification of *E. coli* strains based on serology that identifies the "O" and "H" antigens and the capsular or "K" antigens, is still used (Riemann and Cliver, 1998). This is for practical reasons because serology is useful in tracing the source of disease outbreaks (Riemann and Cliver, 1998).

The *E. coli* genome versatility is conferred by two genetic configurations: virulence-related plasmids and chromosomal pathogenicity islands (Nataro and Kaper, 1998). *E. coli* has been shown to carry at least one virulence-related property on a plasmid that could encode for factors such as host cell attachment, host invasion and toxin production (Levine *et al.*, 1984; Vial *et al.*, 1988; Nataro and Kaper, 1998). The chromosomal virulence genes of some strains of *E. coli* are organized as a cluster referred to as a pathogenicity island (Nataro and Kaper, 1998). Phage DNA (additional virulence factors) carried into the host *E. coli* by toxin-converting phages are incorporated in the host chromosome (O'Brien *et al.*, 1984). The mechanism by which *E. coli* causes disease varies between strains and these strains can be grouped depending on which mechanism is used by a particular strain (Nataro and Kaper, 1998). At present, six groups of pathogenic *E. coli* have been identified (Nataro and Kaper, 1998):

(i) Enterotoxigenic *E. coli* (ETEC)
(ii) Enteropathogenic *E. coli* (EPEC)
(iii) Enteroinvasive *E. coli* (EIEC)
(iv) Enteroaggregative *E. coli* (EAEC)
(v) Enterohaemorrhagic *E. coli* (EHEC)
(vi) Diffusely adherent *E. coli* (DAEC)

2.2 Enterotoxigenic *E. coli*

Enterotoxigenic *E. coli* (ETEC) strains are one of the major causes of travelers' diarrhoea and dehydrating diarrhoeal illnesses in children in less-developed countries (Nataro and Kaper, 1998). ETEC belongs to a range of serogroups that is different to those associated with other groups of pathogenic *E. coli* (Nataro and Kaper, 1998). These organisms adhere to the gut wall by colonization factors, where they produce heat-stable (ST) and heat-labile (LT) enterotoxins (Nataro and Kaper, 1998) (Fig. 2.1).

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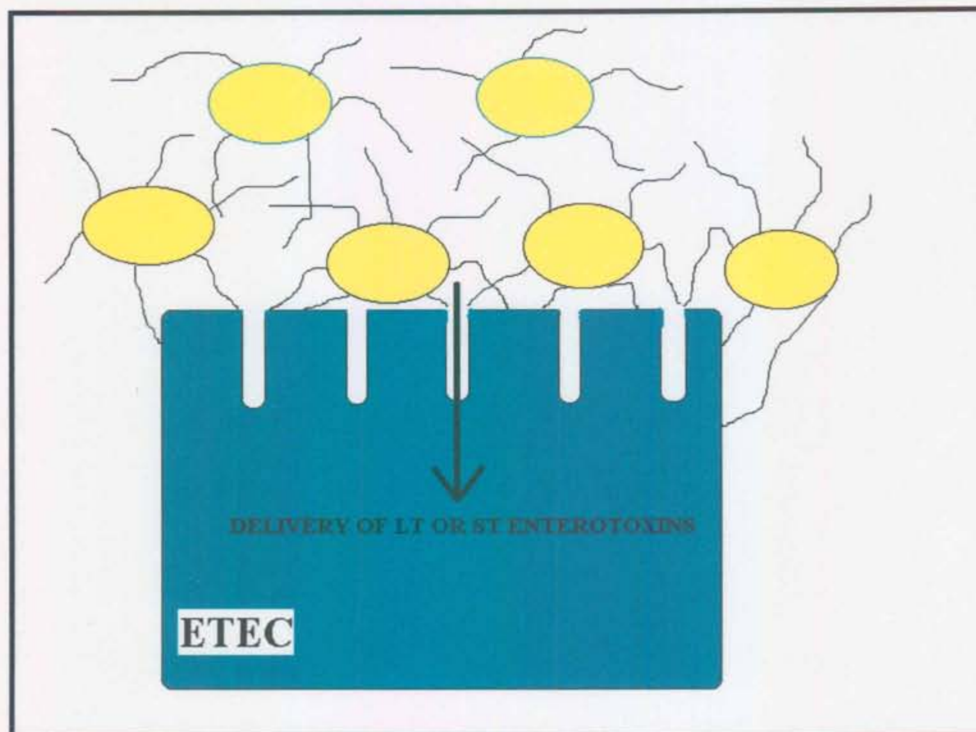

FIGURE 2.1: Schematic representation of the enterotoxigenic *E. coli* pathogenic scheme (Nataro and Kaper, 1998)

2.2.1 Heat-stable enterotoxins

Heat-stable enterotoxins (ST) are almost identical to the *Yersinia enterocolitica* heat-stable enterotoxin and similar to ST produced by *Vibrio cholerae* non-O1 strains (Nataro and Kaper, 1998). These toxins are small, monomeric toxins that contain multiple cysteine residues, whose disulfide bonds account for the heat stability of these toxins (Nataro and Kaper, 1998). The two classes of STs (STa and STb) differ in structure and mechanism of action (De Sauvage *et al.*, 1992; Vaandrager *et al.*, 1994).

(i) STa

Heat-stable enterotoxin-a (STa) is associated with disease in both humans and animals (de Sauvage *et al.*, 1992). STa consists of a 19-amino-acid peptide with a molecular mass of ca. 2 kDa (De Sauvage *et al.*, 1992; Vaandrager *et al.*, 1994). The major receptor for STa is a membrane-spanning enzyme called guanylate cyclase C (GC-C) (De Sauvage *et al.*,

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1992; Vaandrager *et al.*, 1994). The binding of STa to GC-C stimulates guanylate cyclase (GC) activity, leading to increased intracellular cyclic guanylate monophosphate (cGMP) levels (De Sauvage *et al.*, 1992; Vaandrager *et al.*, 1994). The increased cGMP levels lead to stimulation of chloride secretion and inhibition of sodium chloride absorption, resulting in a net intestinal fluid secretion in the host (Crane *et al.*, 1992; Mezoff *et al.*, 1992; Sears and Kaper, 1996) (Fig 2.2).

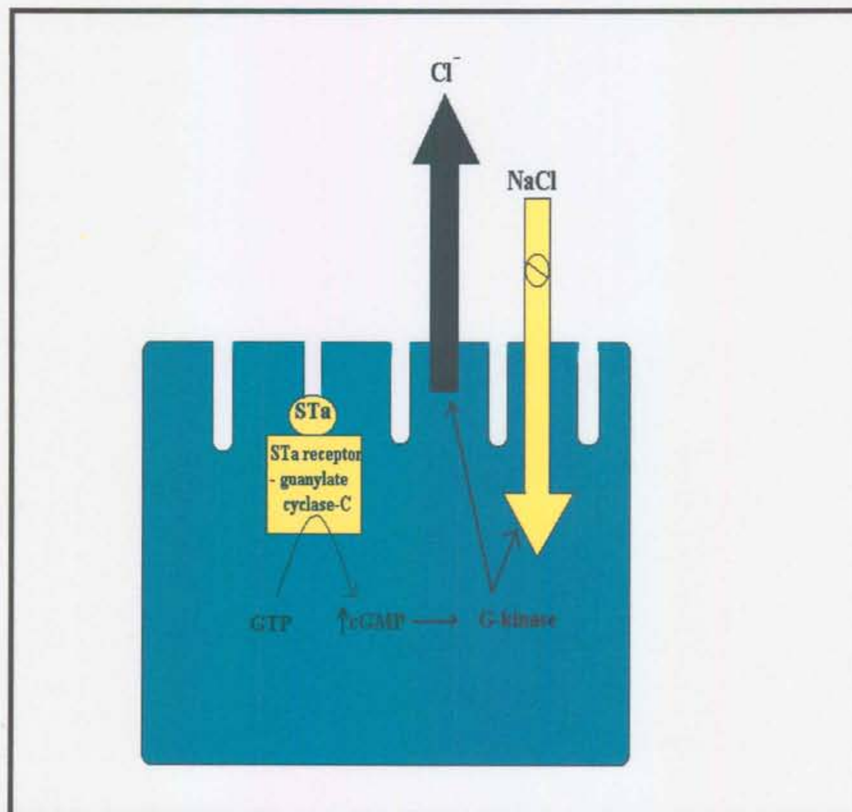

FIGURE 2.2: Schematic representation of the action mechanism of the STa class of heat-stabile enterotoxin of enterotoxigenic *E. coli* (Nataro and Kaper, 1998)

ii) STb

Heat-stable enterotoxin-b (STb) is associated primarily with diarrhoea in piglets, although some human ETEC isolates expressing STb have been reported (Arriaga *et al.*, 1995). STb is synthesized as a 71-amino-acid precursor protein, which is processed to a mature

48- amino-acid protein with a molecular weight of 5.1 kDa (Dreyfus *et al.*, 1993; Arriaga *et al.*, 1995). STb induces histological damage in the intestinal epithelium of the host, with loss of villus epithelial cells and partial villus atrophy (Dreyfus *et al.*, 1993; Arriaga *et al.*, 1995). STb stimulates the secretion of bicarbonate from intestinal cells in the host (Sears and Kaper, 1996).

2.2.2 Heat-labile enterotoxins

The heat-labile enterotoxins (LTs) are related in structure and function to cholera enterotoxin which is expressed by *Vibrio cholerae* O1 strains (Sixma *et al.*, 1993). There are two major serogroups of LT: i) LT-I and ii) LT-II (Sears and Kaper, 1996). LT-I is associated with disease in both humans and animals, while LT-II is found mainly in animals (Sears and Kaper, 1996).

i) LT-I

Heat-labile enterotoxin-I (LT-I) is an oligomeric toxin composed of one 28 kDa A subunit and five identical 11.5 kDa B subunits (Lencer *et al.*, 1995). The A subunit is primarily responsible for the enzymatic activity of the toxin (Lencer *et al.*, 1995). LT-I shares some of the same characteristics as cholera toxin which includes structure, primary receptor identity and enzymatic activity (Lencer *et al.*, 1995). After binding to the host cell membrane the toxin enters the cell by endocytosis and is translocated through the cell membrane (Lencer *et al.*, 1995). LT targets the host enzyme adenylate cyclase which is situated in the intestinal epithelial cells of the host (Lencer *et al.*, 1995). This leads to an increase in the levels of intracellular cyclic AMP (cAMP) (Nataro and Kaper, 1998). cAMP-dependent protein kinase (A kinase) is activated, which leads to phosphorylation of chloride channels (Nataro and Kaper, 1998). The net result is the stimulation of Cl^- secretion and inhibition of NaCl absorption, resulting in osmotic diarrhoea in the host (Nataro and Kaper, 1998) (Fig. 2.3).

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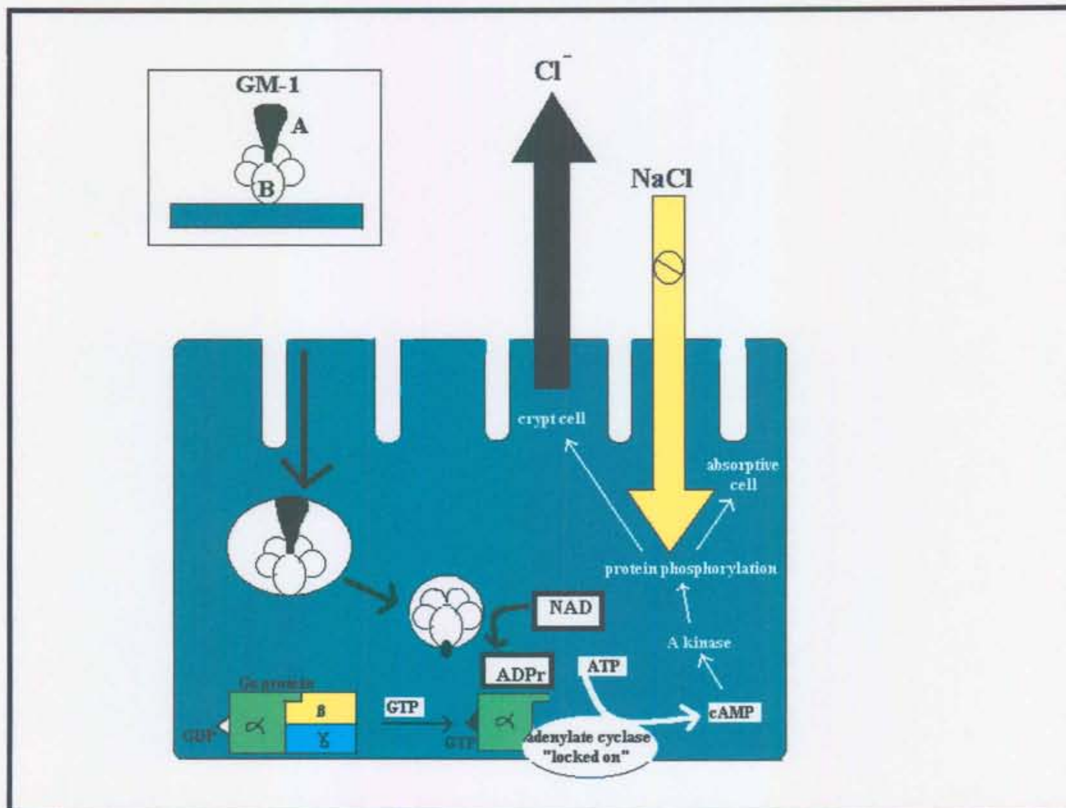

FIGURE 2.3: The LT-I action mechanism of heat-labile enterotoxigenic *E. coli* (Nataro and Kaper, 1998)

ii) LT-II

There are several similarities between heat-labile enterotoxin-II (LT-II) and LT-I except for the fact that LT-II have been isolated primarily from animals and rarely from humans (Sears and Kaper, 1996). The LT-II serogroup showed 55% to 57% identity to LT-I in the A subunit (Sears and Kaper, 1996). The LT-II B subunit showed no homology to the B subunit of LT-I (Sears and Kaper, 1996; Guth *et al.*, 1997). LT-II comprises of two antigenic variants, LT-IIa and LT-IIb, which share 71% and 66% identity in the A and B subunit sequences of the toxin, respectively (Sears and Kaper, 1996; Guth *et al.*, 1997). LT-II increases intracellular cyclic-AMP levels similar to the mechanism of LT-I by activating adenylate cyclase in cell culture systems (Sears and Kaper, 1996). There is no evidence to suggest that LT-II is associated with human or animal disease even though it

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has been isolated from animals and some human individuals (Sears and Kaper, 1996).

2.3 Enteropathogenic *E. coli*

Epidemiological studies indicated that enteropathogenic *E. coli* (EPEC) is one of the major causes of infantile diarrhoea in developing countries (Levine, 1987; Gross, 1990; Nataro and Kaper, 1998). EPEC strains are not associated with diarrhoeal illness in older children or adults (Levine, 1987). The classic histopathological lesion in the human intestine is caused by the close adherence of the bacteria to the enterocyte membrane, colonization of the epithelial cells of the small intestine and destruction of the microvilli (Moon *et al.*, 1983). This lesion is termed the attaching-and-effacing (AE) lesion (Moon *et al.*, 1983). The damage to the microvilli in the small intestine changes the balance between secretion and absorption, resulting in a watery diarrhoea with mucus (Moon *et al.*, 1983). This lesion is quite different to the histopathology of *V. cholerae* and ETEC with regard to adherence to the epithelial cell membrane (Moon *et al.*, 1983). EPEC adherence can be either localized or diffuse and is plasmid mediated (Moon *et al.*, 1983). The plasmid is referred to as the EPEC adherence factor (EAF) plasmid (Baldini *et al.*, 1983; Nataro *et al.*, 1985). Girón and co-workers (1991), reported the identity of the mediated localized adherence factor. He described the 7 nm diameter fimbriae produced by EPEC strains (encoded by 13 genes on the EAF plasmid) which aggregated to form bundles, thereby naming it "Bundle-forming pillus" (BFP) (Girón *et al.*, 1991) (Fig 2.4). BFP are involved in bacterium-bacterium adherence and there is no proof that BFP mediates adherence to epithelial cells (Baldwin *et al.*, 1991). Adherence to the host epithelial is mediated by EAF plasmid (Baldini *et al.*, 1983). Infection with EPEC increases the intracellular calcium levels in the epithelial cells and triggers the release of inositol phosphates (IP_3 and IP_4) in infected cells (Baldwin *et al.*, 1991).

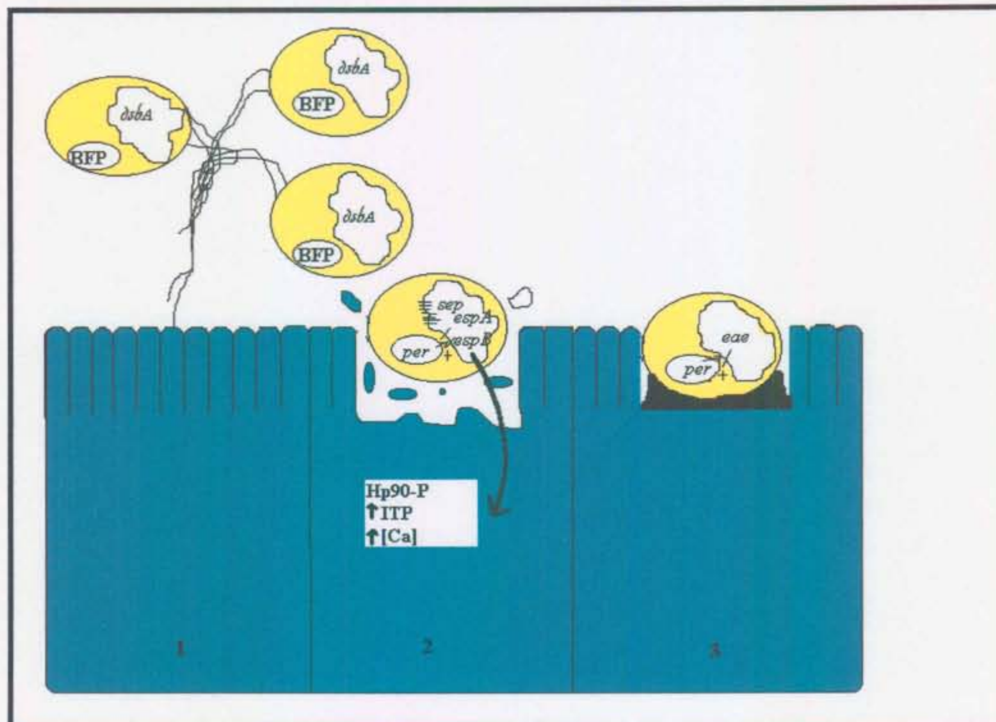

FIGURE 2.4: Three stage model of EPEC pathogenesis. The first stage represents the interaction of the BFP of the bacteria with the enterocyte layer. The second stage represents the *eae* and other genes that are activated, causing dissolution of the microvillar structure. During the third stage the bacterium binds to the epithelial membrane via the protein intimin (Donnenberg *et al.*, 1993; modified by Nataro and Kaper, 1998).

Activation of phosphokinase C (PKC) (one of 2 kinases activated by EPEC adherence to epithelial cells) induces rapid changes in intestinal water and electrolyte secretion (Nataro and Kaper, 1998). The bacterial membrane protein, intimin, mediates the adherence of EPEC to epithelial cells (Nataro and Kaper, 1998). Jerse and co-workers (1990) first reported the gene which codes for intimin (*eae*, for *E. coli* attaching and effacing). The *eae* gene is present in all EPEC, EHEC and other strains capable of producing the AE histopathology (Nataro and Kaper, 1998). All these mechanisms are involved in the onset of diarrhoea due to EPEC (Nataro and Kaper, 1998). The main features which define EPEC are the production of the AE lesion and the absences of shiga toxin. The latter toxin is found in all EHEC strains, many of which also produce AE

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histopathology (Koornhof, 2001).

2.4 Enteroinvasive *E. coli*

Enteroinvasive *E. coli* (EIEC) are similar to *Shigella* species in their ability to cause watery diarrhoea that develops into typical scanty dysenteric stools containing blood and mucus (Nataro and Kaper, 1998). EIEC possesses the biochemical profile of *E. coli* and the phenotypic characteristics of *Shigella* spp.(Nataro and Kaper, 1998). The ability of EIEC to invade colonic mucosal cells is due to virulence genes present on the p*Inv* plasmid (Nataro and Kaper, 1998). The mode of pathogenesis includes: (i) epithelial cell penetration by induced endocytosis, (ii) lysis of the endocytic vacuole, (iii) intracellular multiplication, (iv) directional movement through the cytoplasm and (v) extention into adjacent epithelial cells after lysis of the cells (Sansonetti, 1992; Goldberg and Sansonetti, 1993; Nataro and Deng, 1993) (Fig. 2.5). The incidence of EIEC in developed countries is low (Nataro and Kaper, 1998). Documented outbreaks of EIEC are foodborne or waterborne, although person-to-person transmission does occur (Harris *et al.*, 1985).

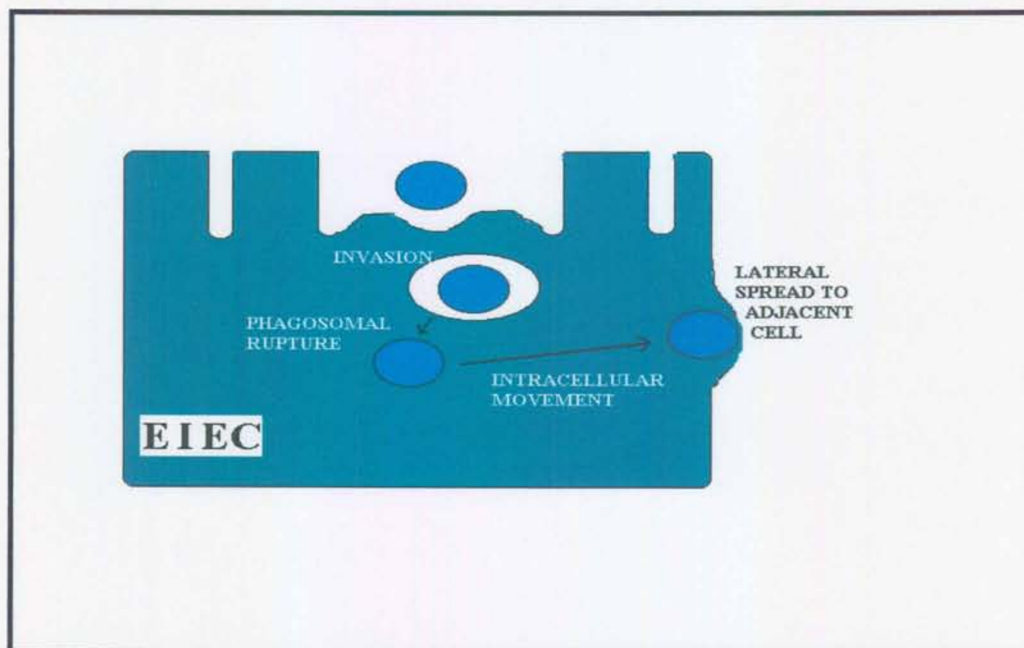

FIGURE 2.5: Schematic representation of the pathogenic scheme of enteroinvasive *E. coli* (Nataro and Kaper, 1998)

2.5 Diffusely adherent *E. coli*

Little is known about diffusely adherent *E. coli* (DAEC) which was initially used to refer to any HEp-2-adherent *E. coli* strain that did not form EPEC-like microcolonies (Nataro and Kaper, 1998). This group of organisms is now recognized as an independent category of diarrhoeagenic *E. coli* (Nataro and Kaper, 1998). Most epidemiological features of DAEC such as mode of transmission and acquisition are undetermined (Nataro and Kaper, 1998).

2.6 Enterohaemorrhagic *E. coli*

Enterohaemorrhagic *E. coli* (EHEC), known as Shiga-like toxin-producing *E. coli* or Vero cytotoxin-producing *E. coli*, is characterized by its ability to produce a toxin that is cytotoxic to HeLa (human cervical cancer cells) and Vero (monkey kidney) cells (Ismaili *et al.*,1995). The primary site of infection in humans is the colon (Ismaili *et al.*, 1995). In animal models, EHEC strains produce the same attachment-effacement (A/E) histopathology that is seen with EPEC strains (Ismaili *et al.*, 1995). According to Ismaili *et al.* (1995), there are some differences between the human cellular response to EPEC and the response to EHEC.

In addition to the AE lesion, EHEC strains produce cytotoxins known as Shiga toxins, which are among the most potent of all bacterial toxins (Takeda *et al.*, 1993). Two distinct types of the toxin, Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) and several variants of both toxins have been reported (Takeda *et al.*, 1993). A single EHEC strain may express Stx1, Stx2, both toxins or even multiple forms of Stx2 (Takeda *et al.*, 1993).

EHEC produces a variety of clinical syndromes including bloody and non-bloody diarrhoea, haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS)(Nataro and Kaper, 1998). EHEC belongs to different O serogroups but those of serogroup O157 are the most important in human disease (Nataro and Kaper, 1998). The *Escherichia coli* O157:H7 strain has tended to dominate the world literature on EHEC. Infections caused by *E. coli* O157:H7 are now recognized more frequently, which reflects increased interest in the incidence and detection of this organism (Nataro and Kaper, 1998).

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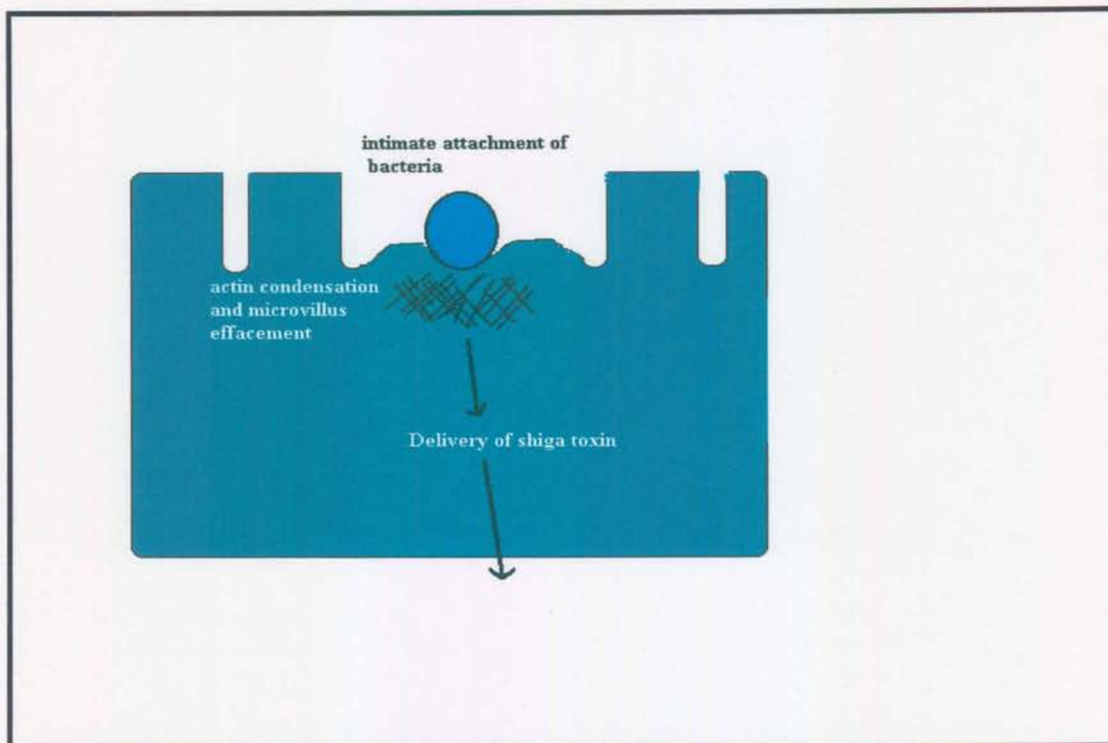

FIGURE 2.6: Schematic representation of the pathogenic scheme of enterohaemorrhagic *E. coli* (Nataro and Kaper, 1998)

2.7 *E. coli* O157:H7

In the late 1970's and early 1980's there was an interest in the pathogenicity of verocytotoxins in strains of enteropathogenic *Escherichia coli* (EPEC) (Konowalchuk *et al.*, 1977; Karmali *et al.*, 1983; Wells *et al.*, 1983). These cytotoxins were first described in 1977 by Konowalchuk and colleagues in Canada (Konowalchuk *et al.*, 1977). Subsequent studies have led to the recognition of a new class of *E. coli*, the enterohaemorrhagic *E. coli* (EHEC) (Nataro and Kaper, 1998).

It was since 1982 that EHEC have been recognized as an important aetiological agent of diarrhoeal diseases in man and animals (Karmali *et al.*, 1983; Wells *et al.*, 1983; Karmali, 1989). *E. coli* O157 was described as a "rare" serotype (Karmali *et al.*, 1983). Studies conducted between 1983 and 1985 in the United States and Canada, have linked EHEC infection to haemorrhagic colitis (HC) and it had a close relation with the classical form of haemolytic uraemic syndrome (HUS) (Karmali *et al.*, 1985). As a result of these and other studies Orskov *et al.* (1987) re-examined

isolates of *E. coli* belonging to the O157 serogroup that had been submitted to the International Escherichia and Klebsiella Centre. Three isolates were found that had the H7 antigen (Orskov *et al.*, 1987). These three isolates were from the faeces of one animal out of a batch of 39 calves with colibacillosis in Argentina (Orskov *et al.*, 1987). Orskov and colleagues (1987) speculated that cattle might be the reservoir for these organisms.

From the first description of *E. coli* O157:H7 in 1982, this serotype has tended to dominate the world literature on EHEC. More than 25 serotypes of EHEC have been isolated, with *E. coli* O157:H7 the predominant EHEC serotype isolated in outbreaks of haemorrhagic colitis in countries world wide (Goldwater and Bettelheim, 1998). It was speculated that *E. coli* O157:H7 were derived from one successful clone of *E. coli* that has spread world wide (Goldwater and Bettelheim, 1998). Outbreaks associated with this strain caused bloody diarrhoea which in some cases was described as "all blood and no stool", thus the symptom being named haemorrhagic colitis (HC) (Ostroff *et al.*, 1989). The haemolytic uremic syndrome (HUS) was first described in 1955 (Gasser *et al.*, 1955). In 1968, Kibel and Barnard suggested, (while describing HUS in South Africa), that a mutant strain of *E. coli* (mutated by a bacteriophage) may be responsible for this syndrome.

The major developments occurred in the 1980's when the clinical symptoms were linked by researchers to *E. coli* O157:H7 infection (Nataro and Kaper, 1998). It was found that *E. coli* O157:H7 did not produce any *E. coli* toxins, such as heat-labile enterotoxin (LT) and heat-stabile enterotoxin (ST), nor was it an enteroinvasive *E. coli* (Takeda, 1997). In the 1980's, it was first recognised by researchers that Stx is encoded by a bacteriophage in *E. coli* (Karmali *et al.*, 1983; Wells *et al.*, 1983; Karmali, 1989). Thus, *E. coli* O157:H7 and the diseases it causes have become prominent.

EHEC was originally defined as organisms that cause disease similar to *E. coli* O157:H7 (Levine, 1987). Today EHEC are defined by: i) the ability to produce one or more cytotoxins called Shiga toxins (Stxs) and ii) the ability to cause attaching and effacing (AE) lesions (Nataro and Kaper, 1998). Our understanding of the mechanisms by which *E. coli* O157:H7 cause disease has increased considerably in the last fifteen years. Considering its importance as a major public

health risk all around the world, epidemiological, clinical and laboratory investigations have been carried out (Nataro and Kaper, 1998). This knowledge has led to the development of improved methods for the isolation and identification of these organisms.

2.7.1 Epidemiology

The epidemiology of *E. coli* O157:H7 infection which includes the incidence, modes of transmission and infectious dose are discussed in this section. All these factors are necessary when proper diagnosis of outbreaks or sporadic *E. coli* O157:H7 infection are reported.

2.7.1.1 Incidence

Sporadic infections comprise the major disease burden of EHEC even though outbreaks involving hundreds of individuals seem to attract the most attention. The failure of clinical laboratories to screen for this organism complicates estimates on the burden of disease caused by *E. coli* O157:H7 and the incidence of this organism (Griffin and Tauxe, 1991). After *E. coli* O157:H7 was identified as a major cause of haemorrhagic colitis, an international laboratory campaign in the United States, Canada and the United Kingdom arose to review their records to look for *E. coli* O157:H7 (Riley *et al.*, 1983). More than 3 000 *E. coli* strains serotyped by the Centers for Disease Control (CDC) in the United States between 1973 and 1983 were screened (Riley *et al.*, 1983). One O157:H7 was isolated in 1975 from a California woman with abdominal cramps and grossly bloody diarrhoea (Riley *et al.*, 1983). The CDC laboratory in Canada reviewed more than 2 000 *E. coli* strains isolated from patients with diarrhoea between1978 and 1982 (Johnson *et al.*, 1983). They isolated the organism from six patients, two of whom had haemorrhagic colitis (Johnson *et al.*, 1983). The Public Health Laboratory in the United Kingdom found one O157:H7 strain among 15 000 *E. coli* that were serotyped between 1978 and 1982 (Day *et al.*, 1983). This suggested that the rate of infection due to *E. coli* O157:H7 was on the increase, although the data were limited and were difficult to interpret because of inadequate laboratory screening methods (Day *et al.*, 1983).

The reported incidence of these infections is likely to change over time as a result of better laboratory methods. The CDC estimated the annual disease burden of *E. coli* O157:H7 in the United States to be more than 20 000 infections and with as many as 250 deaths (Boyce *et al.*,

1995). According to Slutsker *et al.* (1997), *E. coli* O157:H7 is the pathogen most frequently isolated from stool specimens that contain visible blood (Slutsker *et al.*, 1997). The World Health Organization (WHO) is concerned about this organism because bloody diarrhoea is a major cause of morbidity and mortality among children in developing countries in the southern hemisphere, including South Africa (WHO, 1997). Infections caused by this organism are not notifiable in South Africa. Information is limited concerning *E. coli* O157:H7 and other EHEC serotypes in humans and animals.

According to the WHO Consultation report on the Prevention and Control of Enterohaemorrhagic *Escherichia coli* Infections (1997), there have been three cases of *E. coli* O157 identified in Pretoria since 1988. The first case of *E. coli* O157:H7 in South Africa associated with haemorrhagic colitis was reported by Browning *et al.* (1990). One case was associated with eating a hamburger from a fast-food outlet (WHO, 1997). A large number of haemorrhagic colitis cases, caused by non-motile *E. coli* O157 has been identified in Swaziland and adjacent provinces such as Mpumalanga and Kwa-Zulu Natal (WHO, 1997; Effler *et al.*, 2001). Since 1982, 10 *E. coli* O157 organisms were isolated from pigs with haemorrhagic colitis (WHO, 1997). The low incidence of this organism in many countries, including South Africa, is in accordance with studies including those of Cravioto *et al.* (1990) and Albert *et al.* (1995) where EHEC organisms had been actively sought. To reduce the incidence of this organism worldwide, the cooperative efforts of the scientific research community, public- and environmental health agencies and all other concerned parties are of crucial importance.

2.7.1.2 Modes of transmission of *E. coli* O157:H7

Foodborne transmission of *E. coli* O157:H7 is probably the most important source of infection in humans (Griffin and Tauxe, 1991). Transmission mostly occurs by the ingestion of faecally contaminated meat products (Griffin and Tauxe, 1991). Undercooked minced or ground beef used for the preparation of hamburgers, contaminated with this organism allow the survival of the pathogens (Nataro and Kaper, 1998). Cattle are the main reservoir for *E. coli* O157:H7, although it has been isolated from other animals such as chickens, pigs and sheep (Griffin, 1995). Contaminated meat products from these animals might impose a health risk if ingested (Nataro and Kaper, 1998). An outbreak of diarrhoea due to strains of *E. coli* O157:H7 among native

Canadians in the Northern Territories of Canada (Orr *et al.*, 1994) has implicated the ingestion of caribou meat, suggesting the possibility that these animals are carriers of *E. coli* O157:H7. A variety of food sources other than meat products have been implicated in the transmission of *E. coli* O157:H7 such as raw cow's milk and cheese, pasteurized milk, apple cider and contaminated fruit and vegetables (Nataro and Kaper, 1998).

Waterborne transmission of *E. coli* O157:H7 has been reported from both recreational waters and contaminated drinking water supplies (Swerdlow *et al.*, 1992; Keene *et al.*, 1994). Another incident of waterborne transmission was reported in the United States in July 1998, with 200 suspect illnesses in 13 states (DeNileon, 1998). This water-borne outbreak constituted one of the largest US outbreak of *E. coli* O157:H7 linked to municipal water (DeNileon, 1998).

Another issue of concern with this organism is the potential for secondary person-to-person transmission, by a direct faecal-oral route, once it is introduced into the community through a food or water vehicle. This mode of transmission usually occurs in day-care centres, nursing homes or places where there is close contact between individuals affected (Griffin and Tauxe, 1991). In a large outbreak of *E. coli* O157:H7 in a Canadian nursing home, both the patients and the personnel was affected (Griffin and Tauxe, 1991). Some of the residents consumed sandwiches contaminated with the organism (Griffin and Tauxe, 1991). The staff who cared for the sick got sick, which was indicative of person-to-person transmission (Griffin and Tauxe, 1991). The importance of person-to-person transmission in day-care centres should be stressed since small children are particularly susceptible to developing HUS, which is one of the complications of *E. coli* O157:H7 (Griffin and Tauxe, 1991).

The modes of transmission for sporadic *E. coli* O157:H7 infections appear to be similar to those for outbreaks (Griffin and Tauxe, 1991). Persons with high exposure to cattle and ground beef due to their professions, may be at an increased risk. Three cases of laboratory-acquired infection have been reported (Ostroff *et al.*, 1989; Booth and Rowe, 1993; Burnens *et al.*, 1993). The vehicles of transmission of *E. coli* O157:H7 in 75 U.S. outbreaks between 1982 and 1995 are summarized in Table 2.1.

Table 2.1. Vehicles of transmission of *E. coli* O157:H7 in 75 U.S. outbreaks between 1982 and 1995 (Centers for Disease Control and Prevention unpublished data)

Vehicles of transmission	Number of outbreaks (%)
Ground beef	25 (50)
Other beef	5 (9)
Raw milk	2 (4)
Non-cattle foods	17 (30)
Water	3 (5)
Venison	1 (2)

2.7.1.3 Outbreaks

E. coli O157:H7 was first recognized as a pathogen in 1982 with the occurrence of two outbreaks in the United States and one in Canada (Takeda, 1997). Numerous outbreaks of *E. coli* O157:H7 and non-O157:H7, including in the U.S.A, Scotland and Japan (Table 2.2 and Table 2.3) have been reported around the world (Centers for Disease Control and Prevention, 1995). Outbreaks were detected because of a cluster of haemolytic uraemic syndrome or thrombocytopenic purpura cases or because a large number of people were hospitalized simultaneously for severe bloody diarrhoea (Griffin, 1995). Most of these outbreaks have been associated with the consumption of foods of bovine origin (Griffin, 1995).

Undercooked hamburgers, roast beef, raw milk, other meat sources such as porcine, avian and sheep have directly been associated with outbreaks (Griffin and Tauxe, 1991; Griffin, 1995). Outbreaks of *E. coli* associated with mayonnaise (Griffin, 1995), unpasteurized apple juice (Besser *et al.*, 1996; McCarthy, 1996) and salami (Centers for Disease Control, 1995) illustrated the capability of the organism to adapt to low pH conditions. The organism can survive acidic conditions of pH 3.4 for several days (Zhao *et al.*, 1993; Benjamin and Datta, 1995; Leyer *et al.*, 1995).

Table 2.2: Major worldwide outbreaks of enterohaemorrhagic *E. coli* infection (Takeda, 1997)[a]

Year	Location	Setting	Vehicle of transmission	Serotype
1982	USA	Community	Hamburger	O157:H7
1982	USA	Community	Hamburger	O157:H7
1982	Canada	Nursing home	Hamburger	O157:H7
1983	Canada	Community	Non-food	O157:H7
1984	USA	Nursing home	Hamburger	O157:H7
1984	USA	Day-care centre	Non-food	O157:H7
1985	Canada	Nursing home	Cold sandwich	O157:H7
1985	UK	Community	Raw potato	O157:H7
1985	USA	Community	Ground beef	O157:H7
1986	Canada	School	Raw milk	O157:H7
1987	USA	Mentally handicapped inst.	Ground beef	O157:H7
1988	USA	School	Precooked meat patties	O157:H7
1989	USA	Community	Municipal water	O157:H7
1990	USA	Community	Roast beef	O157:H7
1990	USA	Psychogeriatric ward	Non-food	O157:H7
1990	UK	Restaurant	-	O157:H7
1991	USA	Community	Non-food	O157:H7
1991	USA	Community	Fresh apple cider	O157:H7
1991	Canada	Community	Non-food	O157:H7
1991	UK	Community	Yogurt	O157:H7
1992	Germany	Day-care centre	Non-food	O157:H7
1992	Italy	Community	Non-food	O111:H-
1993	USA	Community	Hamburger	O157:H7
1993	Italy	Community	Non-food	O158,O111 and O86
1995	Australia	Community	Metwurst	O111:H-
1996	UK	Community	Cold cooked meat	O157

-: source unidentified

a) Not included in the study is an outbreak in 1996 in Sakai, Japan, where 5727 cases were reported. Smaller outbreaks during the same year occurred in Japan, totalling 9451 reported cases. No direct vehicle of transmission was identified although the organism was isolated from twelve samples of raw beef (WHO, 1997).

Table 2.3: Outbreaks and sporadic disease by non-O157:H7 and non-O111 enterohaemorrhagic *E. coli* (Goldwater and Bettelheim, 1998)

Year	Location	Setting	Nr. of cases	Serotype
1979	UK	BD:sporadic/community	3	O26
1989-91	UK	HC:HUS	-	O26:H-
1987-90	Germany		-	O26:H11
1984-93	Japan	Sporadic/intrafamilial	16	O26:H11
1978-79	New Zealand	Diarrhoea	-	O26:H11
1992	Australia	HUS	1	O26:H11
1988-95	Czech Rep.	HUS	5	O26:H11
1987-90	Germany	HUS	-	O91:H21
1987-89	France	HUS	6	O103:H2
	USA	Urinary infection	1	O103:H2
1994	USA	Outbreak/gastroenteritis	17	O104:H21
1997	Australia	BD TTP	1	O133:H21
1984	Japan	Diarrhoea	100	O145:H-
1991	Japan	School	89	Ont:H19
1984-93	Japan	?	1	Ont:H19
1995	Japan	?	4	O165

BD: Bloody diarrhoea; TTP: Thrombotic thrombocytopenic purpura; HUS: Haemolytic uraemic syndrome; -: Unknown

The first outbreak of *E. coli* O157:H7 in Great Britain was caused by workers packing potatoes in peat which was contaminated with calf manure (Griffin, 1995). One of the major outbreaks in the United States which brought the situation into public focus was the multistate outbreak in 1993 (Griffin, 1995). This outbreak which affected 583 persons was caused by the intake of hamburgers in a restaurant which was part of a popular hamburger restaurant chain (Griffin, 1995). Other outbreaks have occurred in various settings such as day-care centres, schools and nursing homes (Griffin, 1995). With modern food processing procedures and distribution methods used today, it is possible to facilitate large scale outbreaks (Griffin, 1995). Such outbreaks include the outbreak in Sakai City in July 1996 in Japan which affected approximately 6 000 children and the Juner 1996 outbreak in central Scotland affecting 490 persons of whom 18 adults died (ProMed, 1999).

Water-borne infections and outbreaks due to EHEC infections are common. Water sources which include recreational waters (Keene *et al.*, 1994), well water and municipal water systems have been connected to recent outbreaks (Swerdlow *et al.*, 1992; Keene *et al.*, 1994; Jones and Roworth, 1996; Ackman *et al.*, 1997). The ability of these organisms to cause waterborne disease was demonstrated by two large outbreaks which were caused by people drinking unchlorinated municipal water (Swerdlow *et al.*, 1992) and people swimming in a contaminated lake (Ackman *et al.*, 1997). Apart from food- and waterborne transmission of *E. coli* O157:H7, person-to-person transmission is well documented (Griffin, 1995). These outbreaks were reported in day care centres, nursing homes and in institutions for the mentally retarded (Griffin, 1995). *E. coli* O157:H7 may cause disease in all age groups although children are most affected (Griffin, 1995).

2.7.1.4 Infectious dose

The general patterns of transmission in outbreaks (waterborne transmission and person-to-person spread), suggested that *E. coli* O157:H7 has a low infectious dose (Griffin, 1995). The apparent ease of person-to-person transmission together with waterborne outbreaks, where these organisms were diluted out (low number of organisms per volume of water) but still able to cause disease when ingested, supported the notion of a low infectious dose required to cause infection (Griffin and Tauxe, 1991). The low infective dose implied that the organisms had to survive exposure to gastric acidity (Griffin and Tauxe, 1991). *E. coli* O157:H7 attached itself to surfaces in the stomach which made them resistant to inhibitors and inhibitory conditions (such as stomach acidity) and enabled the organisms to tolerate acid (Rowbury, 1997). Certain strains of *E. coli* O157:H7 were inherently acid tolerant or have gained inducible acid tolerance prior to ingestion (Rowbury, 1997). Inducible acid tolerance responses present in the stomach were critical for *E. coli* O157:H7 survival (Rowbury, 1997). Some strains were able to survive in pH conditions as low as 2.0 (Miller and Kaspar, 1994), in cold apple cider (pH 3.7 to 3.9) for up to 31 days (Zhao *et al.*, 1993; Besser *et al.*, 1996) and in mayonnaise with pH levels of 3.6 to 3.9 (Zhao and Doyle, 1994). The estimated infectious dose of *E. coli* O157:H7 (taken in consideration a large number of outbreaks investigated) was in the order of 100 to 200 organisms to cause infection (Nataro and Kaper, 1998).

2.8. Pathogenesis

The pathogenicity of *E. coli* O157:H7 is a multistep process involving different complex interactions between bacteria and host. The organisms have to survive the acid environment of the stomach to colonize the intestines (Paton and Paton, 1998). Colonization of the colon and distal small intestine resulted in the formation of the typical attachment-effacement lesions accompanied by bloody diarrhoea (Nataro and Kaper, 1998). The events of pathogenesis can be summarized as follows: i) the colonization of the gut, ii) the effect of the virulence factors on the host and iii) disease caused by the virulence factors.

2.8.1 Colonization of the gut

The initial event in the pathogenesis of *E. coli* O157:H7 infection is the attachment or adherence of the organism to the wall of the gut (Karmali, 1989). In the case of *E. coli* O157:H7, the main focus of attachment to the intestinal mucosa is the distal small intestine, colon and the rectum (Karmali, 1989). No information is available on the nature or mechanisms of intestinal colonization in humans. Studies on the colonization mechanisms of *E. coli* O157:H7 have been performed in animal models such as gnotobiotic piglets (Tzipori *et al.*, 1989; Donnenberg *et al.*, 1993; McKee *et al.*, 1995; Tzipori *et al.*, 1995), infant rabbits (Pai *et al.*, 1986, while cultured epithelial cells have also been used (Knutton *et al.*, 1989; Ismaili *et al.*, 1995).

After attachment to the intestinal mucosa, *E. coli* O157:H7 colonized and multiplied in the human gut because natural peristaltic mechanisms were ineffective in removing the bacteria (Nataro and Kaper, 1998). This mechanism is called attaching and effacing adherence or A/E adherence (Nataro and Kaper, 1998). This A/E adherence causes a typical A/E lesion similar to that caused by enteropathogenic *E. coli* in animal models (Paton and Paton, 1998). These A/E lesions have not yet been reported for human clinical specimens (Nataro and Kaper, 1998). The cellular responses due to EHEC have not been studied as intensively as they have been with EPEC (Paton and Paton, 1998). Attachment is critical to the survival of *E. coli* O157:H7 because it derives its nutritional requirements from the intestinal mucosa (Beachley, 1981). Attachment to mucosal surfaces prevents the loss of organisms into the environment and it promotes the delivery of toxins to eukaryotic cell surfaces (Beachley, 1981).

Differences between the A/E histopathology of EHEC and EPEC have been observed (Paton and Paton, 1998). High concentrations of polymerized actin are seen in EHEC mucosal lesions, with increased levels of IP_3 and intracellular calcium (Knutton *et al.*, 1989; Ismaili *et al.*, 1995). In contrast to EPEC, EHEC failed to induce tyrosine phosphorylation of epithelial cell proteins (Ismaili *et al.*, 1995) as well as to efficiently invade HEp-2 and Henle 407 cells (Sherman *et al.*, 1987; Oelschlaeger *et al.*, 1994). *E. coli* O157:H7 contains the same 35-kb "locus for enterocyte effacement" (LEE) pathogenicity island as the A/E phenotype for EPEC (Paton and Paton, 1998). This pathogenicity island includes a cluster of genes necessary for the pathogenicity of EHEC (Paton and Paton, 1998). Within this pathogenicity island are genes that code for intimin, which is a 97-kDa outer membrane protein (OMP), known as $intimin_{O157}$ (Louie *et al.*, 1993), the secretory proteins EspA, EspB, EspD and a type III secretion pathway (Jarvis and Kaper, 1996; McDaniel *et al.*, 1995). The EHEC LEE encodes for a translocated intimin receptor (Tir) homolog similar to the Tir that is secreted by EPEC (Paton and Paton, 1998). *E. coli* O157:H7 induces a host inflammatory response similar to that of EPEC, which is linked to the A/E histopathology (Nataro and Kaper, 1998). If this inflammatory response is inhibited, diarrhoea is reduced but not eliminated (Nataro and Kaper, 1998).

The *eae*A gene of *E. coli* O157:H7, which is situated on the LEE pathogenicity island (the same as the *eae*A gene of EPEC), is necessary for attaching and effacing to occur in the intestines (Paton and Paton, 1998). There is a strong association between carriage of *eae*A and the ability of EHEC, especially *E. coli* O157:H7 to cause severe human disease such as HC and HUS (Nataro and Kaper, 1998). A small number of EHEC strains that do not contain *eae*A, were capable of causing HC and HUS (Nataro and Kaper, 1998). This suggested that these non-*eae*A strains might produce additional virulence factors, not yet characterized, to compensate for the absence of *eae*A (Nataro and Kaper, 1998).

2.8.2 Shiga toxins

The precise role of Shiga toxins (Stx) in diarrhoeal illness, specifically haemorrhagic colitis and haemolytic uraemic syndrome has to be elucidated (Nataro and Kaper, 1998). These potent cytotoxins are major factors that lead to death of patients and are responsible for other symptoms in patients infected with *E. coli* O157:H7 (Nataro and Kaper, 1998).

2.8.2.1 Nomenclature

E. coli strains that produce the Stx toxins have been referred to as VT-producing *E. coli* (VTEC), shiga-toxigenic *E. coli* (STEC) and enterohaemorrhagic *E. coli* (EHEC) (Karmali, 1989). These three toxin nomenclatures have been used interchangeably in the literature and were further complicated by the existence of two major types of Stx (Stx1 and Stx2), with substantial sequence variation between them (Paton and Paton, 1998). Calderwood *et al.* (1996) has proposed a justification of nomenclature among the Stx family and it is represented in Table 2.4.

Table 2.4. Nomenclature of members of the Shiga toxin family, proposed by Calderwood *et al.* (1996).

Previous nomenclature	Proposed new nomenclature	
	Gene	Protein
Shiga toxin (Stx)	*stx*	Stx
Shiga-like toxin I (SLT-I) or verotoxin 1 (VT1)	stx_1	Stx1
SLT-II or VT2	stx_2	Stx2
SLT-IIc or VT2c	stx_{2c}	Stx2c
SLT-IIe or VT2e	stx_{2e}	Stx2e

2.8.2.2 Types and structure of Stx

Shiga toxin is a subunit toxin made up of an A (active) subunit (32 kDa) and a number of B (binding) subunits (7.7 kDa monomers) that form a pentameric ring around the C-terminal of the A subunit (Middlebrook and Dorland, 1984; O'Brien and Holmes, 1987; Fraser *et al.*, 1994). The toxins bind to cellular receptors via the B subunits with internalization of the A subunit which interrupts cell function (Karmali, 1989). The molecular weight of the Shiga holotoxin is approximately 70 kDa (Karmali, 1989). The Stx cytotoxins contain two distinct, immunologically non-cross-reactive groups called Stx1 (or VT1) and Stx2 (or VT2) (Nataro and Kaper, 1998).

EHEC strains may express Stx1, Stx2, both toxins or multiple forms of Stx2 (Nataro and Kaper, 1998). The Stx1 toxin is essentially identical to Shiga toxin produced by *Shigella dysenteriae* type I (Nataro and Kaper, 1998). The two toxins, Stx1 and Stx2 have 55% and 57% sequence identity in the A and B subunits, respectively (Jackson *et al.*, 1987).

The eucaryotic cell surface receptor for Stx is globotriaosylceramide (Gb_3) (Lingwood *et al.*, 1987). The exception to this is the globotetraosylceramide (Gb_4) receptor which is recognised by the Stx2e variant (DeGrandis *et al.*, 1989). Stx is cytopathic for a restricted number of cultured cell lines in vitro and cytotoxic for endothelial and epithelial cells which includes human colonic and ileal epithelial cells (Sears and Kaper, 1996). Cell cultures susceptible to the toxins are HeLa and Vero cells (Sears and Kaper, 1996). The susceptibility of CaCo cells to Stx have not yet been described in the literature.

2.8.2.3 Stx-converting bacteriophages

The first criteria for phage taxonomy were described by Bradley in 1967. He introduced six basic morphological types of phages which have been increased to eighteen (Ackermann and Eisenstark, 1974). All phage families and genera established by the International Committee on Virus Taxonomy (ICTV) were derived from their morphological features (Matthews, 1982). Phages with the same morphology may be found in conjunction with different bacterial genera (Ackermann, 1975). If morphological identical phages infect the same host organism they may be serologically related (Ackermann *et al.*, 1978). Phages consist of a nucleic acid molecule surrounded by a protein coat (capsid), while some contain lipids and additional structures such as tails and spikes (Holtzhauzen, 1992). The morphology of the head may vary in length and width and may even be tubular (Goyal *et al.*, 1987). The nucleic acid inside the capsid may be double-stranded DNA, single-stranded DNA, single-stranded RNA or double-stranded RNA (Freifelder, 1987). The tail may be contractile or non-contractile (Goyal *et al.*, 1987).

Toxin synthesis can be stimulated by the induction of integrated toxin-converting phages from the host *E. coli* O157:H7 organism (Kimmit *et al.*, 2000). The most common way to induce/ release phages from *E. coli* O157:H7 is by ultra-violet (UV) exposure of the organisms for a short period (Clay, 1988). The UV-mediated DNA damage of *E. coli* O157:H7 triggers a bacterial SOS response resulting in high-level expression of previously silent bacteriophage genes, including Stx genes (Kimmit *et al.*, 2000). Other SOS-inducing antimicrobial agents include quinolones, trimethoprim, furazolidone and some β-lactam antibiotics eg. amoxycillin, cefuroxane and ceftriaxane, but not agents such as fosfomycin, aminoglycosides, tetracyclones, macrolides or

imipeneum (Yoh and Honda, 1997; Kimmit *et al.*, 2000).

Some phages infecting *E. coli* O157:H7 appear to be lambdoid, which are morphologically similar to lambda (λ-type) phages (Muniesa *et al.*, 1999). Rietra *et al.* (1989), described phages (strain 933 coding for both Stx1 and Stx2) infecting *E. coli* O157:H7 having regular hexagonal heads and short tails. Bacteriophages of *E. coli* O26 strains coding for Stx1 have elongated hexagonal heads and long tails (Rietra *et al.*, 1989). In contrast to this report, O'Brien *et al.* (1984) reported an *E. coli* strain 933 carrying the Stx1-encoding phage (933J) to be indistinguishable from Stx1 phage H19J in *E. coli* O26 strain H19.

The molecular size of the *E. coli* O157:H7 phage DNA is between 66 kb and 68 kb (Rietra *et al.*, 1989) whereas that of lambda phage is 49 kb (Jofre, personal communication, 1997). The shiga toxins are encoded by bacteriophages which are inserted into the chromosome of *E. coli* O157:H7 (Nataro and Kaper, 1998) which was first suggested by Kibel and Barnard in 1968. Other potential virulence factors are encoded in the chromosome and on a plasmid found in all *E. coli* O157:H7 isolates (Nataro and Kaper, 1998). Additional pathogens carrying toxin-converting phages coding for specific virulence factors include species such as *Vibrio cholerae* (cholera toxin) (Waldor and Mekalanos, 1996), group A streptococci (exotoxin A) (Johnson *et al.*, 1986) and EHEC strains (enterotoxin) (Scotland *et al.*, 1983; O'Brien *et al.*, 1984).

There is a lack of knowledge regarding the role of phages infecting *E. coli* O157:H7 in natural conditions (Muniesa and Jofre, 1998). Transduction and phage conversion between bacteria in the environment and in the human gut might prove feasible according to studies performed by Saye *et al.* (1990). The prevalence of phages carrying the gene coding for Stx2 in sewage from different countries has been investigated (Muniesa and Jofre, 1998; Muniesa and Jofre, 2000). It was found that naturally occurring phages that infect *E. coli* O157:H7 and coding for the Stx2 gene persisted more successfully in the natural environment than their host bacteria and may be the main reservoir of Stx2 in the environment (Muniesa *et al.*, 1999).

2.8.2.4 Stx in haemorrhagic colitis

A number of studies have focussed on the role or involvement of Stx in diarrhoeal illness such as

haemorrhagic colitis and enterocolitis which was first demonstrated with experiments done on animals such as rabbits (Nataro and Kaper, 1998). The significance of Stx in intestinal disease can differ according to the animal model used (O'Brien and Holmes, 1987). Purified Stx caused fluid accumulation and histological damage when injected into rabbit ileal loops (O'Brien and Holmes, 1987).

Stx's are "vasculotoxins" targeting vascular endothelial cells with selective killing of adsorptive villus tip intestinal epithelial cells and preservation of secretory crypt cells (Kandel *et al.*, 1989). Stx's are responsible for damaging the microvasculature of the large intestine, damaging the capillary vessels in the colonic mucosa, causing edema, local ischaemia and an influx of inflammatory cells into the mucosa (Fontaine *et al.*, 1988). Stx does not play a significant role in the levels of Na^+ absorption and Cl^- ion secretion (Nataro and Kaper, 1998).

Apart from Stx, pathogenesis of intestinal secretion with EHEC strains involves expression of additional virulence proteins by these organisms which are situated on the LEE pathogenicity island (Nataro and Kaper, 1998). Thus, the formation of an A/E lesion is associated with disease such as the development of diarrhoea (Nataro and Kaper, 1998). The ability of EHEC to produce an A/E lesion without the presence of Stx is sufficient to cause non-bloody diarrhoea but Stx is essential in the development of bloody diarrhoea and haemorrhagic colitis (Nataro and Kaper, 1998).

2.8.2.5 Stx in HUS

It is assumed that through colonic vascular damage, shiga toxins produced in the intestine may be translocated to the bloodstream although Stxs have never been detected in the blood of patients suffering from HUS (Nataro and Kaper, 1998). Stx moves across the epithelial cell monolayer through a transcellular pathway (Acheson *et al.*, 1992). Patients with bloody diarrhoea are more likely to develop HUS than patients with non-bloody diarrhoea (Griffin, 1995) which could be due to damage of the intestinal epithelium, bacterial lipopolysaccharide (LPS) or other inflammatory mediators aiding translocation of Stx to the bloodstream (Griffin, 1995; Nataro and Kaper,1998).

The direct cytotoxic activity or action of Stx on renal endothelial cells with the aid of cytokines

such as tumor necrosis factor alpha (TNF-a) and interleukin 6 (IL-6), is the basic mechanism for the development of HUS (Tesh *et al.*, 1994). HUS is characterized by swollen glomerular endothelial cells with platelet and fibrin deposits (Louise and Obrig, 1995). Shiga toxin inhibits protein synthesis (O'Brien and Holmes, 1987) which causes the endothelial cells in the glomeruli to detach and expose platelets to initiate coagulation. Injured erythrocytes caused by the traversal of the occluded microvasculature and the decreased glomerular filtration rate causes acute renal failure characteristic of HUS (Nataro and Kaper, 1998). It is suggested that Stx2 is more important in the development of HUS than Stx1(Griffin, 1995). *Shigella dysenteriae* type 1, the only *Shigella* species producing Stx, is the only enteric pathogen other than the Stx-producing *E. coli* serotypes that causes HUS (Griffin, 1995).

2.8.3 Enterohaemolysin

Enterohaemolysin present in certain EHEC strains (subsequently designated EHEC-Hly), especially *E. coli* O157:H7, is encoded by the 60-Mda "virulence plasmid"(Schmidt *et al.*, 1995). Nearly all *E. coli* O157:H7 strains contained enterohaemolysin which is distributed among non-O157 Stx-producing *E. coli* strains (Nataro and Kaper, 1998). In a study performed by Beutin *et al.* (1994), 90% of all *E. coli* Stx-positive strains isolated from patients contained genes that code for enterohaemolysin. In another study 88% of patients presenting with HUS contained *E. coli* bacteria with genes coding for enterohaemolysin, while 22.2% of non-HUS patients contained enterohaemolysin-coding genes (Schmidt and Karch, 1996). The manner in which enterohaemolysin contributes to the pathogenicity of EHEC organisms is not fully understood and is subject to speculation. One of these speculations is that haemoglobin released by the action of enterohaemolysin may contribute to the stimulation of EHEC growth in the gut by means of providing a source of iron (Edelman and Levin, 1983). It was found that alpha-haemolysin was cytotoxic to endothelial cells in patients with HUS (Suttorp *et al.*, 1990).

2.8.4 pO157 plasmid

As the name suggests, *E. coli* O157:H7 strains contain a plasmid which is called the pO157 plasmid (Nataro and Kaper, 1998). Its size varies between 93.6 to 104 kb (Schmidt *et al.*, 1996). The pO157 plasmid can be found in almost all Stx-positive *E. coli* strains including the O26:H11

strain (Beutin *et al.*, 1994). The pO157 plasmid is found at high frequencies in human non-O157 Stx-positive isolates (Beutin *et al.*, 1994). The fragment which was developed to aid as a diagnostic probe for EHEC and which encodes the enterohaemolysin, is located on the pO157 plasmid (Levine, 1987). Other fragments present on this plasmid include genes that code for potential adherence factors and catalase-peroxidase whose function is unknown (Brunder *et al.*, 1996). The role of this plasmid in the adherence of the bacterium to epithelial cells is not defined. Evidence suggested that there was a strong correlation between the pO157 plasmid and the development of HUS rather than diarrhoea (Nataro and Kaper, 1998). It is uncertain what the significance of this pO157 plasmid is in pathogenesis of EHEC and disease caused by these organisms.

2.8.5 Other potential virulence factors

The 104 kDa extracellular serine protease (EspP) encoded on the pO157 plasmid is considered to be a potential virulence factor of EHEC organisms (Brunder *et al.*, 1997). EspP has 70% homology to the 110 kDa EPEC secreted protein, called EspC and is cytotoxic to Vero cells (Djafari *et al.*, 1997). Brunder *et al.* (1997) suggested that secretion of EspP by the organism in the gut could cause haemorrhagic disease. Brundel *et al.* (1997) found EspP in four of six *E. coli* O157 isolates, one of two *E. coli* O26 isolates and none of two *E. coli* O103 EHEC organisms tested. One can speculate that EspP is not an universal EHEC virulence factor but it could be involved in assisting other virulence factors in causing disease (Brundel *et al.*, 1997). Another EHEC virulence factor worth mentioning, which might contribute to the pathogenesis of watery diarrhoea, is the 39-amino acid heat-stabile enterotoxin referred to as EAST1 (encoded by *astA*) seen in certain strains of EAEC (Nataro and Kaper, 1998).

2.9 Clinical presentation

EHEC, especially *E. coli* O157:H7, is responsible for a variety of clinical manifestations that varies from asymptomatic infection to mild uncomplicated non-bloody diarrhoea or gross bloody diarrhoea (Nataro and Kaper, 1998). The clinical syndrome which causes bloody diarrhoea is better known as haemorrhagic colitis (HC) and is characteristic of EHEC infections (Nataro and Kaper, 1998). The incubation period of EHEC diarrhoea is between 3 and 4 days but could be as

long as 5 to 8 days or as short as 1 to 2 days in exceptional cases (Nataro and Kaper, 1998). Systemic complications of *E. coli* O157:H7 include syndromes such as haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Nataro and Kaper, 1998). The onset of EHEC infection is firstly characterized by abdominal cramps with little or no diarrhoea, progressing to watery diarrhoea (a frequent early manifestation) and subsequently bloody diarrhoea (Karmali, 1989). Complications such as HUS and TTP mainly occur in children and the elderly infected with *E. coli* O157:H7 (Karmali, 1989). The incidence of *E. coli* O157:H7 in various disease states is summarized in Table 2.5.

Table 2.5: Incidence of *E. coli* O157:H7 infection in various disease states (Greenwald and Brandt, 1997)

Disease state	Incidence of *E. coli* O157:H7 (%)
In all diarrhoea	0.6-2.4
In bloody diarrhoea	15-36
In haemorrhagic colitis	38-61
In haemolytic uraemic syndrome	46-58

2.9.1 Asymptomatic infection and non-bloody diarrhoea

Asymptomatic carriage of *E. coli* O157:H7 has been detected in screening exercises of persons not affected in areas of *E. coli* O157:H7 outbreaks (Greenwald and Brandt, 1997). These asymptomatic infections could lead to non-bloody diarrhoea without progression to haemorrhagic colitis (Greenwald and Brandt, 1997). These patients are more likely than HC patients to have a mild case of illness and are less likely to develop HUS or TTP (Greenwald and Brandt, 1997). More than 95% of all patients investigated with sporadic *E. coli* O157:H7 infection had bloody diarrhoea (Ostroff *et al.*, 1989). This very high percentage (95%) could be the result of under-reporting of non-bloody diarrhoeal cases (Ostroff *et al.*, 1989). Most physicians do not perform stool cultures on patients presenting with non-bloody diarrhoea which could indicate asymptomatic *E. coli* O157:H7 infection. The same goes for outbreak investigators who tend to focus on persons with bloody diarrhoea and subsequently failing to detect a number of asymptomatic *E. coli* O157:H7 carriers.

2.9.2 Haemorrhagic colitis

E. coli O157:H7 is recognised as the most important aetiological agent of HC (Greenwald and Brandt, 1997). Common symptoms of HC caused by *E. coli* O157:H7 are abdominal cramps and watery diarrhoea followed by a bloody discharge resembling lower gastrointestinal bleeding (Ostroff *et al.*,1989; Griffin and Tauxe, 1991; Greenwald and Brandt, 1997). This bleeding is sometimes accompanied by right lower quadrant pain, nausea, vomiting, little or no fever and chills (Greenwald and Brandt, 1997). There is evidence of colonic mucosal edema in HC patients (Griffin and Tauxe, 1991). HC is distinguishable from inflammatory colitis by less severe fever and the lack of an inflammatory exudate (Karmali, 1989). Disease subsides within a week (3 to 7 days) of onset with no further complications (Greenwald and Brandt, 1997). In about 10% of children and elderly patients the HC would develop into complications such as HUS (Greenwald and Brandt, 1997).

2.9.3 Haemolytic uraemic syndrome

Most patients infected with *E. coli* O157:H7 will not develop any complications after infection (Greenwald and Brandt, 1997). However, some develop HUS with secondary complications like TTP (Greenwald and Brandt, 1997). Gasser *et al.* (1955) described the first case of HUS. He investigated five young children with a fatal disorder characterised by acquired haemolysis, thrombocytopenia and renal failure (Gasser *et al.*, 1955). Between 1957 and 1960, Dr. Renée Habib and her colleagues wrote five papers on HUS in France (Kaplan, 1998). One of her most important contributions was the recognition that the severity of the renal clinical findings could be correlated with the predominant histopathologic lesion and that typical and atypical HUS cases had different histopathological changes (Habib *et al.*, 1982). In 1964 Gianantonio and colleagues defined the clinical features of diarrhoea-associated HUS (Gianantonio *et al.*, 1964).

The first reports on HUS in South Africa were from Griffiths and Irving (1961) as well as Javett and Senior (1962). Kibel and Barnard (1968) were the first to differentiate between typical and atypical HUS. Other clinical manifestations of HUS include oligouria or anuria, oedema, pallor and in some cases, seizures (Nataro and Kaper, 1998). When HUS was first described, HUS and

TTP were considered to be the same disease (Proesmans, 1996). In the 1980's, HUS and TTP were considered to be two distinct entities (Proesmans, 1996). HUS was generally associated with infants and young children whereas TTP was mostly linked to adult patients, especially women (Proesmans, 1996). The mortality rate of patients with HUS used to be in the order of 50% but this number has dropped considerably over the past few years with the increase in knowledge regarding this syndrome and because of the availability of better treatment (Greenwald and Brandt, 1997). With the mortality rate today between 5% and 10%, infants, children and the elderly seem to be most affected by this syndrome and it is the common cause of acute renal failure in these patients (Greenwald and Brandt, 1997).

Classical HUS presents a few days after the onset of acute bloody diarrhoea (Karmali, 1989). The rate of progression from *E. coli* O157:H7 infection to HUS is between 2% and 7% (Greenwald and Brandt, 1997). It is important to distinguish between diarrhoea-associated and non-diarrhoea-associated HUS. Diarrhoea-associated HUS (D(+)HUS) was shown to be an entity on its own with a specific etiology, while non-diarrhoea-associated HUS (D(-)HUS) is being regarded as a mixed group of several entities including familial and idiopathic cases and TTP (Proesmans, 1996).

The terminology of D(+)HUS and D(-)HUS (Trompeter *et al.*, 1983) has been changed to typical HUS and atypical HUS respectively. A number of other factors has been implicated in the development of this disease like drugs, chemicals, toxins, other microbes and even pregnancy (Greenwald and Brandt, 1997). The study performed by Kibel and Barnard (1968) in South Africa suggested that idiopathic HUS appeared to be substantially more common in white than in black children. In another study performed by Trompeter *et al.* (1983) in England, the HUS appeared to be more common in rural areas than in urban areas. It is not certain what the role of regional and socio-economical differences are in the prevalence of HUS. The recurrence of HUS is very uncommon (Siegler *et al.*, 1993). In a US study that reported patients who had HUS more than once over a period of 20 years, 2.6% had a recurrent episode of the syndrome (Siegler *et al.*, 1993).

2.9.4 Thrombotic thrombocytopenic purpura

Thrombotic thrombocytopenic purpura (TTP) is manifested by thrombocytopenia, renal failure, microangiopathic haemolytic anaemia, fever and neurologic symptoms (Greenwald and Brandt, 1997). Some cases are associated with fever (Griffin and Tauxe, 1991). TTP was first described by Moschcowich in 1924. This illness resembles HUS in some features but differs in that neurological signs and fever are more prominent in TTP and renal failure is less frequent (Karmali, 1989). Cases of TTP are characterized without any preceding illnesses whereas prodromal diarrhoea is an essential feature of classical HUS (Karmali, 1989). The presence of TTP points to the recognition of an *E. coli* O157:H7 outbreak in a community. The progression of haemorrhagic colitis to TTP does not readily occur in children, but it has been reported in adults (Greenwald and Brandt, 1997). The distinguishable features of HUS and TTP are summarized in Table 2.6.

Table 2.6. Distinguishable features of HUS and TTP (Greenwald and Brandt, 1997)

HUS	TTP
Thrombocytopenia	Thrombocytopenia
Renal failure	Renal failure
Haemolytic anaemia	Haemolytic anaemia
Renal thrombi	Diffuse thrombi
	Neurological changes
	Fever

2.10 Diagnosis and detection of *E. coli* O157:H7

Immunomagnetic separation, culture techniques, cytotoxic activity, immunoassays, ELISA methods, DNA probes and PCR are among the most common *E. coli* O157:H7 detection methods used (Nataro and Kaper, 1998). Proper detection of *E. coli* O157:H7 is crucial in diagnosing illness and in recognizing the causative agent in outbreak investigations.

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2.10.1 Immunomagnetic separation

Immunomagnetic separation (IMS) is the method of choice used for isolating *E. coli* O157:H7 from clinical and food samples (Chapman and Siddons, 1996; Cubbon *et al.*, 1996; Vernozy-Rozand *et al.*, 1997; Tomoyasu, 1998). The effectiveness of the IMS method (using magnetic beads coated with antibodies against *E. coli* O157) as a selective enrichment method for *E. coli* O157 strains, has been well established (Wright *et al.*, 1994 ; Bennet *et al.*, 1996). Dynabeads® anti-*E. coli* was designed for selective enrichment of *E. coli* O157 by IMS directly after a sample was pre-enriched in a culture broth. Dynabeads® anti-*E. coli* is made of uniform, superparamagnetic, polystyrene beads with adsorbed and affinity purified anti-*E. coli* O157 antibodies covalently bound to the bead particle surfaces (Figure 2.7).

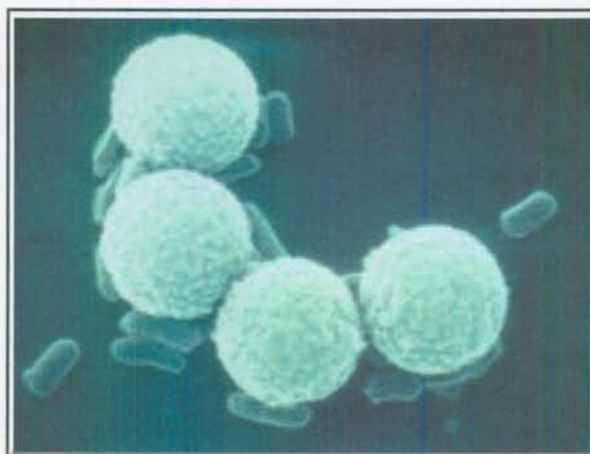

Figure 2.7: *E. coli* O157:H7 bound to anti-*E. coli* O157 antibodies on superparamagnetic, polystyrene Dynabeads® (Dynal website, 2000)

These particles are suspended in phosphate buffered saline (PBS) with 0.1% human serum albumin (HSA) and 0.02% sodium azide (NaN_3) (Dynal, 1995). The pH of this suspension is 7.4 (Dynal, 1995). These beads are incubated with the pre-enrichment culture and the magnetic antibody-coated beads will bind to the target bacteria. Electrolytes present in the culture suspention could have an effect on the initial bacterial adsorption to the solid bead surfaces (Marshall *et al.*, 1971; Uyen *et al.*, 1988). These bead-bacteria complexes are separated with a magnetic particle concentrator (MPC), the Dynal MPC-M®. Presumptive results are obtained

within 24 h by plating these bead-bacteria complexes onto selective *E. coli* O157:H7 media such as Cefixime-Tellurite Sorbitol MacConkey agar (CT-SMAC), CHROMagar O157 and Rainbow agar O157. All these media are commercially available.

2.10.2 Culture techniques

Several methods have been developed for the isolation of *E. coli* O157:H7 from food and clinical samples based on the knowledge that sorbitol-fermenting enteric bacteria produce β-glucuronidase (Doyle and Schoeni, 1987). A variety of selective and differential plating media have been developed for the isolation of *E. coli* O157:H7. The medium which is commonly used for experimental and routine screening of samples for *E. coli* O157:H7, is Sorbitol MacConkey (SMAC) agar (Difco Laboratories, Detroit, Mich.) supplemented with an *E. coli* O157-resistant cefixime-tellurite (CT) antibiotic solution (Figure 2.8.).

Figure 2.8: ***E. coli*** **O157:H7 incubated on CT-SMAC agar indicating its non-sorbitol fermenting properties**

This medium is used for a number of reasons: i) *E. coli* O157:H7 does not ferment sorbitol, and for this reason it is distinguishable as colourless colonies from other *E. coli* organisms that do ferment sorbitol ii) it is inexpensive when compared to other *E. coli* O157 selective media, and is thus suitable when screening large numbers of samples. However, as many as 20% of *E. coli*

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strains may be sorbitol-negative and other species of enteric bacteria can grow as colourless colonies on SMAC (Biolog website, 1999). SMAC is not useful in screening for other EHEC strains.

CHROMagar O157 (Dynal®, UK, Ltd) is a non-inhibitory *E. coli* O157 selective medium specially designed to differentiate *E. coli* O157 from other *E. coli* organisms because of its specific chromogenic properties. This medium can differentiate *E. coli* O157 by its pink-mauve colony colour, from sorbitol negative background micro-organisms such as *Proteus* and *Pseudomonas* found on SMAC (CHROMagar website, 2000). CHROMagar O157 was designed by Dynal® to be used as plating medium after IMS with Dynabeads® anti-*E. coli* O157. The vast majority of other bacterial species are inhibited or give blue or colourless colonies (CHROMagar website, 2000) (Figure 2.9).

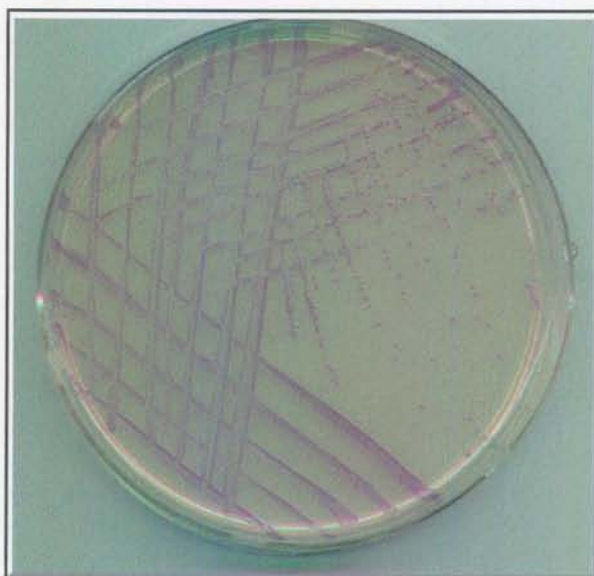

Figure 2.9: *E. coli* O157:H7 streaked out on CHROMagar O157 to illustrate the chromogenic properties of this medium

Rainbow® Agar O157 has both selective and chromogenic properties that make it particularly useful for isolating pathogenic *E. coli* such as *E. coli* O157:H7 and other enterohaemorrhagic or verocytotoxin-producing *E. coli* (VTEC). The medium contains chromogenic substrates that are

specific for two *E. coli*- associated enzymes: β-galactosidase (blue-black substrate) and β-glucuronidase (red substrate). When plated onto this medium, *E. coli* strains yield colonies ranging in colour from red, magenta, purple, violet, blue, grey to black. *E. coli* O157:H7 is typically glucuronidase negative so it forms unique charcoal grey or black colonies on Rainbow® Agar O157 (Figure 2.10).

Figure 2.10: Rainbow agar O157 with typical glucuronidase negative charcoal grey/ black colonies of *E. coli* O157:H7

Other bacterial species are either inhibited on this medium or they grow as white or cream-coloured colonies. Non O157-EHEC strains tend to overproduce β-galactosidase relative to β-glucuronidase on this medium and they are typically coloured purple, violet or blue. The selectivity of Rainbow® Agar O157 can be increased by the addition of novobiocin to inhibit background growth of species found in stool such as enterococci (Biolog website, 1999) .

2.10.3 Cytotoxic activity

The tissue culture cytotoxicity assay is a sensitive method to detect the presence of Stx- producing organisms (Nataro and Kaper, 1998). This was first observed by Konowalchuk *et al.* (1977), using

Vero cells to determine Stx's cytotoxic effect. The high number of Gb receptors (Gb_3 and Gb_4) on Vero cell plasma membranes (the preferred receptors for Stx) can be used to detect all Stx variants (Paton and Paton, 1998). HeLa cells have been used to detect Stx-producing organisms, but because these cells lack the Gb_4 receptor on its plasma membranes it is not as sensitive to some variants of Stx (Paton and Paton, 1998). Cytotoxic activity involves the treatment of cell monolayers with diluted stool filtrates and examining cells for cytopathogenic effect after 3 days of incubation (Karmali, 1989). Any cytotoxic activity is confirmed by neutralization with specific anti-Stx serum (Karmali, 1987). This method, although effective in detecting Stx-producing organisms, is labour-intensive and time-consuming. It is therefore not practical when rapid diagnosis of Stx-producing organisms is required (Paton and Paton, 1998).

2.10.4 Immunoassays

Specific immunoassays used in detecting *E. coli* O157:H7 are directed at the O157 lipopolysacharide (LPS) and H7 flagellar antigens of *E. coli* O157:H7 (Nataro and Kaper, 1998). Suspect colonies are directly screened for the O157 antigen from the selective media but the H7 antigen requires passage through motility medium (Nataro and Kaper, 1998). Enzyme-linked immunosorbent assay (ELISA) methods offered significant advantages over tissue culture-based methods for the detection of shiga toxins in patients with suspected Stx infection (Karmali, 1989). This included a shorter identification time than using DNA hybridization methods (Karmali, 1989). ELISA kits available to detect *E. coli* O157:H7 antigen directly from stool samples are rapid, accurate and easy (Dylla *et al.*, 1995). The ELISA detects the presence of shiga toxin-producing *E. coli* (STEC) (or other Stx-producing bacteria) regardless of the serogroup (Paton and Paton, 1998). This method could be used to detect Stx-producing organisms where cell culture facilities are not available. ELISA's are less sensitive than the verocytotoxicity assay (Downes *et al.*, 1989). A number of commercial diagnostic kits are currently available on the market. This include kits for latex agglutination, ELISA and gold-labelled antibodies. Most of these kits seem to produce similar Stx-detection results when compared (Sowers *et al.*, 1996).

2.10.5 DNA probes and PCR

Specific DNA probes and PCR, directed to detect the Stx-encoding genes of *E. coli* O157:H7 are

available (Newland and Neill, 1988; Nataro and Kaper, 1998). These DNA probes have been used extensively in diagnostic procedures in different laboratories (Wilshaw *et al.*, 1985; Wilshaw *et al.*, 1987). Some of these oligonucleotide probes were based on highly conserved sequence regions among the various toxin genes, allowing the detection of all types of Stx (Paton and Paton, 1998). Other probes with less conserved regions allow distinction between different types of Stx genes (Stx1, Stx2 and Stx2e) (Brown *et al.*, 1989). PCR has been extensively used to detect Stx, either Stx-only or in multiplex form catering for the detection of other genes like *eae*, *ehx*, *uidA*, or *fliC* (Nataro and Kaper, 1998). A few primer pairs designed for Stx2 can distinguish between its variants (Stx2 and Stx2e) (Ramotar *et al.*, 1995). Although specific primers have been designed to distinguish between Stx2c and Stx2e, PCR combined with restriction fragment length polymorphism (RFLP) is normally used (Tyler *et al.*, 1991; Rüssmann *et al.*, 1994; Caprioli *et al.*, 1995). The PCR technique proved to be more sensitive than SMAC agar for the detection of *E. coli* O157:H7 but less sensitive than cell culture assays for the detection of free faecal cytotoxin (Nataro and Kaper, 1998). DNA probes and PCR can be used for the detection of other *E. coli* O157:H7 virulence factors such as the *eae* gene, the pO157/haemolysin gene, *fliC* gene encoding the H7 antigen and other genes (Nataro and Kaper, 1998).

2.10.6 Serodiagnosis

Serological techniques proved valuable in supplying diagnostic information on EHEC infections because many cases of HUS are not recognised until after faecal shedding of the organism has ceased (Nataro and Kaper, 1998). Due to the small numbers of EHEC present in faeces the diagnosis of EHEC-related disease by PCR can be problematic when patients present late in the course of disease (Paton and Paton, 1998). Antibodies to Stx and the lipopolysaccharide (LPS) have been detected in the sera of patients with recent *E. coli* O157:H7 infection (Chart *et al.*, 1991; Paton and Paton, 1998). Variable results have been obtained by numerous researchers on the production of antibodies to Stx (Barret *et al.*, 1991; Karmali *et al.*, 1994; Yamada *et al.*, 1994). It appeared that the serological responses to Stx were not sensitive enough to be used in diagnostic tests (Paton and Paton, 1998). The O157 LPS antibody response seemed to indicate a higher percentage of seropositive patients (Barret *et al.*, 1991). Although the O157 LPS is useful for serodiagnostic purposes it is difficult to prepare and there may be cross reactions with other

organisms because O157 LPS shares epitopes with *E. coli* O44 LPS, O55 LPS and the LPS of some serogroups of *Salmonella* spp., *Yersinia enterocolitica*, *Brucella abortus* and *Vibrio colerae* non-O1 strains (Yamada *et al.*, 1994).

2.10.7 Strain subtyping

A number of different techniques have been used to differentiate between different strains of the *E. coli* O157:H7 serotype and EHEC strains of serotypes other than *E. coli* O157:H7 for epidemiological studies (Nataro and Kaper, 1998). The sequence variation that exists within Stx has been used to characterize strains of *E. coli* O157:H7 by using specific Stx oligonucleotide probes and PCR techniques (Johnson *et al.*, 1990; Hii *et al.*, 1991; Tyler *et al.*, 1991; Thomas *et al.*, 1993; Rüssmann *et al.*, 1994; Caprioli *et al.*, 1995; Franke *et al.*, 1995).

2.10.7.1 Phage typing

Phage types are determined by the lysis pattern obtained when the isolate is subjected to a range of lytic phages (Strockbine *et al.*, 1998). Phage typing can be used for subtyping *E. coli* O157:H7 (Nataro and Kaper, 1998). According to the phage typing method *E. coli* O157:H7 strains can be separated into 66 different phage types according to plaque morphology after host infection (Frost *et al.*, 1993). A combination of Stx-specific gene probes and phage typing has been used successfully in epidemiological studies in the United Kingdom (Frost *et al.*, 1993). Phage typing is relatively easy to perform and allows large numbers of isolates to be analysed (Strockbine *et al.*, 1998).

2.10.7.2 Random amplified polymorphic DNA PCR

Random amplified polymorphic DNA PCR (RAPD-PCR) has been used to differentiate between different O157:H7 strains by using low-stringency PCR amplification conditions with randomly chosen oligonucleotide primers with small random core sequences (8-10 nucleotides) (Heuvelink *et al.*, 1995; Birch *et al.*, 1996; Larson *et al.*, 1999). Relaxed PCR conditions allow multiple unspecific priming sites on each DNA strand (Larson *et al.*, 1999). Primer sites are randomly distributed along the *E. coli* O157:H7 genome and flank both conserved and highly variable regions (Birch *et al.*, 1996). RAPD typing has

significant advantages in its relative simplicity and the benefit of the rapid analysis of large sample numbers (Birch *et al.*, 1996). This method eliminates the need for complex DNA extraction and purification techniques by simply boiling and freezing pure cultures (Birch *et al.*, 1996). The major disadvantage of the RAPD method is the lack of reproducibility due to small primer core sequences that randomly bind to different locations on the genome, giving different patterns after each experiment (Tyler *et al.*, 1997; Kock, 2000). If the reproducibility of RAPD between different laboratories can be improved, it could be implemented as a useful subtyping method (Strockbine *et al.*, 1998).

2.10.7.3 Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) can be used for strain subtyping of *E. coli* O157:H7 using Stx probes (Samadpour, 1995). This method is based on the use of nucleic acid probes from Stx1 and Stx2 structural genes to generate RFLP patterns resulting from digestion of arbitrary isolated genomic DNA with different restriction enzymes (RE) (Samadpour, 1995). Restriction enzymes (RE) cut DNA at precise recognition sites within the bacterial genome defined by the specific sequence of nucleotides (4-6 bp) (University College, London website, 2000). Restriction enzymes used in RFLP's include *Bam*HI, *Eco*RI, *Hind*III and *Pvu*II (Samadpour, 1995). RFLP is a sensitive method for interstrain differentiation of *E. coli* O157:H7 but yields complex patterns that can complicate the analysis of large numbers of strains (Samadpour, 1995). The disadvantages of RFLP is the labour-intensive requirement for blotting and probing the gel and the specialized reagents (Strockbine *et al.*, 1998).

2.10.7.4 Pulse-field gel electrophoresis

Pulse-field gel electrophoresis (PFGE) is a sensitive method for molecular *E. coli* O157:H7 epidemiological studies but is labour- and equipment-intensive (Barret *et al.*, 1994). PFGE is a derivative of the traditional DNA agarose gel electrophoresis in which the orientation of the electric field is changed repeatedly (Meng *et al.*, 1995). This change in electric field allows the resolution of large DNA fragments generated by digesting the *E. coli* O157:H7 chromosome with restriction endonucleases that cleave the DNA

infrequently (Meng *et al.*, 1995). Interpretation of results with PFGE has been shown to be difficult when isolates differ by only a few fragments (Meng *et al.*, 1995). Aside from the lack of sensitivity for interstrain differentiation of *E. coli* O157:H7, a major disadvantage of PFGE is the difficulty in accurate sizing, tabulation and comparing a large number of isolates (Samadpour, 1995).

2.10.7.5 Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) is another PCR-based assay for bacterial and plant DNA fingerprinting which is easy to perform and can identify significant polymorphisms (Lin and Kuo, 2000). AFLP is used for digesting total genomic DNA randomly by restriction endonucleases such as *Eco*R I and *Mse* I, ligated to *Eco*R I and *M*se I adapters and amplified by PCR using primers that contain the sequences of the adapters and one to three random nucleotides as selective sequences (Lin and Kuo, 2000). The high cost factor is the major drawback of this method.

2.10.7.6 Repetitive sequence analysis

Repetitive sequence analysis can be used to detect short intergenic repeated sequences in *E. coli* organisms (De Bruijn, 1992). These sequences contain highly conserved central inverted repeats which can be divided into three groups: i) class I that consists of repetitive extragenic palindromic elements (REPs) (Stern *et al.*, 1984), ii) class II consisting of the enterobacterial repetitive intergenic consensus (ERIC) sequences (Hulton *et al.*, 1991) and iii) the recently discovered BOX element (Martin *et al.*, 1992). These techniques allowed fingerprinting and the determination of the phylogenetic relationships of individual genera, species and strains (De Bruijn, 1992). Amplification-based methods such as ERIC often suffer from the same lack of reproducibility (Tyler *et al.*, 1997). However, the advantages of this method includes the absence of any probes, Southern blotting or DNA hybridization, the use of a single set of primers and simple agarose gels for separation of PCR products (De Bruijn, 1992).

2.10.7.7 Toxin sequence analysis

Sequencing of PCR products of *E. coli* O157:H7 positive isolates and phages carrying the Stx2 genes allows the comparison of the nucleotide sequences between different strains of *E. coli* O157:H7. Differences that may be present in the DNA of the bacterial or phage genome can be determined with the sequence analysis of shiga toxins. The method can distinguish between the two major types of Stx (Stx1 and Stx2), with additional sequence variation between them (Paton and Paton, 1998). Toxin sequence analysis of an isolate has limited value by itself but can add useful information when combined with other analysis (Strockbine *et al.*, 1998). The restricted value of sequencing is due to the limited number of possible toxin types combined with the prevalence of a single toxin type in a specific geographical area (Strockbine *et al.*, 1998). The presence of a variant Stx gene may be an useful marker (Willshaw *et al.*, 1997).

2.11 Summary

The recognition of new virulence factors has led to the classification of *E. coli* organisms into various groups according to their pathogenicity mechanisms (Nataro and Kaper, 1998). It has been 20 years since EHEC had been separated from the other *E. coli* groups by their distinguished feature of producing shiga toxins (Konowalchuk *et al.*, 1977). One serotype from the EHEC group, *E. coli* O157:H7, has been the focus of this study. *E. coli* O157:H7 has been considered the most dangerous enteric pathogen that clinical microbiologists in developed countries were likely to encounter (Nataro and Kaper, 1998). The disease-causing mechanism of *E. coli* O157:H7 was found to be mediated by shiga-like toxins which were introduced into the bacteria by lysogenic lambdoid toxin-converting bacteriophages which contained the structural genes for shiga toxins (Stx1 and Stx2) and were found to be chromosomally integrated (Karmali, 1989). Shiga toxins with the assistance of additional virulence factors such as enterohaemolysin, *eae*A- and the EAST1 genes (which are situated on a pathogenicity island in the organism) are responsible for the clinical disease presentation of haemorrhagic colitis, haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura (Paton and Paton, 1998).

Significant progress has been made in understanding the pathogenesis and epidemiology of disease

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entities caused by *E. coli* O157:H7 and other enterohaemorrhagic *E. coli*, as well as improving their laboratory diagnosis. It was concluded that the pathogenicity of *E. coli* O157:H7 was a multistep process involving different complex interactions between bacteria and host (Paton and Paton, 1998). Firstly, the organisms had to survive the harsh acid environment of the stomach and then compete with other organisms to colonize the gut (Paton and Paton, 1998). Colonization of the colon and distal small intestine gave the bacteria the opportunity to target specific toxin receptors on the cell surfaces causing the typical attachment-effacement lesions accompanied by bloody diarrhoea (Karmali, 1989). Further complications such as HUS and TTP were common among children and the elderly (Nataro and Kaper, 1998). A low infectious dose for *E. coli* O157:H7 infection has been estimated from data obtained from outbreak studies worldwide (Griffin and Tauxe, 1991). Foodborne transmission was the most common vehicle of infection among humans (Griffin and Tauxe, 1991; Griffin, 1995). Research indicated that cattle are the principal reservoir of *E. coli* O157:H7 organisms although they have been isolated from other animals such as pigs, sheep, goats, dogs and cats (Nataro and Kaper, 1998). *E. coli* O157:H7 enters the human food chain through a number of sources such as contaminated meat products and milk and other non-animal foods including apple cider, mayonnaise and radish sprouts contaminated with manure (Nataro and Kaper, 1998). Other transmission routes included waterborne- and person-to-person transmission (Griffin, 1995).

Advances in the development of laboratory detection methods to detect the organisms in human faeces have been crucial in the proper diagnosis of *E. coli* O157:H7 infection. Immunomagnetic separation, culture techniques, cytotoxic activity, immunoassays, ELISA methods, DNA probes and PCR for the detection of genes coding for Stx, *eae*A, and enterohaemolysin are among the most common *E. coli* O157:H7 detection methods currently used (Nataro and Kaper, 1998). Serological and *E. coli* O157:H7 strain subtyping techniques proved valuable in diagnostic and epidemiological studies (Nataro and Kaper, 1998). Most of these methods were not optimized to detect *E. coli* O157:H7 in water but were exclusively used for diagnostical purposes. Waterborne transmission studies used direct culture techniques for *E. coli* O157:H7 isolation (Swerdlow *et al.*, 1992; Keene *et al.*, 1994; Jones and Roworth, 1996; Ackman *et al.*, 1997).

It can be concluded that *E. coli* O157:H7 was and still is globally recognised as an important cause of both epidemic and sporadic disease. More extensive studies are needed to evaluate *E. coli* O157:H7 isolation methods from water environments. This study has focused on immunomagnetic separation as an isolation method for *E. coli* O157:H7. Methods for detecting phages infecting *E. coli* O157:H7 and carrying the Stx1 and Stx 2 genes in natural environments in South Africa have been investigated. Future research should include studies to determine the possibility of phage conversion in the environment and in the human intestines between *E. coli* O157:H7 and other enterobacteriaceae requires further research.

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CHAPTER 3

The Occurrence of *E. coli* O157:H7 in South African Water Sources Intended for Direct and Indirect Human Consumption

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3.1 Abstract

The occurrence of *Escherichia coli* O157:H7 in selected sewage and river water samples in South Africa was investigated. The chromogenic Rainbow™ agar O157 medium designed for the rapid identification of *E. coli* O157:H7 was used for the detection of these organisms in various river-water samples in the Vaal Barrage Reservoir drainage basin of South Africa. A total of 204 samples were obtained from 15 sites where the water was used for direct and indirect human consumption. Samples were filtered through Gelman filter-membranes and incubated on Rainbow™ agar O157 that produced different colours according to the bacterial chromogenic properties. Six hundred and sixty-three suspected *E. coli* O157:H7 colonies, with colours ranging between dark blue, grey and black, were subcultured onto sorbitol-MacConkey agar and screened for different virulence factors specific for *E. coli* O157:H7 and agglutination with anti-*E. coli* O157 antiserum. The results indicated that none of the suspected colonies contained all of the virulence factors necessary to classify them as *E. coli* O157:H7. None of these organisms agglutinated with antisera against *E. coli* O157. The probability of being infected with *E. coli* O157:H7 from direct or indirect consumption of these river water sources is therefore low. Some isolates did, however, contain enterohaemorrhagic *E. coli* virulence properties, such as Stx1, Stx2 and enterohaemolysin, which might impose a health risk if ingested.

Key words - Escherichia coli O157:H7, Rainbow™ agar O157, virulence factors, agglutination, river water

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Abbreviations - EHEC-Enterohaemorrhagic *Escherichia coli*, Stx-Shiga toxin, HC-Haemorrhagic colitis, HUS-Haemolytic uraemic syndrome, A/E-Attaching and effacing, kb-kilo base pairs, LEE-Locus for enterocyte effacement, EPEC-Enteropathogenic *Escherichia coli*, kDa-kilo Dalton, OMP- Outer membrane protein, EspA-*Escherichia coli* secretory protein A, EspB-*Escherichia coli* secretory protein B, EspD-*Escherichia coli* secretory protein D, Tir-Translocated intimin receptor, *eaeA-Escherichia coli* attaching and effacing gene, VT-Vero cytotoxin, EHEC-Hly-Enterohaemorrhagic *Escherichia coli* haemolysin, Mda-Mega Dalton, P- Plasmid

3.2 INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) is a worldwide cause of infection in humans and animals. The most important component of their virulence is the production of one or both Shiga-like toxins (Stx) 1 and 2 (O'Brien and Holmes, 1987). Strains belonging to the O157 serogroup, especially O157:H7 were associated with outbreaks and sporadic cases throughout the world. EHEC produces a variety of clinical syndromes including bloody and non-bloody diarrhoea, haemorrhagic colitis (HC), and haemolytic uraemic syndrome (HUS). The *Escherichia coli* O157:H7 strain has tended to dominate the world literature on EHEC. Infections caused by *E. coli* O157:H7 were recognised frequently, which reflects increased interest in the incidence and detection of this organism. The failure of clinical laboratories to screen for this organism, (which leads to under-reporting of EHEC infections), complicated estimates on the burden of disease caused by *E. coli* O157:H7 and the incidence of this organism. The World Health Organisation (WHO) was particularly concerned about *E. coli* O157:H7 because bloody diarrhoea was a major cause of morbidity and mortality among children in developing countries in the southern hemisphere, including South Africa (WHO, 1997). Infections caused by this organism are not notifiable in South Africa.

According to the WHO Consultation report on the Prevention and Control of Enterohaemorrhagic *Escherichia coli* Infections (1997), there have been three cases of *E. coli* O157 identified in Pretoria, South Africa since 1988. The first case of *E. coli* O157:H7 in South Africa associated with haemorrhagic colitis was reported by Browning *et al.* (1990). Another case was associated

with eating a hamburger from a fast-food outlet (WHO, 1997). Haemorrhagic colitis cases, caused by non-motile *E. coli* O157 have been identified in provinces such as Mpumalanga, Kwa-Zulu Natal and the adjacent Swaziland (WHO, 1997). A total of 10 isolates of *E. coli* O157 have been obtained from pigs with haemorrhagic colitis in South Africa (WHO, 1997). The WHO report and studies by Cravioto *et al.* (1990) and Albert *et al.* (1995) indicate an infrequent incidence of *E. coli* O157:H7 in South Africa.

E. coli O157:H7 have been implicated in waterborne infections (Dev *et al.*, 1991; Geldreich *et al.*, 1992; Swerdlow *et al.*, 1992). Waterborne transmission of *E. coli* O157:H7 from sources such as recreational waters (Keene *et al.*, 1994), well water (Nataro and Kaper, 1998) and municipal water systems (Swerdlow *et al.*, 1992; DeNileon, 1998) have been associated with recent outbreaks. The ability of these organisms to cause waterborne disease was demonstrated by two large outbreaks resulting from people drinking unchlorinated municipal water (Swerdlow *et al.*,1992) and people swimming in contaminated lake water (Keene *et al.*, 1994). The most recent outbreak of *E. coli* O157:H7 occurred in the water supply system of the small farming community of Walkerton, Ontario in Canada in May 2000 (ProMed website, 2000). This outbreak resulted in 6 deaths with 2000 sickened. According to recorded outbreaks, the apparent ease of water-borne transmission, where these organisms were diluted out, but still able to cause disease when ingested, indicated that a low infectious dose could result in infection (Griffin and Tauxe, 1991).

The initial event in the pathogenesis of the infection was the attachment or adherence of the organism to the wall of the gut. *E. coli* O157:H7 attached to the intestinal mucosa of the distal small intestine, colon and the rectum (Karmali, 1989). Once the organism was attached to the intestinal mucosa, natural peristaltic mechanisms were ineffective in getting rid of the bacteria and the organisms were able to multiply and colonize (Ismaili *et al.*, 1995). This mechanism was called attaching and effacing adherence or A/E adherence (Ismaili *et al.*, 1995). Attachment was critical to the survival of *E. coli* O157:H7 because it derived its nutritional requirements from the intestinal mucosa (Beachley, 1981). *E. coli* O157:H7 contained the same 35-kb A locus for enterocyte effacement (LEE) pathogenicity island as the A/E phenotype for EPEC (Nataro and Kaper, 1998). The EHEC pathogenicity island included a cluster of genes necessary for the

pathogenicity of these organisms (Nataro and Kaper, 1998). It included genes that code for intimin, which is a 97-kDa outer membrane protein (OMP), known as $intimin_{O157}$ (Louie *et al.*, 1993), the secretory proteins EspA, EspB and EspD and a type III secretion pathway (Jarvis and Kaper, 1996; McDaniel *et al.*, 1995). The EHEC LEE encodes a translocated intimin receptor (Tir) homolog similar to the Tir that was secreted by EPEC (Nataro and Kaper, 1998). The *eae*A gene of *E. coli* O157:H7 (which was situated on the LEE pathogenicity island) was the same as the *eae*A gene of EPEC (Nataro and Kaper, 1998).This *eae*A gene was necessary for attaching and effacing to occur (Nataro and Kaper, 1998). There was a strong association between carriage of *eae*A and the ability of EHEC, especially *E. coli* O157:H7 to cause severe human disease such as HC and HUS (Nataro and Kaper, 1998).

The role of Stx in diarrhoeal illness, specifically HC and HUS have not been fully elucidated. This potent cytotoxin was the major factor that lead to death and other symptoms in patients infected with EHEC and *E. coli* O157:H7 (Nataro and Kaper, 1998). The Stx cytotoxins contained two distinct immunologically non-cross-reactive groups called Stx1 [(also called Verocytotoxin 1(VT1)] and Stx2 [(or Verocytotoxin 2 (VT2)] (O'Brien and Holmes, 1987). As previously mentioned EHEC strains may express Stx1, Stx2, both toxins or multiple forms of Stx2. The Stx1 toxin was essentially identical to Shiga toxin produced by *Shigella dysenteriae* type I (O'Brien and Holmes, 1987).

Enterohaemolysin present in certain EHEC strains (designated EHEC-Hly), especially *E. coli* O157:H7 was encoded by the 60-Mda virulence plasmid (Schmidt *et al.*, 1995). All *E. coli* O157:H7 strains contained enterohaemolysin and were widely distributed among non-O157 Stx-producing *E. coli* strains (Beutin *et al*, 1994). Schmidt and Karch (1996) found that 88% of patients presenting with HUS contained *E. coli* bacteria with genes coding for enterohaemolysin, while only 22.2% of non-HUS patients contained enterohaemolysin-coding genes.

The objectives of this study were to test water samples intended for direct and indirect consumption of humans for *E. coli* O157:H7 virulence factors and to determine the occurrence

of *E. coli* O157:H7 in these water sources.

3.3 MATERIALS AND METHODS

3.3.1 Samples investigated

Serial dilutions of 1 ml water samples filtered through Gelman GN-6 Metricel* Grid (0.45 μm) and incubated for 24 h at 37 °C on Rainbow™ agar O157 (Biolog) were supplied by Rand Water from 15 collection sites in the Vaal Barrage Reservoir drainage basin in the Gauteng region of South Africa on a weekly basis. These sample points were selected, based on their position relative to potential sources of contamination or being representative of a specific catchment area. The position of the sample points can be summarised as follows:

1. Klip River System
   The Klip River contributed the largest volume of water downstream of the Vaal Dam to the Vaal River Barrage (contribution of Vaal Dam excluded). The sample point at the end of the Klip River system was C-K19. Other sample sites situated in the Klip River system included C-N8, C-R5, C-R6, C-K.H_2O, C-K25, C-K18, OCA and OCB.
2. The input of the Vaal Dam to the Vaal River Barrage was represented by C-V2, a sample point in the Vaal River.
3. Suikerbosrant and Rietspruit Catchments
   C-S2 and C-RV2 represented the Suikerbosrant and Rietspruit rivers just before the confluence with the Vaal River, in the Vaal River Barrage. C-B10, a sample site in the Blesbokspruit, (tributary of the Suikerbosrant River) was known to be subject to mine and treated sewage effluent.
4. ERWAT sewage water samples were supplied by the East Rand Waterboard.

3.3.2 Culture medium

Rainbow™ agar O157 has recently been introduced to the market by Biolog, Inc. (3938 Trust Way, Hayward, Calif., U.S.A.) for the enhanced detection of EHEC and *E. coli* O157. It contained

chromogenic substances, specific for two enzymes, ß-glucuronidase (a red chromogenic substrate) and ß-galactosidase (a blue-black chromogenic substrate) associated with *E. coli*. Depending on the strain of *E. coli*, colony colours ranged from pink, through purple, violet, and blue to black.

E. coli O157:H7 was typically glucuronidase negative (unique and distinctive charcoal grey or black colonies). Suspect colonies were subcultured onto sorbitol-MacConkey (SMAC) agar (Oxoid) for confirmation because of its non-sorbitol fermenting properties. *E. coli* O157:H7 produced colourless colonies on SMAC agar. Other *E. coli* strains were glucuronidase positive on Rainbow™ agar O157 and they were distinguishable as red or magenta colonies. It appeared that many other non-O157 EHEC strains overproduced ß-galactosidase relative to ß-glucuronidase on this medium and consequently they were typically coloured purple, violet or blue. Other bacterial species were either inhibited or grew as white or cream-coloured colonies.

3.3.3 Molecular detection

Six to eight presumptive *E. coli* O157:H7 colonies from each membrane incubated on Rainbow agar O157 (supplied by Rand Water), were subcultured onto sorbitol-MacConkey agar plates supplemented with a cifixime-tellurite (Dynal) antibiotic solution as described by the manufacturer. A loopful of non-sorbitol fermenting colonies was dispersed in 500 µl of distilled water and boiled for 10 minutes at 99°C to release the bacterial DNA. The samples were centrifuged at 10 000 x *g* for 1 min (Eppendorf Centrifuge 5402) and 10 µl volumes were used for DNA amplification without further treatment. Oligonucleotide primers (Sigma-Genosys Ltd.) (Table 3.1) specific for Stx1 (VT1), Stx2 (VT2), EHEC 1 and 2 (*E. coli* O157 specific primers), EHEC P1 and P2 (Enterohaemolysin plasmid primers) were used in the polymerase chain reaction (PCR) (Pollard *et al.*, 1990; Gannon *et al.*, 1993; Fratamico *et al.*, 1995). The PCR cycle for all reactions consisted of an initial 5 min DNA denaturation cycle at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C (Pharmacia LKB Thermocycler). The amplicons (20 µl aliquots from each amplification) were detected with gel electrophoreses using a 2% agarose (SeaKem[7] LE) gel suspension stained with ethidium bromide (Sigma). The amplified products

were visualised by UV-transillumination (UVP -Transilluminator) and the image was captured using the UVP Image store 5000 gel documentation system with 100 bp DNA (Promega) as molecular size marker. Suspected *E. coli* O157:H7 colonies were individually tested for agglutination using a commercially available *E. coli* O157 slide agglutination kit with antisera against *E. coli* O157(MAST ASSURE, Mono factor O157, code M12030).

3.4 RESULTS AND DISCUSSION

A total of 204 water samples were tested with 196 (96%) failing to show evidence of any *E. coli* O157 virulence factors. However, 8 isolates from 8 samples demonstrated the presence of one or more virulence factors (Table 3.2). Haemolysin plasmid was detected in the majority of positive isolates (77.7%) whilst Stx1was detected twice and Stx2 only once. One sample of water (C-V2) was positive for both Stx1 and haemolysin factors and one (C-R5) for Stx2 and haemolysin. One sample (C-K19) was positive for Stx1. No isolate agglutinated with antiserum specific for *E. coli* O157. As a consequence, none of the isolates analysed further in this study showed the full range of virulence factors necessary to confirm the presence of *E. coli* O157. When Rainbow™ agar O157 was used, the wide range of interfering background growth made it difficult to select potential *E. coli* O157:H7 colonies. The variation in colony colour, in particular, complicated the choice of candidate colonies. This problem in recognising potential *E. coli* O157 isolates was a major shortcoming of this agar for routine assessment of the pathogen in water samples.

The results obtained in the study indicated that there was an infrequent incidence of *E. coli* O157 in the water examined, suggesting that the likelihood of acquiring disease through the ingestion of these waters was low. The water that contained some of the EHEC virulence factors might constitute a health risk if ingested.

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Table 3.1 Primer sequences and predicted sizes of PCR amplified products for the detection of EHEC O157, the haemolysin plasmid and Stx (VT)-specific genes of *E. coli* O157:H7

Primer	Oligonucleotide sequence (5'-3')	Target(s)	Size of amplified product (base pairs)	Reference
VT1a	GAAGAGTCCGTGGGATTACG	Stx 1	130	Pollard *et al.* (1990)
VT1b	AGCGATGCAGCTATTAATAA			
VT2a	TTAACCACACCCACGGCAGT	Stx2	346	Pollard *et al.* (1990)
VT2b	GCTCTGGATGCATCTCTGGT			
EHEC 1*	CAGGTCGTCGTGTCTGCTAAA	*eaeA*	1087	Gannon *et al.* (1993)
EHEC 2*	TCAGCGTGGTTGGATCAACCT			
EHEC/P1#	ACGATGTGGTTTATTCTGGA	60-MDa plasmid	166	Fratamico *et al.* (1995)
EHEC/P2#	CTTCACGTCACCATACATAT			

* = EHEC genes specific for *E. coli* O157
# = Haemolysin plasmid
Primers were obtained from Sigma-Genosys Ltd. London Road, Papisford, Cambridgeshire, CB2 4EF, UK

Table 3.2 Detection of *E. coli* O157:H7 virulence factors in river water samples in the Vaal Barrage Reservoir drainage basin in the Gauteng region of South Africa

Site	Number of		Number of isolates (per sample) with virulence factor			
	Samples	Isolates	Stx1	Stx2	Haemolysin	EHEC O157
C-K19	6	42	1(1)	0	0	0
C-N8	15	108	0	0	0	0
C-R5	18	135	0	1(1)	1(1)	0
C-R6	17	109	0	0	0	0
C-K21	14	98	0	0	1(1)	0
C-KH$_2$O	17	104	0	0	2(2)	0
C-K25	14	93	0	0	0	0
C-K18	15	129	0	0	2(2)	0
C-V2	14	95	1(1)	0	1(1)	0
C-S2	18	115	0	0	0	0
C-RV2	16	99	0	0	0	0
C-B10	17	108	0	0	0	0
ERWAT	19	102	0	0	0	0
OCA	2	14	0	0	0	0
OCB	2	12	0	0	0	0

CHAPTER 4

Application of the Immunomagnetic Separation Method in the Isolation of *Escherichia coli* O157:H7 from Sewage, River Water, Beef and Milk

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4.1 Abstract

Recreational and drinking water supplies polluted with sewage have become an important source of *E. coli* O157:H7 infection. Immunomagnetic separation (IMS) has been extensively used for the isolation of *E. coli* O157:H7 from food and stool samples but not for samples such as wastewater. In this study the IMS method was used in combination with the *E. coli* O157:H7 selective media, immunoassays, biochemical tests and PCR, to assess the prevalence of *E. coli* O157:H7 in selected sewage and environmental waters in South Africa. Environmental water, wastewater as well as beef and milk samples were seeded with *E. coli* O157:H7 to determine the sensitivity and selectivity of the enrichment-IMS-selective agar method. Natural occurring *E. coli* O157:H7 organisms were recovered from selected samples by means of IMS. The IMS concentrates were plated on 3 selective *E. coli* O157:H7 media. *E. coli* O157:H7 were detected in seeded sewage and river water samples with numbers as low as 1.2 cfu.ml^{-1}. In both the seeded beef and milk samples the lowest average count of *E. coli* O157:H7 in test samples from which the organisms were recovered was 9.7 cfu.ml^{-1}. The IMS procedure was used to investigate the prevalence of *E. coli* O157:H7 in randomly selected sewage, river water, beef and milk samples in South Africa. A total of 91 sewage- and 40 river water samples were tested and 17.6% and 20% yielded suspected *E. coli* O157:H7 colonies on CT-SMAC respectively. None of the 25 beef and 25 milk samples yielded any suspect colonies on CT-SMAC. PCR was used to confirm the presence of genes coding for Stx1, Stx2, *eae*A and enterohaemolysin (*hly*). Standard immunoassay kits specific for the O157 and H7 antigen and a biochemical indole test were used for further *E. coli* O157:H7 confirmation. Three colonies from one sewage sample (1.1 % of all sewage samples) agglutinated with anti-*E. coli* O157- and H7 antiserum and contained the genes

coding for Stx2, *eae*A and *hly*. None of the colonies isolated from the river water samples were positive for *E. coli* O157:H7. CT-SMAC proved to have limited *E. coli* O157:H7 selective capabilities from samples such as sewage with high bacterial counts. Seeded sample experiments indicated that IMS is a suitable method for isolating *E. coli* O157:H7 from samples with high bacterial interference and low numbers of *E. coli* O157:H7. Evidence has been presented that the enrichment-IMS-selective agar procedure substantially increased the sensitivity of *E. coli* O157:H7 isolation compared to direct plating of test samples onto selective agar generally practised in the past.

Keywords - Beef, *Escherichia coli* O157:H7, immunomagnetic separation, milk, river water, sewage

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Abbreviations - A/E - attachment-effacement, CT-SMAC - Cefixime-tellurite Sorbitol-MacConkey agar, cfu - colony forming units, EHEC - Enterohaemorrhagic *Escherichia coli*, EPEC - Enteropathogenic *Escherichia coli*, IMS - Immunomagnetic separation, HC - Haemorrhagic colitis, HUS - Haemolytic uraemic syndrome, PBS - Phosphate buffered saline, PCR - Polymerase chain reaction, Stx - Shiga toxin, VCC - Vancomycin-Cefixime-Cefsulodin,

4.2 INTRODUCTION

Escherichia coli O157: H7 is characterised by its ability to produce shiga toxins that are cytotoxic to Vero- and HeLa-cells (1). *E. coli* O157:H7 produces a variety of clinical syndromes including bloody and non-bloody diarrhoea, haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (2). Infections caused by *E. coli* O157:H7 are recognised frequently, which resulted in an increased interest in the incidence and detection of this organism. A considerable number of epidemiological, clinical and laboratory investigations have been carried out on *E. coli* O157:H7 infections (3). The failure of clinical laboratories to screen for this organism, with subsequently under-reporting of EHEC infections, complicates estimates on the burden of disease caused by *E. coli* O157:H7. The US Center for Disease Control (CDC) estimated the annual disease burden of *E. coli* O157:H7 in the United States to be more than 73 000 infections and as many as 61

deaths (4). *E. coli* O157:H7 is the pathogen most frequently isolated from stool specimens that contain visible blood (5). The World Health Organization (WHO) is particularly concerned about this because bloody diarrhoea is a major cause of morbidity and mortality among children in developing countries in the southern hemisphere, including South Africa (6).

Waterborne transmission of *E. coli* O157:H7 has been reported from both recreational waters and contaminated drinking water (7; 8; 9). One of the most recent outbreaks of *E. coli* O157:H7 occurred in the water supply system of the small farming community of Walkerton, Ontario in Canada in May 2000 where 6 people died and more than 2000 people were left sickened (9). The high number of enterohaemorrhagic *E. coli* organisms isolated from the faeces of patients, has led to the concern that these organisms, especially *E. coli* O157:H7, could pose a significant health risk when sewage leaks into water supplies. Food-borne transmission of *E. coli* O157:H7 is another important source of infection in humans (10). The most common vehicle of transmission is through the ingestion of faecally contaminated meat products (10). Cattle are the main reservoirs of *E. coli* O157:H7, although it has been isolated from other animals such as chickens, pigs and sheep (10; 11; 12). A variety of food sources other than meat products have been implicated in the transmission of *E. coli* O157:H7: raw cow's milk and cheese, pasteurised milk, mayonnaise, apple cider, fruit and vegetables (3; 10; 13; 14). Once *E. coli* O157:H7 is introduced into the community through food or water it can be transmitted from person-to-person (15). Person-to-person transmission occurs in day-care centres, nursing homes or where there is close contact between individuals (10; 16). The modes of transmission for sporadic *E. coli* O157:H7 infections appear to be similar to those for outbreaks (10). Three cases of laboratory-acquired *E. coli* O157:H7 infection have been reported (17; 18; 19).

None of the previous waterborne transmission studies (7; 8; 20) used IMS for concentrating *E. coli* O157:H7 from water sources. Direct plating of water samples on *E. coli* O157 selective media was used to detect *E. coli* O157:H7 in these cases. This study will focus on using the enrichment-IMS-selective agar method for the isolation of *E. coli* O157:H7 from river water and sewage as well as beef- and milk samples. *E. coli* O157:H7 was confirmed with molecular and biochemical techniques.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial strains

Shiga toxin 2 (Stx2)-positive strain *E. coli* O157:H7 (ATCC 43889) and Stx2-negative strain *E. coli* O157:H7 (ATCC 43888) were used as Stx2 positive and negative controls respectively (21). Stx1-positive *E. coli* C600 and Stx1-negative *E. coli* C600 were used as Stx1 positive and negative controls. Prof. J. Jofre from the University of Barcelona, Spain, supplied all *E. coli* control cultures.

4.3.2 Sewage and river water

Weekly, 500 ml sewage and environmental water samples were collected over a period of one-year (September 1998 to August 1999). Sewage sampling sites included Daspoort-, Zeekoegat- and Baviaanspoort water purification plants near Pretoria, Gauteng, South Africa. River water samples were collected from the Levuvhu River in the Northern Province, Pienaars- and Apies Rivers, situated near Pretoria, Gauteng, and Klip River (south of Johannesburg, Gauteng) in South Africa. Samples were kept at 4 °C to 10 °C and examined within 24 h after collection.

4.3.3 Beef and milk

The grounded beef (200 g) and milk (250 ml) samples were randomly obtained from different retail outlets. Upon receipt at the laboratory, the samples were analysed immediately or held at 4°C for 48 h before analysis.

4.3.4 Immunomagnetic separation (IMS) of E. coli O157:H7

The effectiveness of the immunomagnetic separation (IMS) method for the selective recovery of *E. coli* O157:H7 from food and stool specimens has been well established (22; 23). Dynabeads® anti-*E. coli* (Dynabeads anti-*E. coli* O157; Dynal, Oslo) are made of uniform, superparamagnetic, polystyrene beads with adsorbed and affinity purified anti-*E. coli* O157 antibodies covalently bound to the bead particle surfaces. These magnetic antibody-coated beads are incubated with the pre-enrichment culture to allow the target bacteria to bind onto it. The IMS concentrates are

plated on selective media and suspected colonies were confirmed with molecular and biochemical techniques.

Municipal sewage: Settled sewage samples (100 µl) and samples seeded with *E. coli* O157:H7 ($1.2 - 1.2 \times 10^3$ cfu.ml^{-1}) were inoculated in 50 ml of buffered peptone-saline water (Oxoid, CM509) supplemented with vancomycin (8 mg.l^{-1}), cefixime (0.05 mg.l^{-1}) and cefsulodin (10 mg.l^{-1}) (VCC) antibiotic solution (MAST® Diagnostics) to inhibit the growth of gram-positive organisms as well as *Aeromonas* and *Proteus* spp. The sewage enrichment broth suspensions were incubated in a shaker incubator (Hub-O-Mat) for 6 h at 37°C while rotating at 100 rpm.

River water: River water samples (100 ml) and samples seeded with *E. coli* O157:H7 ($1.2 - 1.2 \times 10^3$ cfu.ml^{-1}) were filtered through 0.45 µm Gelman GN-6 Metricel filter membranes (Prod no. 66191) and incubated in VCC-supplemented buffered peptone-saline water (Difco) for 6 h at 37°C.

Beef: Ten grams of grounded beef samples and samples seeded with *E. coli* O157:H7 ($9.7 - 9.7 \times 10^3$ cfu.ml^{-1}) were mixed with 100 ml of VCC-supplemented buffered peptone-saline water and incubated for 6 h at 37°C.

Milk: Milk samples (1 ml) and samples seeded with *E. coli* O157:H7 ($9.7 - 9.7 \times 10^3$ cfu.ml^{-1}) were directly inoculated in 50 ml VCC- buffered peptone-saline broth. The milk enrichment broth suspensions were incubated in a shaker incubator (Hub-O-Mat) for 6 h at 37°C while rotating at 100 rpm.

Aliquots (1 ml) of the pre-enriched samples with the addition of 20 µl of Dynabead® suspension were incubated at room temperature for 10 min with continuous mixing. This step was performed

to allow the O157-specific antibodies coated onto the beads to bind to the target bacteria. The bead-bacteria complexes were separated using a magnetic particle concentrator, Dynal® MPC-M for 3 min (Dynal, Oslo). After discarding the supernatant and resuspending the bead-particles in PBS-Tween (Sigma), the process was repeated 4 times (Dynal® product brochure, 1995). The final bead-bacteria complexes were resuspended in 100 µl washing buffer (PBS-Tween).

4.3.5 Isolation of E. coli O157

After immunomagnetic separation, 10 µl and 50 µl volumes of each sewage, river water, milk and beef IMS concentrates were transferred to *E. coli* O157 selective media. The *E. coli* O157:H7 selective media used in this study were Cefixime-tellurite Sorbitol-MacConkey agar (CT-SMAC) (Oxoid), CHROMagar O157 (Dynal®) and Rainbow agar O157 (Biolog). *E. coli* O157:H7 strains produced typical colourless colonies on CT-SMAC, red/pink colonies on CHROMagar O157 and grey/black colonies on Rainbow O157 agar after 24 h of incubation at 37°C. All suspect colonies were subcultured on CT-SMAC to confirm its non-sorbitol fermenting properties. Non-sorbitol fermenting colonies were examined for the presence of the genes coding for Stx1, Stx2, enterohaemolysin and *eae*A. A loopful of these colonies was dispersed in 500 µl ultra high quality (UHQ) water and boiled at 99°C for 10 min without further treatment to obtain bacterial DNA for amplification with PCR (24).

Oligonucleotide primers (Sigma-Genosys Ltd.) specific for Stx1 (VT1), Stx2 (VT2), EHEC 1 and 2 (*eae*A primers) and EHEC P1 and P2 (Enterohaemolysin plasmid primers) were used in the polymerase chain reaction (PCR) (25; 26; 27) (Table 4.1). Each 90 µl PCR reaction mixture contained: 100 pmol of VT1 and VT2 primers; 10 µl of template DNA; 2 µl of 10 mM dNTP (Promega); 10 µl of Mg-free 10x amplification buffer (Promega); 6 µl of 25 mM $MgCl_2$ (Promega) and 2.5 U of *Taq* DNA polymerase (Promega). The mixture was overlaid with one drop of sterile mineral oil and placed in an automated thermocycler (Hybaid). The PCR cycle consisted of an initial 5 min DNA denaturation cycle at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C (Pharmacia LKB Thermocycler). The amplicons (20 µl aliquots from each

amplification) were detected by gel electrophoresis using a 2% agarose (SeaKem® LE) gel suspension stained with ethidium bromide (Sigma). A 100 base pair DNA molecular size marker (Promega) was used. The amplified products were visualised by UV-transillumination (UVP - Transilluminator) and the image was captured using the UVP Image store 5000 gel documentation system (Fig 4.1). Suspect *E. coli* O157:H7 colonies were individually tested for agglutination using a commercial *E. coli* O157 slide agglutination kit with antisera against *E. coli* O157 (Mast Assure, Mono Factor O157, code:M12030). All colonies were biochemically confirmed as *E. coli* by their ability to produce indole from tryptophan using Kovac's reagent (28).

4.4 RESULTS

Assessment of the sensitivity of the enrichment-IMS-selective agar procedure revealed that *E. coli* O157:H7 colonies were recovered from sewage and river water samples with average counts of seeded *E. coli* O157:H7 as low as 1.2 $cfu.ml^{-1}$. In both the seeded beef and milk samples the lowest average count of *E. coli* O157:H7 in test samples from which the organisms were recovered was 9.7 $cfu.ml^{-1}$. The enrichment procedure increased average counts of *E. coli* O157:H7 in seeded sewage samples from 1.2 to 45 $cfu.ml^{-1}$ (3 650%), in seeded river water samples from 1.2 to 72 $cfu.ml^{-1}$ (5 900%), in seeded grounded beef samples from 9.7 to 54 $cfu.ml^{-1}$ (457%), and seeded milk samples from 9.7 to 67 $cfu.ml^{-1}$ (591%). In the case of samples seeded with higher numbers of *E. coli* O157:H7 the percentage increase in counts of *E. coli* O157:H7 accomplished by enrichment was lower (Table 4.2). In the case of sewage seeded with *E. coli* O157:H7 to average counts of 1 200 $cfu.ml^{-1}$, the percentage increase was only 45%.

After the above determination of the sensitivity of the enrichment-IMS-selective agar method, the procedure was applied in studies on the incidence of naturally occurring *E. coli* O157:H7 in selected samples. A total of 91 sewage, 40 river water, 25 beef and 25 milk samples was examined. Suspect *E. coli* O157:H7 colonies were isolated from 16 sewage (17.6%) and 8 river water (20%) samples using the 3 selective media. No *E. coli* O157:H7 colonies were isolated when sewage, river water, beef and milk samples were directly plated on the selective media due to interfering growth and overwhelming numbers of non-pathogenic strains of *E. coli*. Three colonies isolated on CT-SMAC from one sewage sample of the Daspoort West Intake (Table 4.2)

agglutinated with the anti-*E. coli* O157 antisera, tested positive for indole using Kovac's reagent and contained the genes coding for Stx2, hly and *eae*A after PCR. These three isolates imply that 6.25% of suspect *E. coli* O157:H7 colonies isolated from sewage were confirmed to be *E. coli* O157:H7, and that *E. coli* O157:H7 colonies were isolated from 1.1% of sewage samples analysed. No suspect *E. coli* O157:H7 colonies were isolated from any of the 40 river water samples, 25 grounded beef samples or 25 milk samples analysed.

Although not confirmed by statistically meaningful results, CT-SMAC proved the agar medium of choice for the selective cultivation of *E. coli* O157:H7. The choice is predominantly based on the morphology and colour of suspect colonies, and the extent to which their detection was obscured by background growth.

4.5 DISCUSSION

Evidence has been presented that the enrichment-IMS-selective agar procedure substantially increased the sensitivity of *E. coli* O157:H7 isolation compared to direct plating of test samples on selective agar. Comparative tests revealed that the enrichment step increased counts of *E. coli* O157:H7 seeded into samples of sewage, river water, grounded beef and milk and varied between 13% and 5 900% (Table 4.2). The higher percentage increase in counts of *E. coli* O157:H7 in samples seeded with low numbers of the organism, is probably due to normal population dynamics and maximum numbers of organisms attainable in a steady state culture. This phenomenon serves the objectives of the enrichment procedure because initial low numbers of *E. coli* O157:H7 require higher levels of enrichment for detection. Since *E. coli* O157:H7 colonies were recovered from all seeded samples, the lowest numbers of the organisms detectable in the samples concerned has unfortunately not been established. Determination of the lowest number of *E. coli* O157:H7 detectable would require tests in which samples are seeded with numbers of *E. coli* O157:H7 even lower than those in Table 4.2. However, the results indicated that *E. coli* O157:H7 would be detectable when present in numbers as low as 1.2 $cfu.ml^{-1}$. Although not confirmed by statistically meaningful results, the CT-SMAC Agar medium seemed to yield the best results of the three media used for the selective cultivation of *E. coli* O157:H7 bacteria. In addition, CT-SMAC agar was less expensive than Rainbow Agar O157 and CHROMagar O157.

The higher efficiency of the enrichment-IMS-selective agar procedure for the recovery of naturally occurring *E. coli* O157:H7 in sewage and river water would appear to be supported by the isolation of at least one *E. coli* O157:H7 organisms from a sewage sample in this study. In comparison, the survey described by Müller *et al.* (2001) (29) using conventional plating of test samples on selective media, failed to recover the pathogen from any of the samples analysed.

Enterohaemorrhagic *E. coli* bacteria have been isolated from sewage in Germany by direct plating of test samples onto selective media (30). *E. coli* O157:H7 has been isolated from patient stool specimens as well as samples of meat products and milk associated with infections (3). However, as far as can be established we report here for the first time the isolation of *E. coli* O157:H7 from sewage in South Africa, and the first application of the enrichment-IMS-selective agar procedure for the isolation of *E. coli* O157:H7 from wastewater anywhere.

One out of 16 samples (6.3%) of suspect *E. coli* O157:H7 were confirmed as *E. coli* O157:H7. This confirms the shortcomings of the agar media for the selective cultivation of *E. coli* O157:H7. The three *E. coli* O157:H7 colonies isolated from the same sewage sample were probably the offspring from the same original organism which multiplied during the initial enrichment stage. This was confirmed by identical features such as the toxicity factors detected which they carried (Table 4.3). Shortcomings of media for the selective cultivation of *E. coli* O157:H7 in test samples with heavy background growth such as sewage and river water has also been reported by other researchers (31). Bettelheim (1998) pointed out that the black colonies of *E. coli* O157:H7 were difficult to distinguish on Rainbow Agar O157 in the presence of large numbers of other *E. coli* colonies of different colours. These observations call for further improvement of methods for the selective cultivation of *E. coli* O157:H7 in the presence of large numbers of wild type *E. coli* and other bacteria capable of growing on the selective media. Resistance to the antibiotics used for the suppression of bacteria other than *E. coli* O157:H7 may largely be accountable for the problem. One solution may therefore be to find antimicrobial agents which more efficiently suppress background growth and strains of *E. coli* other than *E. coli* O157:H7.

Despite shortcomings of the growth media for the selective cultivation of *E. coli* O157:H7, evaluation of the sensitivity of the enrichment-IMS-selective agar procedure in seeding experiments confirmed that it was capable of detecting numbers of *E. coli* O157:H7 as low as one cfu per ml in any of the sewage, river water, grounded beef and milk samples analysed (Table 4.2). These results confirmed that *E. coli* O157:H7 bacteria did occur in low numbers in the samples under investigation.

Data reported on the isolation of at least one *E. coli* O157:H7 organism from sewage and results of the seeding experiments, confirmed that a procedure is available for the relatively sensitive isolation of *E. coli* O157:H7 from a variety of environmental samples with heavy background growth and large numbers of wild type *E. coli* and related bacteria. This enrichment-IMS-selective agar technique could be applied for purposes such as analysis of samples related to *E. coli* O157:H7 infections. The enrichment-IMS-selective agar procedure developed in this study was superior to methods used in the past, but there is potential for further improvement. This refers in particular to the final step for the selective cultivation of *E. coli* O157:H7 isolates. Selection may be improved by using alternative antimicrobial agents which will more efficiently suppresses interfering background growth and wild type *E. coli*.

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Table 4.1 Primer sequences and predicted sizes of PCR amplified products for the detection of EHEC O157, the haemolysin plasmid and Stx (VT)-specific genes of *E. coli* O157:H7

Primer	Oligonucleotide sequence (5'-3')	Target(s)	Size of amplified product (base pairs)	Reference
VT1a	GAAGAGTCCGTGGGATTACG	Stx 1	130	25
VT1b	AGCGATGCAGCTATTAATAA			
VT2a	TTAACCACACCCACGGCAGT	Stx2	346	25
VT2b	GCTCTGGATGCATCTCTGGT			
EHEC 1*	CAGGTCGTCGTGTCTGCTAAA	*eaeA*	1087	26
EHEC 2*	TCAGCGTGGTTGGATCAACCT			
EHEC/P1#	ACGATGTGGTTTATTCTGGA	60-MDa plasmid	166	27
EHEC/P2#	CTTCACGTCACCATACATAT			

* = EHEC genes specific for *E. coli* O157
# = Haemolysin plasmid

Primers were obtained from Sigma-Genosys Ltd. London Road, Papisford, Cambridgeshire, CB2 4EF, UK

Table 4.2 Assessment of the sensitivity of the enrichment-IMS-selective agar method using tests on seeded samples

Seeded samples	Count of seeded *E. coli* O157:H7 cfu .ml^{-1}		
	Before Enrichment	**After Enrichment**	**Percentage Increase**
Sewage	1 200	1 740	45
	120	170	42
	12	52	333
	1.2	45	3650
River water	1 200	2 950	146
	120	890	641
	12	140	1 067
	1.2	72	5900
Grounded beef	9 700	17 400	79
	970	1 700	75
	97	520	436
	9.7	54	457
Milk	9 700	25 000	158
	970	1 100	13
	97	520	436
	9.7	67	590

Enrichment was on CT-SMAC Agar. Counts are averages of tests carried out in triplicate.
E. coli O157:H7 was detected in all seeded samples.

Table 4.3 Isolation of *E. coli* O157:H7 from selected samples of sewage, river water, beef and milk

Samples	Total number of samples	Number of isolates (Percentage of Total)		Toxicity Factors		
		Suspect Isolates	Confirmed Isolates	Stx2	Hly	*eae*A
Sewage	91	16 (17.6 %)	1 (1.1 %)	1	1	1
River water	40	0	0	0	0	0
Beef	25	0	0	0	0	0
Milk	25	0	0	0	0	0
Total	181	16 (8.8 %)	1 (0.6 %)	1	1	1

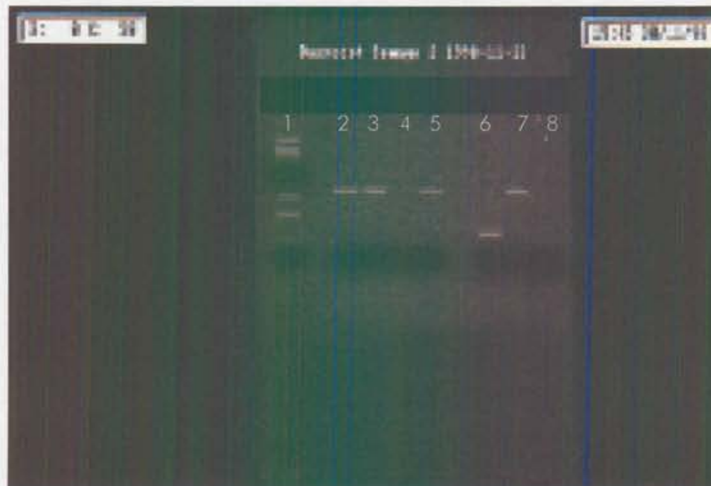

Figure 4.1 **The detection of Stx1(130 bp product) and Stx2 (346 bp product) amplicons of *E. coli* O157:H7 isolates from sewage (Daspoort water purification plant, Pretoria, South Africa) using Gel Electrophoresis.**

Lane 1: 100 bp ladder

Lane 2: *E. coli* colony 1 from Daspoort sewage west intake

Lane 3: *E. coli* colony 2 from Daspoort sewage west intake

Lane 4: *E. coli* colony 3 from Daspoort sewage west intake

Lane 5: *E. coli* colony 4 from Daspoort sewage west intake

Lane 6: Stx1 positive control (130 bp)

Lane 7: Stx2 positive control (346 bp)

Lane 8: Negative control

CHAPTER 5

Characterization of *Escherichia coli* O157:H7 and Shiga Toxin - converting Bacteriophages isolated from water sources and animal reservoirs

Submitted for publication in: Water, Science and Technology

5.1 Abstract

Toxin-converting bacteriophages encoding the Stx2 gene were induced from strains of *Escherichia coli* O157:H7 isolated from sewage, bovine and porcine faeces. Toxin synthesis can be stimulated by the induction of integrated toxin-converting phages from the host *E. coli* O157:H7 organism by ultra-violet (UV) exposure. The UV-mediated DNA damage of *E. coli* O157:H7 triggers a bacterial SOS response resulting in phage release. Free ranging phages outside their *E. coli* O157:H7 hosts were detected but could not be isolated directly from environmental samples such as sewage and river water. *E. coli* O157:H7 colonies carrying the genes coding for Stx2 were isolated from 1 sewage sample (0.76% of positive samples), 8 cattle faecal samples (16.67% of positive samples) and 2 pig faecal samples (14.28% of positive samples). Characterization of *E. coli* O157:H7 was done by repetitive sequence analysis using ERIC-PCR to determine the relationships between the individual *E. coli* O157:H7 strains. The ERIC-PCR analysis of 31 *E. coli* O157:H7 strains revealed two distinct sections with two groups in each section. DNA sequencing of all the *E. coli* O157:H7 positive isolates carrying the Stx2 genes were performed confirming the amplified DNA nucleotide sequences of Stx2. Electron microscopic analysis revealed, for the first time in South Africa, that Stx2- converting phages induced from *E. coli* O157:H7 have different morphologies to that of phage lambda which was previously described. Apart from Stx-phages with lambdoid structure, phages were induced with long hexagonal heads and long non-contractile tail structures. The role of the induced integrated Stx2 phages in natural environments such as river and dam water remains unclear. With the induction of Stx2-converting phages from environmental *E. coli* O157:H7 isolates, it is now

possible to determine the potential of these phages to convert non-pathogenic *E. coli* strains and other enterobacteriaciae into pathogenic strains.

Keywords: *E. coli* O157:H7; enterobacterial repetitive intergenic consensus; immunomagnetic separation; toxin-converting bacteriophages

5.2 Introduction

Escherichia coli serotype O157:H7, which is associated with outbreaks of haemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopaenic purpura, embodies shiga toxins (Stx) whose genes are encoded by toxin-converting bacteriophages (phages) (Scotland *et al.*, 1983; O'Brien *et al.*, 1984). The toxin-converting phages are responsible for the transfer of genetic information (Stx genes and other virulence factors) between bacteria (Plunkett *et al.*, 1999). *Escherichia coli* O157:H7 strains may express Stx1, Stx2, both toxins or multiple forms of Stx2 (Nataro and Kaper, 1998). The Stx1 toxin is essentially identical to Shiga toxin produced by *Shigella dysenteriae* type I (Cantey, 1985; O'Brien and Holmes, 1987). Stx2 is heterogeneous and differs immunologically from Stx1 (Nakao and Takeda, 2000).

Phages infecting *E. coli* O157:H7 appear to be lambdoid, which are morphologically similar to lambda (λ-type) phages (Muniesa *et al.*, 1999). Lambda-type phages are temperate phages, which could show lytic or lysogenic growth characteristics. Lytical growth is characterised by the phage producing many copies of its genome inside the host organism, packaged into new phage particles, and lysis of the host cell releasing progeny phage (Sambrook *et al.*, 1989). When it grows lysogenically, the phage DNA is inserted into the DNA of the host organism, replicating with the host cell (Sambrook *et al.*, 1989). The plaques formed by λ-phages are turbid because some cells are lysed by phages growing lytical and some cells are spared from lysis because they have become lysogens. Rietra *et al.* (1989), described phages (strain 933 coding for both Stx1 and Stx2) infecting *E. coli* O157:H7 having regular hexagonal heads and short tails. *E. coli* O26 strains coding for Stx1 have elongated hexagonal heads and long tails (Rietra *et al.*, 1989).

However, O'Brien *et al.* (1984) reported *E. coli* strain 933 carrying the Stx1-encoding phage (933J) to be indistinguishable from Stx1 phage H19J in *E. coli* O26 strain H19.

Numerous studies have been carried out on the isolation of stx-producing *E. coli* (STEC), especially *E. coli* O157:H7, from a variety of sources world wide (Willshaw *et al.*, 1994; Bielaszewska *et al.*, 1997; Chapman *et al.*, 1997; Mechie *et al.*, 1997; Rahn *et al.*, 1997; Itoh *et al.*, 1998; Colombo *et al.*, 1998; Höller *et al*, 1999; Zschöck *et al.*, 2000; Müller *et al.*, 2001). Zschöck *et al.* (2000) studied the occurrence of STEC in healthy dairy ruminants (13 552 *E. coli* colonies from 726 cows, 28 sheep and 93 goats) and found 0.66% of *E. coli* strains investigated positive for the genes coding for Stx1, 0.47% positive for Stx2 and 0.42% positive for Stx1 and Stx2. None of these isolates belonged to the *E. coli* O157:H7 serogroup (Zschöck *et al.*, 2000).

The prevalence of *E. coli* O157:H7 and phages carrying the gene coding for Stx2 in sewage from different countries have been investigated (Muniesa and Jofre, 1998; Höller *et al.*, 1999; Muniesa and Jofre, 2000). Muniesa *et al.* (2000) demonstrated that Stx2e, a variant of Stx2, can be encoded in the genome of an infectious bacteriophage. It was found that naturally occurring phages that infect *E. coli* O157:H7 and carrying the Stx2 gene persisted more successfully in the natural environment than their host bacteria and may be the main reservoir of Stx2 in the environment (Muniesa *et al.*, 1999). Osawa *et al.* (2000) demonstrated that Stx2-converting phages of different genotypes of *E. coli* O157:H7 were quite similar, indicating the possibility of horizontal transfer of Stx2-converting phages under some circumstances. Acheson *et al.* (1998) validated the ability of Stx1 lysogens to transduce an *E. coli* recipient strain in the murine gastrointestinal tract, giving rise to infectious *E. coli* progeny within the host. Further studies could substantiate the possibility of Stx2 phage conversion of *E. coli* flora in the human gastrointestinal tract into pathogenic strains. In this paper we report the prevalence of *E. coli* O157:H7 and Stx2 toxin-converting phages from water sources and animal reservoirs. The genetic and morphological characteristics for both *E. coli* O157:H7 and the Stx2 converting phages were compared.

5.3 Materials and methods

5.3.1 Sampling

Settled sewage samples used in this study were collected from various sewage purification plants including the Daspoort (400 000 inhabitants), Baviaanspoort (400 000 inhabitants) and Zeekoegat (200 000 inhabitants) sewage purification plants and the Pretoria Academic Hospital in Pretoria, South Africa. Daspoort sewage purification plant were divided into two groups, Daspoort East Intake (urban sewage, 200 000 inhabitants) and Daspoort West Intake (urban and industrial sewage, 200 000 inhabitants). A total of 244 river and dam water samples were collected from the Apies and Pienaars rivers, Hartebeespoort and Roodeplaat dam near Pretoria. Additional river water samples were collected from sites in the Vaal Barrage Reservoir drainage basin in the Gauteng region of South Africa. These sample points were selected, based on their position relative to potential sources of contamination. Cattle (48) and pig (14) faecal samples were collected from the Pretoria Zoological Gardens in Pretoria and from feed lot operators near Delmas and Bronkhorstspruit in the Gauteng region of South Africa. Human faecal samples (6) that contained visible blood were obtained from the Department of Microbiology at the University of Pretoria, Pretoria, South Africa.

5.3.2 Bacterial strains

Shiga toxin 2 (Stx2)-positive strain *E. coli* O157:H7 (ATCC 43889) and Shiga toxin 2 (Stx2)-negative strain *E. coli* O157:H7 (ATCC 43888) were used as Stx2 positive and negative controls (Muniesa and Jofre, 1998). Shiga toxin 1 (Stx1)-positive *E. coli* C600 and Shiga toxin 1 (Stx1)-negative *E. coli* C600 were used as Stx1 positive and negative controls. Prof. Juan Jofre supplied all *E. coli* control cultures, from the University of Barcelona in Spain.

5.3.3 Isolation of E. coli O157:H7 using Immunomagnetic separation (IMS)

Five gram faecal samples (human, bovine and porcine) and 100 µl sewage samples were inoculated in 50 ml of buffered peptone-saline water (Oxoid, CM509) supplemented with vancomycin (8

mg.l^{-1}), cefixime (0.05 mg.l^{-1}) and cefsulodin (10 mg.l^{-1}) (VCC) antibiotic solution (MAST® Diagnostics) to inhibit the growth of interfering bacteria. River water samples (100 ml) were filtered through 0.45 µm Gelman GN-6 Metricel filter membranes (Prod no. 66191) before enrichment. The suspensions were incubated in a shaker incubator (Hub-O-Mat) for 6 h at 37°C while rotating at 100 rpm. IMS was performed on the enrichment cultures as described by the product manufacturer (Dynal® product brochure, 1995). The final bead-bacteria complexes were resuspended in 100 µl washing buffer (PBS-Tween). After IMS, 100 µl of faecal IMS concentrates were transferred to Cefixime-tellurite Sorbitol-MacConkey agar (CT-SMAC) (Oxoid). *E. coli* O157:H7 strains produced typical colourless colonies on CT-SMAC after 24 h of incubation at 37°C. Suspect *E. coli* O157:H7 colonies were individually tested for agglutination using a commercial *E. coli* O157 slide agglutination kit with antisera against *E. coli* O157 (Mast Assure, Mono Factor O157, code: M12030). All colonies were biochemically confirmed as *E. coli* by their ability to produce indole from tryptophan using Kovac's reagent (ISO, 2001).

5.3.4 Molecular detection of E. coli O157:H7

Presumptive *E. coli* O157:H7 colonies obtained from CT-SMAC were examined for the presence of the integrated genes coding for Stx1, Stx2, *eae*A and the additional plasmid-mediated enterohaemolysin which is present in almost all *E. coli* O157:H7 organisms. The bacterial colonies were suspended in 500 µl distilled water and boiled in 1.5 ml microcentrifuge tubes for 10 min at 99°C. The samples were centrifuged at 10 000 *g* (Eppendorf Centrifuge 5402) for 1 min and 10 µl volumes of the supernatant containing genomic DNA was amplified. Oligonucleotide primers (synthesized by Sigma-Genosys Ltd., London Road, Pampisford, Cambridgeshire, CB2 4EF, UK) specific for Stx1 (VT1) (Pollard *et al.* 1990), Stx2 (VT2) (Pollard *et al.* 1990), *eae*A (Gannon *et al.*, 1992) and the haemolysin plasmid (Fratamico *et al.*, 1995) were used in the polymerase chain reaction (PCR) (Table 5.1). Each 90 µl PCR reaction mixture contained: 100 pmol of VT1 and VT2 primers; 10 µl of template DNA; 2 µl of 10 mM dNTP (Promega); 10 µl of Mg-free 10x amplification buffer (Promega); 6 µl of 25 mM $MgCl_2$ (Promega) and 2.5 U of *Taq* DNA polymerase (Promega). The mixture was overlaid with one drop of sterile mineral oil and placed in an automated thermocycler (Hybaid).

The PCR cycle for all reactions consisted of an initial 5 min DNA denaturation cycle at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C (Pharmacia LKB Thermocycler). The amplicons (20 µl aliquots from each amplification) were detected with gel electrophoresis using a 2% agarose gel (SeaKem® LE) stained with ethidium bromide (Sigma). A 100 base pair DNA (Promega) was used as molecular size marker.

5.3.5 Sequencing of genome integrated Stx2

Sequencing of PCR products of *E. coli* O157:H7 positive isolates carrying the Stx2 genes were carried out according to the T7 $\text{Sequenase}_{v2.0}$ protocol described by Amersham Life Science (prod. nr. US70170). Sequencing was performed to confirm the amplification of Stx2. Dimethyl sulfoxide (DMSO) was added to the annealing template to enhance primer specificity and amplification. Forward toxin sequences of the 346 bp fragment were compared to the gene sequence of Stx2 on GenBank (AF291819.1; GI= 9858182) (Appendix B).

5.3.6 Repetitive sequence analysis

In addition, genomic DNA extraction and purification of environmental *E. coli* O157:H7 isolates and the 3 reference strains (*Shigella dysenteriae*, *Salmonella cholerasuis* and *Proteus mirabilis*) for repetitive sequence analysis (RSA) was performed as follows: All isolates were grown on LB agar and several colonies were dispersed in 4.5 ml of sterile distilled water. The optic density of the suspensions was measured (wavelength 620 nm) (Spectro 22 Spectro photometer) to obtain a standardised number of cells. The formula used to calculate the number of *E. coli* O157:H7 cells was $V_{(ml)} = 0.2/OD_{620}$. The cell suspensions were centrifuged (Sorvall Super T21) at 13 000 *g* for 5 min at 4°C. Supernatants were discarded and excess media blotted away. Volumes of 100 µl of ultra high quality (UHQ) water and Tris-HCl (10 mM, pH= 8.2) were added to the cell pellets. Proteinase K (1 $mg.ml^{-1}$) were added to the suspensions and incubated at 55°C overnight. Boiling the samples at 99°C for 10 min inactivated the proteinase K. Cell lysates were stored at -20 °C. The following was added to each 90 µl ERIC-PCR mixture: 12 µl of 10x Mg-free buffer (Promega); 6 µl of 10 mM $MgCl_2$ (Promega); 1.5 µl of dNTP mixture (Promega); 0.2 µl of Taq DNA Polymerse (Promega); 25 pmol of each ERIC1R and ERIC2 primer (Sigma-Genosys Ltd.) (Table 1) and 10 µl of purified template DNA. The PCR was performed with a Hybaid OmniGene

Thermocycler. The use of ERIC sequences and PCR was based on the study performed by De Bruijn (1992). The PCR cycle consisted of one cycle at 95°C for 7 min, followed by 35 cycles of 1 min at 94°C, 1 min at 40°C and 8 min at 65°C and a final cycle of 16 min at 65°C (Hybaid OmniGene Thermocycler). The amplicons (20 µl aliquots from each amplification) were detected with polyacrylamide gel electrophoreses (PAGE) using a 4% acrylamide suspension stained with ethidium bromide (Sigma). Marker XIV (Roche) was used as a 100 bp molecular size marker. A dendrogram was constructed using the Gel Compar® version 4.1 software (Applied Maths, Kort Rijk, Belgium).

5.3.7 E. coli O157:H7 phage induction

Phages were induced from *E. coli* O157:H7 cultures, carrying the Stx2 genes, isolated from sewage, cattle and pigs. Induction of phages using ultraviolet (UV) light from the positive *E. coli* O157:H7 isolates was done according to methods described by O'Brien *et al.* (1984). The *E. coli* O157:H7 strains were inoculated in 5 ml of LB medium with a single colony of *E. coli* O157:H7 and incubated overnight at 37 °C with shaking (100 rpm). These culture suspensions were diluted 1:20 in 100 ml of LB medium, incubated at 37 °C with shaking (100 rpm) for 2-3 hours until the beginning of the exponential growth phase (OD_{600} = 0.1). The cells were harvested by centrifugation and supernatants discarded. The *E. coli* O157:H7 cells were resuspended in a 10 ml solution containing 10 mM $MgCl_2$ and 10 mM $CaCl_2$. The time of UV exposure quoted by O'Brien *et al.* (1984) proved to be too long and killed all cells. A killing curve experiment was carried out to establish optimum radiation time and to standardise the induction procedure. According to this experiment all the solutions were exposed to UV light for 2 seconds. After UV exposure all manipulations were carried out in the dark to prevent photo repair of the UV induced damage. The UV exposed cells were diluted 1:10 in LB medium. These suspensions were incubated overnight at 37 °C with shaking (Hub-O-Mat) (100 rpm). Phages were extracted by chloroform treatment (1:10) for 30 min, vortexed and centrifuged for 15 min at 2000 *g*. Supernatants were filter sterilised through 0.22 µm Millex GV (Millipore) filter-units. Phage production was confirmed using the double agar layer plaque assay as described below. All phage lysates were stored at -70 °C for phage characterisation.

5.3.8 Phage enumeration

E. coli O157:H7 phages were enumerated by the double agar layer procedure using Modified Scholtens Agar (MSA) (Muniesa and Jofre, 1998). Stx2-negative strain *E. coli* O157:H7 (ATCC 43888) was used as host culture, incubated at 37°C until an appropriate logarithmic growth phase for phage enumeration was obtained. A total of 1 ml of each direct sample and dilutions with phosphate-buffered saline solution (PBS) was inoculated with the host culture, mixed with semisolid agar and poured onto solid agar plates. After settling of the semisolid agar mixture, plates were incubated overnight at 37°C.

5.3.9 Free-ranging phage enrichment cultures

Sewage and environmental water samples (30 ml) were centrifuged at 3 000 *g* (Beckman GS-6R) for 15 min to remove debris. This step was followed by the filtration of the supernatants through 0.22 µm-pore-size low-protein-binding Millex-GV membrane filter-units (Millipore Corporation) to eliminate bacteria but allowing phages to pass through. The filtered samples were treated with DNase (10 $U.ml^{-1}$) to eliminate free DNA in suspension. Bacteriophage enrichment cultures were prepared in Modified Scholtens broth (MSB) as described by Muniesa and Jofre (1998). Sample volumes of 10 ml were added to 100 ml cultures of Stx-negative *E. coli* O157:H7 (ATCC 43888) at the logarithmic growth phase. MSB was added to a final volume of 250 ml (Muniesa and Jofre, 1998). Enrichment cultures were shake incubated (Hub-O-Mat) overnight at 37°C (100 rpm). Volumes of 45 ml were centrifuged (Beckman GS-6R) at 3000 *g* for 30 min and the supernatants were filtered through the same 0.22 µm-pore-size low-protein-binding Millex-GV membrane filter-units. Phage supernatants were stored at –70°C for phage enumeration, phage DNA extraction and electron microscopy.

5.3.10 Free-ranging phage DNA extraction and amplification for Stx1 and Stx2

Phage DNA was extracted from liquid culture suspensions obtained after phage enrichment using the High Pure™ Lambda Isolation Kit as described by the product (Roche- product nr. 2012 871). This extraction kit is specific for the purification of Lambda DNA from liquid cultures and from plate lysates. The eluted DNA suspensions were used in PCR amplification. The same Stx1- and

Stx2 primers and amplification procedures were followed as described for the detection of integrated Stx1 and Stx2.

5.3.11 Electron microscopy

Phage preparations for electron microscopy (EM) were prepared as follows: the Stx2-phage plaque selected from the MSA plate was inoculated in 8 ml of LB-medium containing 1-2 ml of logarithmic growth phase *E. coli* O157:H7 (ATCC 43888 - Stx2 negative) host culture. This suspension was incubated overnight at 37°C without shaking, mixed with chloroform (1:10) and centrifuged at 1 000 *g* (Beckman GS-6R) for 15 min. The phage supernatant was transferred to a 1.5 ml microcentrifuge tube and centrifuged at 100 000 *g* (Sorvall TL-100) for 1 h, washed with 1.5 ml of UHQ water and again centrifuged at 100 000 *g* (Sorvall TL-100) for 1 h. The supernatant was discarded and the phage pellet mixed with 20-50 µl of 2% phosphotungstic acid (PTA; pH 6.8) and placed on a 400-mesh Formvar carbon-coated grid (Taylor *et al.*, 1993). The dried grid was examined in a Philips 300 EM.

5.4 Results and discussion

5.4.1 Isolation of E. coli O157:H7

Screening of human and animal stool specimens for *E. coli* O157:H7 has increased due to the severe effect of illness in affected patients (Griffin, 1995). Griffin (1995) reported that 0.4% of 30 000 human stool specimens yielded *E. coli* O157:H7 while Höller *et al.* (1999) reported a 3.2% *E. coli* O157:H7 detection rate in municipal sewage. Zschöck *et al.* (2000) studied the occurrence of EHEC in healthy dairy ruminants (13 552 *E. coli* colonies from 726 cows, 28 sheep and 93 goats) and found 0.66% of *E. coli* strains investigated positive for the genes coding for Stx1, 0.47% positive for Stx2 and 0.42% positive for Stx1 and Stx2. None of these isolates belonged to the *E. coli* O157:H7 serogroup (Zschöck *et al.*, 2000). Beutin and Muller (1998) summarized that the percentage detection rate of Stx-producing *E. coli* O157:H7 among 567 pig stool specimens examined to be $<0.2\%$. Henton and Engelbrecht (1997) described 10 *E. coli* O157 (H-type unknown) organisms isolated in South Africa over a period of 20 years (1971 to 1991).

In this study a low percentage (0.76%) of *E. coli* O157:H7 positive isolates were found in municipal- and industrial sewage, although the detection rate of the bacterium in cattle (12.5%) and pigs (14.29%) was higher when compared to the previously mentioned studies (Henton and Engelbrecht, 1997; Beutin and Muller, 1998; Zschöck *et al.*, 2000). A total of 91 sewage-, 244 river water-, 6 human-, 48 bovine-, and 14 porcine faecal samples were analysed for the presence of *E. coli* O157:H7 using IMS and molecular techniques. The following samples tested positive for *E. coli* O157:H7: 3 strains from 1 sewage sample (0.76% sewage samples), 26 strains from 6 bovine samples (12.50% bovine samples) and 3 strains from 2 porcine samples (14.29% porcine samples). None of the human faecal samples or river water samples were positive for *E. coli* O157:H7. All the positive *E. coli* O157:H7 strains contained the genes coding for Stx2, *eae*A and the enterohaemolysin plasmid according to the PCR results. These positive *E. coli* O157:H7 isolates were used for phage induction procedures.

5.4.2 Partial sequencing of Stx2

The nucleotide sequence of *E. coli* O157:H7 Stx2 as described by Chen *et al.* (2000) were compared to the sequences of 31 Stx2-positive *E. coli* O157:H7 strains obtained in this study. The 346 bp nucleotide sequences of all 31 environmental showed similar sequences as the Stx2 sequence (located within the 219 –564 bp region of the Stx2 gene) of GenBank (AF291819.1; GI: 9858182), confirming the amplification of Stx2.

5.4.3 Repetitive sequence analysis

RSA was used to detect highly conserved short intergenic repeated sequences in the *E. coli* O157:H7 genome (De Bruijn, 1992). This is the first study in South Africa in which the ERIC-PCR was used to compare different strains of *E. coli* of the same O157:H7 serotype. ERIC-PCR analysis is extremely sensitive and this study proved that it could detect differences between different strains of the same bacterial serotype. Total chromosomal DNA was extracted from 31 positive *E. coli* O157:H7 environmental isolates which included 2 from sewage, 26 from cattle and 3 from pigs. ERIC-PCR generated multiple amplification products ranging between 50 bp and

3000 bp. The ERIC-PCR analysis revealed diverse patterns for all *E. coli* O157:H7. According to the dendrogram (Fig 5.1) the 31 *E. coli* O157:H7 strains can be divided into 3 major sections (Section I, II and III). Section I, with a 56% correlation, included 15 strains. Two groups were distinguished in this section. Group A, with 11 strains at 60% correlation, was divided into two subgroups (Subgroup 1 with 65% correlation and Subgroup 2 with 78% correlation). Subgroup 1 included 6 bovine strains from one specific cattle feedlot (72% correlation), another bovine strain and 1 porcine strain. Subgroup 2 included 3 strains from one feedlot, indicating a high correlation between the strains. Group B consisted of 4 bovine strains with 61% correlation. Section II, with 14 strains at 48% correlation were grouped into Group C and Group D. Group C, with 62% correlation, was divided into Subgroup 3 (8 strains) and Subgroup 4 (3 strains). Subgroup 3 had 6 cattle isolates from different feedlots, 1 porcine isolate and 1 sewage isolate. Subgroup 4 consisted of 3 cattle isolates from different feedlots. Group D, with 63% correlation consisted of 3 bovine isolates and 1 sewage isolate. The third section included the control cultures and an *E. coli* O157:H7 porcine isolate. Most cattle *E. coli* O157:H7 isolates from the same feedlots grouped together (> 70% correlation) although some were scattered among the other groups. The RSA patterns of the porcine *E. coli* O157:H7 isolates differed quite extensively (30% correlation) while the sewage isolates shared a 50% correlation. The results of RSA revealed the highly discriminating features of the *E. coli* O157:H7 ERIC-patterns among strains of diverse and similar origin.

5.4.4 Free-ranging phage detection

Seventy-eight sewage samples were analysed with PCR for phages carrying the genes coding for Stx1 and Stx2. The average DNA yield after DNA extraction of phage enrichment cultures was between 20 and 160 ng.μl^{-1}. The mean number of phages infecting *E. coli* O157:H7 found in sewage was $3.05x10^{2}$ pfu.ml^{-1}. Two samples (Daspoort West sewage intake and Hartebeespoort Dam) (2.56% of all samples) confirmed the presence of phages carrying the genes coding for Stx2 with PCR. Although they were confirmed positive with PCR these phages could not be isolated directly from the original 2 samples by using the standard double agar layer plaque assay described by Muniesa and Jofre (1998). Direct detection of phages in sewage was low when compared to

the study performed by Muniesa and Jofre (2000), where free-ranging phages were detected in South African sewage but could not be directly isolated. This could be due to different primer pairs used or the lack of an extra nested-PCR step.

5.4.5 E. coli O157:H7 phage induction

Environmental strains of *E. coli* O157:H7 were exposed to ultraviolet radiation at different time intervals to establish the optimal phage release conditions. From a total of 31 strains of *E. coli* O157:H7 subjected to the induction procedure only 5 strains (15.62%) produced phages after induction with UV. Phage titres of phages obtained from the 5 induced *E. coli* O157:H7 organisms were increased with overnight incubation of phage suspensions in a host *E. coli* O157:H7 culture lacking the Stx2 genes. Plaques obtained from all induced cultures (Stx2 positive) were small (<1 mm in diameter). Sergeant (1998) demonstrated the same phenomenon of small plaques and reduced burst sizes when compared to wild-type or laboratory strains of phage λ. UV induction experiments of *E. coli* O157:H7 revealed that the maximum number of Stx2-converting phages was released when the bacterial cells were exposed to UV for 2 seconds and thereafter the phage titre gradually decreased when exposed for longer intervals. This differs from the findings of O'Brien *et al.* (1984) where longer UV exposure times were used during phage induction. Some strains were subjected to mitomycin C but UV-radiation proved superior when phage titres were compared after induction. DNA damage, caused by the UV radiation, triggers a bacterial SOS response which increases phage production and expression of Stx and other phage genes (Yoh and Honda, 1997; Kimmit *et al.*, 2000). The difficulty to induce Stx2-converting phages from the environmental strains of *E. coli* O157:H7 was confirmed by the low titres obtained after UV radiation. Sergeant (1998) described that most Stx-positive *E. coli* strains failed to produce phages and therefore presumably harboured defective prophages. The same conclusion could be reached according to the results obtained in this study, a phenomenon commonly encountered among toxigenic phages (Saunders *et al.*, 1999).

5.4.6 Electron microscopy

EM was performed on phages isolated from sewage, cattle and pigs. Stx2-converting phages

induced from *E. coli* O157:H7 isolated from Daspoort West Intake sewage (Dp2(4)) had elongated heads and a long non-contractile tail structure (Fig 5.2). Rietra *et al.* (1989) described Stx1- converting phages induced from *E. coli* O26:H11, strain H19 and E40877 with a similar structure to phages induced from Dp2(4) in this study. Phages from *E. coli* strain 933, serotype O157:H7 that coded for the production of Stx1 had lambda-like phage morphology (regular hexagonal heads with a short non-contractile tails). *E. coli* O157:H7 strain 933 coding for Stx2 had the same morphology as strain 933 coding for Stx1. O'Brien *et al.* (1984) described the same morphologies for the *E. coli* strain 933 (Stx1 and Stx2) as was demonstrated in this study. Stx2-converting phages induced from environmental *E. coli* O157:H7 isolates revealed for the first time in South Africa that these phages have different morphologies to the previously described phage lambda. We couldn't find any specific phage family to group these phages (with long hexagonal heads and long non-contractile tails), although they have got some Sipho and Myo-viridae phage properties.

5.5 Conclusions

In summary, the data obtained in this study (the low numbers of *E. coli* O157:H7 bacteria isolated from humans and municipal sewage) support the findings of the WHO (1997) that the incidence of *E. coli* O157:H7 infections among humans in South Africa is low when compared to countries in the northern hemisphere. An interesting observation from data obtained in this study is the high percentage of *E. coli* O157:H7 organisms isolated from healthy cattle and pigs, when only 10 isolates of *E. coli* O157 have been isolated in South Africa from pigs with haemorrhagic colitis in the past 20 years (WHO, 1997). Repetitive sequence analysis of the 31 *E. coli* O157:H7 isolates revealed the highly discriminating features of ERIC-PCR among strains of diverse and similar origin. Data obtained from phage induction experiments seem to correspond with other studies (Sergeant, 1998; Jofre, 2001), which indicated a low induction success rate. Future research of these Stx2-converting phages induced from environmental *E. coli* O157:H7 isolates will be used to determine host specificity and toxin conversion among other members of the enterobacteriaciae family.

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Table 5.1 Primer sequences and predicted sizes of PCR amplified products for the detection of ERIC1R, ERIC2, EHEC O157, the haemolysin plasmid and Stx (VT)-specific genes of *E. coli* O157:H7

Primer	Oligonucleotide sequence (5'-3')	Target(s)	Size of amplified product (base pairs)	Reference
ERIC 1R	CACTTAGGGGTCCTCGAATGTA	Conserved repeated sequences	50-3000	De Bruijn (1992)
ERIC 2	AAGTAAGTGACTGGGGTGAGCG			
VT1a	GAAGAGTCCGTGGGATTACG	Stx 1	130	Pollard *et al.* (1990)
VT1b	AGCGATGCAGCTATTAATAA			
VT2a	TTAACCACACCCACGGCAGT	Stx2	346	Pollard *et al.* (1990)
VT2b	GCTCTGGATGCATCTCTGGT			
EHEC 1*	CAGGTCGTCGTGTCTGCTAAA	*eae*A	1087	Gannon *et al.* (1993)
EHEC 2*	TCAGCGTGGTTGGATCAACCT			
EHEC/P1#	ACGATGTGGTTTATTCTGGA	60-MDa haemolysin plasmid	166	Fratamico *et al.* (1995)
EHEC/P2#	CTTCACGTCACCATACATAT			

* = EHEC genes specific for *E. coli* O157

= Haemolysin plasmid

Primers were obtained from Sigma-Genosys Ltd. London Road, Pampisford, Cambridgeshire, CB2 4EF, UK

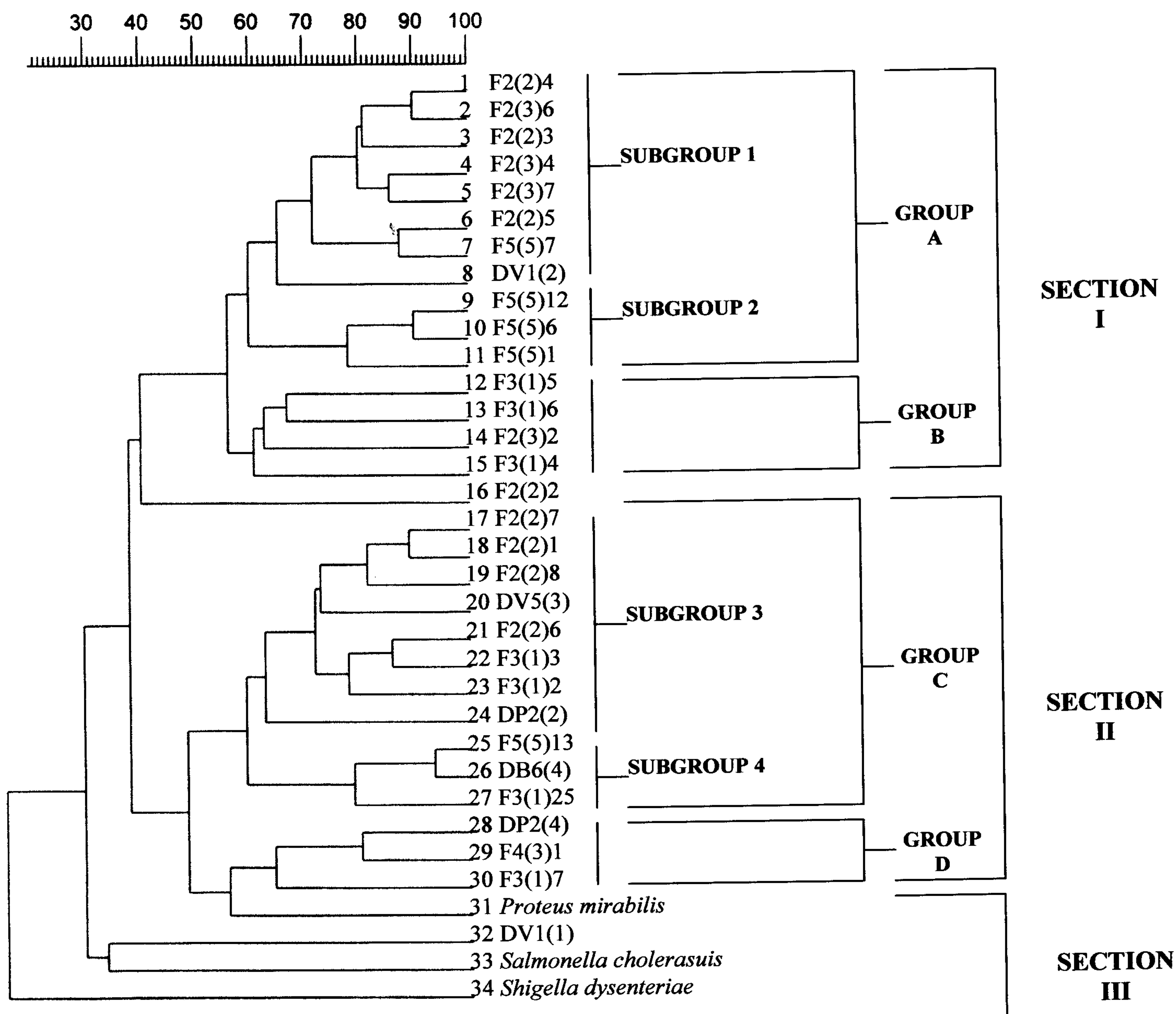

Figure 5.1 **Dendrogram of 31 environmental *E. coli* O157:H7 isolates (lanes 1-30 and 32) and reference cultures (lanes 31, 33 and 34). DV – Zoo pig; DB – Zoo cattle; F – Feedlot cattle; DP – Daspoort sewage.**

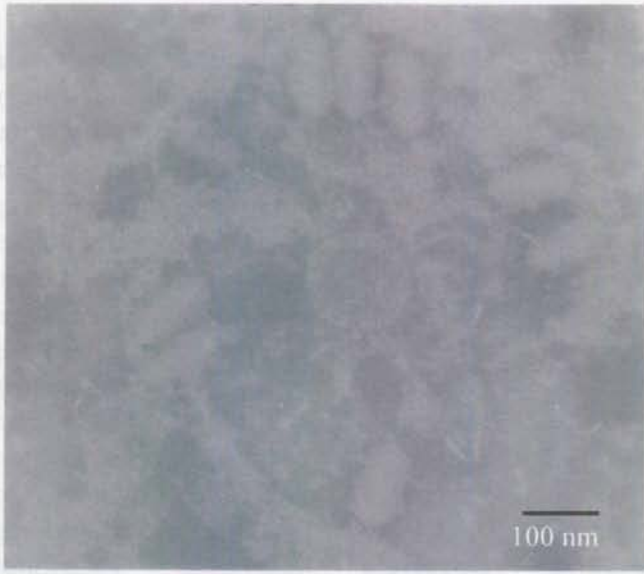

Figure 5.2 Electron micrograph of Stx-converting phages isolated from Daspoort sewage West Intake (Dp2)

CHAPTER 6

Host Range Susceptibility of Toxin-converting Bacteriophages Infecting *Escherichia coli* O157:H7

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6.1 Abstract

The ability of Shiga toxin 2 (Stx2)-encoding bacteriophages isolated from environmental strains of *Escherichia coli* O157:H7 to infect non-pathogenic *E. coli* and other enterobacteriaceae and to develop infectious progeny from these strains were investigated. Phages encoding the production of Stx2, isolated from wild strains of *E. coli* O157:H7 of sewage and bovine origin, were used in this study. A variety of wild-type and laboratory strains of non-pathogenic *E. coli* and other enterobacteriaceae species including *Shigella*, *Salmonella*, *Klebsiella*, *Proteus* and *Enterobacter* were used as host cultures to determine the susceptibility of these phages. Plaque formation and lysogenic conversion among these host cultures were examined. All phage isolates were obtained by the induction of environmental *E. coli* O157:H7 isolates with ultraviolet irradiation. Only one phage (Dp2(4)) out of 5 induced Stx2-encoding phages supported the formation of plaques on *Salmonella cholerasuis.* None of the other phages were able to produce plaques on any of the host cultures except for the control host *E. coli* O157:H7 (Stx2 negative) culture. Phage Dp2(4) isolated from the infected *Salmonella cholerasuis* culture were able to multiply in both *E. coli* O157:H7 and *Salmonella cholerasuis* and were able to re-infect the Stx2-negative *E. coli* O157:H7 strain. Plaques obtained on the lawns of all *E. coli* O157:H7 host cultures were small and clear ($<$1 mm in diameter) while plaques obtained from the lawns of the *Salmonella cholerasuis* cultures were larger and turbid ($>$2 mm in diameter). It is unclear whether or not Dp2(4) phage infection resulted in the *Salmonella cholerasuis* strain

expressing the genes coding for Stx2 and therefore confirming the integration of the Stx2 phage into the *Salmonella* host's chromosome. The results of our study demonstrated that these induced phages were highly host specific and that Stx-phage conversion rarely occurred among members of the enterobacteriaceae family.

6.2 Introduction

Bacteriophages act as vehicles for transferring certain genes or bacterial fragments between different bacterial hosts in the environment (Tòth, 2001). Phage-encapsulated DNA is protected against nucleases and other solvents in the environment and for this reason phages are thought to be an important component in the evolution of bacteria (Brunder and Karch, 2000). A number of bacterial species (*Corynebacterium diphteriae*, *Clostridium botulinum*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Pseudomonas aeroginosa*, *Vibrio cholerae*, *Dichelobacter nodosus* and *Escherichia coli*) contain these prophage genes which code for certain virulence factors (Tòth, 2001). Shiga toxin 1 (Stx1) and shiga toxin 2 (Stx2) are the key virulence factors in the pathogenesis of *E. coli* O157:H7 (O'Brien *et al.*, 1984). These genes are considered to form part of the genome of lambdoid phages (Schmidt *et al.*, 1999). Stx-converting phages are involved in the regulation and expression of Stx genes and in the distribution of these genes (Schmidt *et al.* 2000). These Stx-converting phages contain the Stx genes as an integral part of the phage genome and are considered to be morphologically and genetically distinct (Brunder and Karch, 2000). However, Müller *et al.* (2001) and Jofre (2001) found different morphologies among Stx2-converting phages in the environment. The position of the Stx genes within the phage DNA seems to be highly conserved (Brunder and Karch, 2000).

Several investigators demonstrated phage-mediated gene transfer between different species or even genera. Transduction occurs at considerable rates in simulated natural conditions (Saye *et al.*, 1990).

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Scotland *et al.* (1983) and Smith *et al.* (1983) were the first to demonstrate the ability of Stx genes to be transferred from one *E. coli* bacteria to another confirming the phage origin of Stx genes. Acheson *et al.* (1998) indicated the possibility of phage transduction through horisontal transfer in the gastrointestinal tract of mice. Muniesa *et al.* (1998) investigated the abundance of Stx2-converting phages in natural environments to determine the possibility of infection of the existing human *E. coli* flora by direct ingestion of phage contaminated water. The high number of Stx2 phages obtained from environmental samples indicated that these phages play a role as a reservoir of the Stx2 gene in nature and that they persist more successfully in natural water environments than their host bacteria (Muniesa and Jofre, 1998; Muniesa *et al.*, 1998; Muniesa and Jofre, 2000). In this study we investigate the possibility of Stx2-converting phages to infect and convert wild-type and laboratory strains of *E. coli* and other members of the Enterobacteriaceae family *in vitro* into pathogenic toxin-producing strains.

6.3 Materials and methods

6.3.1 Bacterial strains

The bacterial strains used in this study are listed in Table 6.1. Shiga toxin 2 (Stx2)-positive strain *E. coli* O157:H7 (ATCC 43889) and Shiga toxin 2 (Stx2)-negative strain *E. coli* O157:H7 (ATCC 43888) were used as Stx2 positive and negative controls respectively.

6.3.2 Phage induction

Phages were induced from *E. coli* O157:H7 cultures, carrying the Stx2 genes, isolated from sewage, cattle and pigs. The *E. coli* O157:H7 strains were inoculated in 200 ml of LB medium, incubated at 37 °C with shaking (100 rpm) (Hub-O-Mat) for 2-3 hours until the beginning of the exponential growth phase (OD_{600} = 0.1). The cells were harvested by centrifugation at 1 000 *g* (Beckman GS-6R) and supernatants discarded. The *E. coli* O157:H7 cells were resuspended in a solution containing 10 mM $MgCl_2$ and 10 mM $CaCl_2$. The solutions were exposed to UV light

for 2 seconds and incubated at 37°C with shaking (100 rpm) (Hub-O-Mat). After UV exposure all manipulations were carried out in the dark to prevent photo repair of the UV induced damage. The UV exposed cells were diluted 1:10 in LB medium and adjusted to a final volume of 200 ml of LB. Phages were extracted by chloroform treatment (1:10) for 30 min, vortexed and centrifuged (Beckman GS-6R) for 15 min at 1000 *g*. Supernatants were filter sterilized through 0.22 μm Millex GV (Millipore) filter-units. Phage production was confirmed using the double agar layer plaque assay as described below.

6.3.3 Phage enumeration

E. coli O157:H7 phages were enumerated by the double agar layer procedure using Modified Scholtens Agar (MSA) (Muniesa and Jofre, 1998). Bacterial host cultures were incubated at 37°C to an appropriate logarithmic growth phase ($OD_{600} = 0.5$) for phage enumeration. A total of 1 ml of each direct sample and serial dilutions of these samples were inoculated with the host culture, mixed with semisolid MSA and poured onto solid agar plates. After settling of the semisolid agar mixture, plates were incubated overnight at 37°C. Phages were enumerated as single plaque forming units (pfu) per ml on the plates after incubation.

6.3.4 Molecular detection of Stx2 from phages and bacteria

PCR amplification of genes specific for Stx1 and Stx2 was performed on bacterial strains using the oligonucleotide primers described in Table 6.2. Each 90 μl PCR reaction mixture contained: 100 pmol of VT1 and VT2 primers; 10 μl of template DNA; 2 μl of 10 mM dNTP (Promega); 10 μl of Mg-free 10x amplification buffer (Promega); 6 μl of 25 mM $MgCl_2$ (Promega) and 2.5 U of *Taq* DNA polymerase (Promega). The mixture was overlaid with one drop of sterile mineral oil and placed in an automated thermocycler (Hybaid).The PCR cycle consisted of an initial 5 min DNA denaturation cycle at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C (Pharmacia LKB Thermocycler). The amplicons (20 μl aliquots from each amplification) were detected with gel electrophoresis using a 2% agarose (SeaKem® LE)

gel suspension stained with ethidium bromide (Sigma). A 100 base pair DNA (Promega) was used as molecular size marker. The amplified products were visualised by UV-transillumination (UVP -Transilluminator) and the image was captured using the UVP Image store 5000 gel documentation system.

6.4 Results and discussion

6.4.1 Induction of Stx2-converting phages from E. coli O157:H7

Stx2-converting phages were induced with UV-irradiation from 5 environmental *E. coli* O157:H7 isolates. These phages were designated Dp2(4)ϕ, F3(1)39 ϕ, DB(1)ϕ, F5(5)12 ϕ and Dp2(4)ϕ according to the environmental *E. coli* O157:H7 isolates they were induced from. The titres of these phages were the following ($pfu.ml^{-1}$): Phage Dp2(4)ϕ = 6.5 x 10^6 $pfu.ml^{-1}$; Phage F3(1)39 ϕ = 6.2 x 10^6 $pfu.ml^{-1}$; Phage DB1(1)ϕ = 1.2 x 10^7 $pfu.ml^{-1}$; Phage Dp2(1)ϕ = 1.4 x 10^6 $pfu.ml^{-1}$; Phage F5(5)12ϕ = 8.0 x 10^6 $pfu.ml^{-1}$. All induced phages produced plaques that was clear and smaller than 1 mm in diameter on lawns of *E. coli* O157:H7 Stx2-negative strain (ATCC 43888).

6.4.2 Bacterial strain infection with Stx2-converting phages

Data (Tables 6.3 to 6.7) obtained from Stx2-phage susceptibility experiments on different *E. coli* and enterobacteriaceae host cultures are represented. Phage Dp2(4)ϕ was only once able to infect laboratory strain *Salmonella cholerasuis* (ATCC 6994). Plaques obtained on the *Salmonella cholerasuis* host was turbid and large (>2 mm in diameter). The phage isolated from *Salmonella cholerasuis* was designated phage Dp2(4)ϕSC1. We ruled out the possibility of phage contamination because the Dp2(4)ϕSC1 phage was able to re-infect *E. coli* O157:H7 (Stx2 negative control) and produce infectious progeny and these phages were Stx2 positive. No plaque hybridisation was done because phages were confirmed Stx2-positive by using the High Pure Lambda Isolation extraction kit and PCR . None of the other bacterial strains were susceptible to

phage Dp2(4)ϕ or any other induced phage. This observation proves that Dp2(4)ϕSC1 phage multiplication did occur in the broth containing the *Salmonella cholerasuis* host culture (Table 6.6 and 6.7). However, the plaques obtained from the re-infected *E. coli* O157:H7 host strain was again clear and smaller than 1 mm in diameter.

These results indicated that phages induced from *E. coli* O157:H7 could infect other members of the enterobacteriaceae family under certain conditions that is favourable for phage infection. It is still unclear why the Dp2(4) phage was able to infect *Salmonella cholerasuis* only once. The number of host cells and phage titres were the same for each experiment as determined by the optical densities. Specific host cell receptor sites could have had an effect on bacteria-phage susceptibility. It can be concluded that these Stx2-converting phages are for the most part highly host specific with rarely occurring phage conversion among different enterobacteriaceae species.

6.5 References

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Host Range Susceptibility of Toxin-converting Bacteriophages Infecting *Escherichia coli* O157:H7

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Table 6.1 Bacterial strains of the family Enterobacteriaceae used to determine stx-phage susceptibility

Bacterial strain	**Reference**
E. coli O157:H7 (Stx2 negative)	ATCC 43888[a]
E. coli O157:H7 (Stx2 positive)	ATCC 43889[a]
E. coli C600	ATCC 13706[a]
Salmonella typhimurium	Clinical isolate (human)[b]
Salmonella cholerasuis	ATCC 6994[a]
Proteus mirabilis	Clinical isolate (human)[b]
Proteus mirabilis	ATCC 4630[a]
Enterobacter cloacae	Clinical isolate (human)[b]
Enterobacter cloacae	NCTC 10005[c]
Klebsiella pneumoniae	Clinical isolate (human)[b]
Klebsiella pneumoniae	ATCC 4208[a]
Shigella dysenteriae	Clinical isolate (human)[b]
Shigella dysenteriae	ATCC 9199[a]

[a] = Strains supplied by the American Type Culture Collection
[b] = Strains supplied by the Dept. of Medical Microbiology, University of Pretoria
[c] = Strains supplied by the National Collection of Type Cultures

Table 6.2 Primer sequences and predicted sizes of PCR amplified products for the detection of Stx (VT)-specific genes of *E. coli* O157:H7

Primer	Oligonucleotide sequence (5'-3')	Predicted size of amplified product (base pairs)	Target(s)	Reference
VT1a	GAAGAGTCCGTGGGATTACG	130	Stx1	Pollard *et al.* (1990)
VT1b	AGCGATGCAGCTATTAATAA	346	Stx2	Pollard *et al.* (1990)

Primers were obtained from Sigma-Genosys Ltd. London Road, Pampisford, Cambridgeshire, CB2 4EF, UK

Table 6.3 Phages induced from 5 environmental isolates of *Escherichia coli* O157:H7 infecting *Salmonella choleraesuis* (ATCC 6994), *Proteus mirabilis* (ATCC 4630), *Klebsiella pneumoniae* (ATCC 4208), *Shigella flexneri* (ATCC 9199), *Enterobacter cloacae* (NCTC 10005), *Escherichia coli* C600 (ATCC 13706) and *Escherichia coli* O157:H7 (ATCC 43888).

Phage	Experiment	Host cultures						
		Salmonella choleraesuis	***Proteus mirabilis***	***Klebsiella pneumoniae***	***Shigella flexneri***	***Enterobacter cloacae***	***Escherichia coli* C600**	***Escherichia coli* O157:H7**
Dp2(4)ϕ	1	POS	-	-	-	-	-	POS
	2	-	-	-	-	-	-	POS
	3	-	-	-	-	-	-	POS
Dp2(1)ϕ	1	-	-	-	-	-	-	POS
	2	-	-	-	-	-	-	POS
	3	-	-	-	-	-	-	POS
F5(5)12 ϕ	1	-	-	-	-	-	-	POS
	2	-	-	-	-	-	-	POS
	3	-	-	-	-	-	-	POS
F3(1)39 ϕ	1	-	-	-	-	-	-	POS
	2	-	-	-	-	-	-	POS
	3	-	-	-	-	-	-	POS
DB1(1)ϕ	1	-	-	-	-	-	-	POS
	2	-	-	-	-	-	-	POS
	3	-	-	-	-	-	-	POS

Salmonella choleraesuis cell density at 640 nm - 0.427 (1.2 x 10^8 cells. ml^{-1}); *Proteus mirabilis* (ATCC 4630) cell density at 640 nm - 0.760 (1.1 x 10^8 cells. ml^{-1}); *Klebsiella pneumoniae* (ATCC 4208) cell density at 640 nm - 0.510 (2.0 x 10^7 cells. ml^{-1}); *Shigella flexneri* (ATCC 9199) cell density at 640 nm - 0.451 (2.1 x 10^9 cells. ml^{-1}); *Enterobacter cloacae* (NCTC 10005) cell density at 640 nm - 0.510 (4.1 x 10^9 cells. ml^{-1}); *Escherichia coli* C600 (ATCC 13706) cell density at 640 nm - 0.670 (9.2 x 10^9 cells. ml^{-1}); *E. coli* O157:H7 (ATCC 43888) cell density at 640 nm - 0.480 (6.5 x 10^8 cells. ml^{-1})

Phage Dp2(4)ϕ = 6.5 x 10^6 pfu.ml^{-1}; Phage F3(1)39 ϕ = 6.2 x 10^6 pfu.ml^{-1}; Phage DB1(1)ϕ = 1.2 x 10^7 pfu.ml^{-1}; Phage Dp2(1)ϕ = 1.4 x 10^6 pfu.ml^{-1}; Phage F5(5)12ϕ = 8.0 x 10^6 pfu.ml^{-1}

Table 6.4: Phage Dp2(4)ϕSC1 grown in *Salmonella choleraesuis* (ATCC 6994)

Phage	Date	Phage titer				
		1	-1	-2	-3	-4
Dp2(4)ϕSC1	37150	-	-	-	-	-
	2001-09-23	-	-	-	-	-

Salmonella choleraesuis (ATCC 6994) cell density at 640 nm: Absorbance of 0.427 (1.2×10^8 cfu. ml^{-1})

Table 6.5: Phage Dp2(4)ϕSC1 grown in *Escherichia coli* O157:H7 (ATCC 43888)

Phage	Date	Phage titer				
		1	-1	-2	-3	-4
Dp2(4)ϕSC1	37150	+	+	+	+	+
	2001-09-23	+	+	+	+	+

Escherichia coli O157:H7 (ATCC 43888) cell density at 640 nm: Absorbance of 0.427 (1.2×10^8 cfu. ml^{-1})

Table 6.6: *Salmonella choleraesuis* (ATCC 6994) infected with Dp2(4)SC1 phages grown in *E. coli* O157:H7

Phage	Run 2001-09-17	Phage titer				
		1	-1	-2	-3	-4
Dp2(4)ϕSC1	1 (direct)	-	+ (>2 mm)	-	-	-
	2 (24 h)	-	-	-	-	-

Table 6.7: ***E. coli*** **O157:H7 (ATCC 43888) infected with Dp2(4)ϕSC1 phage grown in *Salmonella choleraesuis* (ATCC 6994)**

Phage	**Run 2001-09-17**	**Phage titer**				
		1	**-1**	**-2**	**-3**	-4
Dp2(4)ϕSC1	1 (direct)	+ (<1 mm)	+ (<1 mm)	-	-	-
	2 (24 h)	+ (<1 mm)	-	-	-	-

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CHAPTER 7

Conclusions

The literature survey outlined general characteristics and public health risks constituted by *E. coli* O157:H7. The *E. coli* O157:H7 organisms are known to occur in South Africa as they do in the rest of the world. Waterborne transmission has not been recorded in South Africa, and the isolation of the *E. coli* O157:H7 from water resources in the country has not been described. Final conclusions and future research are summarised in this chapter:

7.1 Assessment of techniques for the isolation of *E. coli* O157:H7

An assessment of the efficiency of the new enrichment-IMS-selective agar procedure revealed that it was sensitive enough to isolate seeded *E. coli* O157:H7 from sewage, river water, grounded beef and milk samples at levels as low as average counts of one per ml. The new enrichment-IMS-selective agar procedure was then used in a survey for the presence of *E. coli* O157:H7 in selected samples of sewage and river water collected over a period of one year, as well as representative samples of grounded beef and milk. *E. coli* O157:H7 was successfully recovered from a sewage sample.

The prevalence of *E. coli* O157:H7 as reflected by the results in this report would still appear low. The enrichment-IMS-selective agar procedure used had certain shortcomings, particularly with regard to the selective cultivation of *E. coli* bacteria on selective agar media in the final step. *E. coli* O157:H7 colonies proved difficult to distinguish from background growth and other *E. coli* colonies. Consequently the incidence of *E. coli* O157:H7 among suspect colonies was low. It is therefore possible that *E. coli* O157:H7 colonies were not detected and that the actual incidence was higher than reflected by the single isolate from a sewage sample.

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It maybe possible that the incidence of *E. coli* O157:H7 in the waters under investigation was indeed low. Unfortunately it was difficult to assess the potential presence of these pathogens in the waste water concerned because no meaningful data were available on the incidence of *E. coli* O157:H7 infections in the communities from which these wastes originated. In addition, there were no meaningful data in the rest of the world to which these results could be compared. Although *E. coli* O157:H7 has been recovered from wastewater in countries such as Germany (Höller *et al.*, 1999), no quantitative data on the incidence of the pathogens in the waters under investigation were available. The apparently low incidence of *E. coli* O157:H7 in the South African waste water investigated may perhaps be explained by data which suggested that the incidence of *E. coli* O157:H7 infections were lower among humans in the South Africa than in countries of the northern hemisphere (WHO, 1997).

Based on indications that the minimal infectious dose of *E. coli* O157:H7 may be more than 100 organisms, the results obtained in Chapter 3 would seem to indicate that the water under investigation do not constitute a meaningful risk of *E. coli* O157:H7 infection. This would apply to exposure to water in the Vaal River during recreational activities such as boating and fishing, and probably even swimming. The water may not even constitute a meaningful risk of *E. coli* O157:H7 infection when used for domestic purposes. Utilisation of the water in the Vaal River barrage for the production of drinking water supplies by appropriately applied conventional treatment and disinfection procedures would appear to adequately reduce risks of infection to acceptable limits.

However, data on waterborne outbreaks of *E. coli* O157:H7 and the isolation of these pathogens from sewage in South Africa and other parts of the world, warrants attention and caution. This refers in particular to high risk communities which include the very young and very old, under-nourished people, and immuno-compromised patients. The latter include AIDS patients and patients under immuno-suppression for conditions such as cancer and organ transplants. *E. coli* pathogens, like most other pathogens, constitute risks of life-threatening complications and

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implications in these individuals which are much more severe than in healthy people. The high risk component of many communities in South Africa would appear to be substantial and increasing as it does in many other parts of the world.

A major objective of this project has been successfully accomplished. This is the development of technology and expertise on a potentially important waterborne pathogen. The enrichment-IMS-selective agar procedure developed in this project proved relatively sensitive and efficient. The same applies to molecular techniques established for the characterisation of *E. coli* O157:H7 and other *E. coli* pathogens. An infrastructure for services and further research on these pathogens has been established. The work resulted in the first isolation of *E. coli* O157:H7 from water in South Africa. The new enrichment-IMS-selective agar procedure for the isolation of *E. coli* O157:H7 is the first of its kind in the world and according to data presented in this project more sensitive than any methods used for this purpose to date.

7.2 Characterisation of *E. coli* O157:H7

This is the first study in South Africa in which repetitive sequence analysis was used to compare the genotypic relationships of environmentally isolated *E. coli* O157:H7 organisms. The unique locations of ERIC elements made this method extremely sensitive, which allowed for the discrimination at genus, species and strain levels. ERIC-PCR generated multiple amplification products ranging between 50 bp and 3000 bp and revealed distinct patterns for all *E. coli* O157:H7 strains. The *E. coli* O157:H7 strains from the same feedlots grouped together while some were scattered among the other groups. The RSA patterns of the porcine *E. coli* O157:H7 isolates differed extensively while the sewage isolates shared a 50% correlation. The results of RSA revealed the highly discriminating features of the *E. coli* O157:H7 ERIC-patterns among strains of diverse and similar origin.

No strain-specific probes and DNA hybridisation methods were involved, allowing a single set of primers and simple agarose gels sufficient for the analysis of all *E. coli* O157:H7 strains. ERIC-PCR proved to be a rapid, highly discriminating and

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reproducible method for fingerprinting *E. coli* O157:H7 and other enterobacterial strains.

7.3 Induction of Stx2-converting phages from environmental *E. coli* O157:H7 isolates and phage morphology analysis

UV induction experiments of *E. coli* O157:H7 revealed that the maximum number of Stx2-converting phages was released when the bacterial cells were exposed to UV for 2 seconds and thereafter the phage titre gradually decreased when exposed for longer intervals. This differs from the findings of O'Brien *et al.* (1984) who described longer UV exposure times for phage induction. The difficulty to induce Stx2-converting phages from the environmental strains of *E. coli* O157:H7 was confirmed by the low titres obtained after UV radiation. Some strains were subjected to Mitomycin C but UV-radiation proved superior when phage titres were compared after induction. Phage titres after UV exposure were 3 logs higher when compared to exposure with Mytomycin C.

Stx2-converting phages induced from environmental *E. coli* O157:H7 isolates revealed for the first time in South Africa that these phages have different morphologies to the previously described phage lambda (O'Brien *et al.*, 1984). Most induced phages were lambda-like belonging to the family Siphoviridae. We found it difficult to classify the sewage-induced phage isolates which presented with long hexagonal heads and long non-contractile tails. We couldn't find any specific phage family to group these phages, although they resembled some Sipho and Myo-viridae phage properties.

7.4 Host range and phage susceptibility studies

One phage from a total of 5 induced Stx2-encoding phages supported the formation of plaques on *Salmonella cholerasuis*. None of the other phages were able to produce plaques on any of the host cultures except for the control host *E. coli* O157:H7 (Stx2 negative) culture. The phage isolated from the infected *Salmonella cholerasuis* culture were able to multiply in both *E. coli* O157:H7 and *Salmonella cholerasuis* and re-infecting the Stx2-negative *E. coli* O157:H7 strain. Plaques obtained on the lawns

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of all *E. coli* O157:H7 host cultures were small and clear (<1 mm in diameter) while plaques obtained from the lawns of the *Salmonella cholerasuis* cultures were larger and turbid (>2 mm in diameter). The results of this study demonstrated the ability of environmentally induced Stx2-encoding phages to infect certain members of the enterobacteriaceae family and to re-infect *E. coli* O157:H7. This phenomenon of phage conversion rarely occurred in this study and overall, these Stx2-encoding phages seemed to be highly host specific.

There is reason to believe that currently available techniques can be substantially improved with regard to the qualitative and quantitative isolation of *E. coli* O157:H7, particularly from water and food which generally contain these pathogens in low numbers. Molecular techniques for the genetic characterisation of *E. coli* O157:H7 can be improved, particularly with regard to cost, reliability, accuracy, and genetic diversity. Meaningful data on the prevalence of *E. coli* O157:H7 infections will make it possible to assess the public health impact and burden of disease constituted by *E. coli* O157:H7 in South Africa for the first time. Determining the prevalence of *E. coli* O157:H7 in animal reservoirs such as cattle and pigs will cast valuable light on the epidemiology of the pathogens in South Africa. The work is particularly important in South Africa because low socio-economic communities are at highest risk of *E. coli* O157:H7 infections. These efforts are duly justified by the devastating public health impact of *E. coli* O157:H7 on record in other parts of the world.

Future research

In addition, important areas for further research have been identified. These include further improvement of the isolation procedure. Shortcomings in this procedure have been identified. The enrichment-IMS-selective agar procedure developed in this project is superior to methods used in the past, but there is potential for further improvement. This refers in particular to the final step for the selective cultivation of *E. coli* O157:H7 isolates. Selection may perhaps be improved by using alternative antimicrobial agents, which will be more efficient in the suppression of interfering background growth and wild type *E. coli*.

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More data are required on the incidence and behaviour of *E. coli* pathogens in sewage and in raw and treated water supplies in order to assess health risks to a meaningful extent that is required for the formulation of control strategies. Data are required on the incidence of *E. coli* pathogens in animal reservoirs, notably cattle, but also other animals such as pigs. This information is essential for research on the epidemiology of the pathogens, and will cast light on the role of animals in human infections. Information is needed on the incidence of pathogenic *E. coli* infections among humans in South Africa. These details are required for calculations of the burden of disease, which is essential for the formulation of control strategies. Data on the incidence of infections in humans and animals will also facilitate estimation of the numbers of these pathogens that can be expected in wastewater. This information is important for the formulation of strategies to control waterborne transmission.

Research on the phages that transmit the genetic elements coding for toxicity factors to harmless E. coli bacteria is of particular importance. These phages serve as reservoirs for the genetic elements, which convert *E. coli* bacteria into serious pathogens. Details on these phages are likely to hold the key to meaningful strategies for the control of *E. coli* pathogens, which constitute major public health risks. A preliminary survey carried out in collaboration with co-workers at the University of Barcelona revealed the presence of these phages in sewage samples from a number of sources in and around Pretoria. Information is required on the incidence and behaviour of these phages in water environments, and the conditions under which they transfer the toxicity genes to *E. coli* bacteria need to be elucidated.

7.6 References

Höller C, Koshinsky S and Witthuhn D. (1999) Isolation of enterohaemorrhagic *E. coli* from municipal sewage. *Lancet* **353,** 2039.

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O'Brien AD, Newland JW, Miller SF, Holmes RK, Smith HW and Formal SB (1984) Shiga-like toxin-converting phages from *Escherichia coli* strains that cause haemorrhagic colitis or infantile diarrhea. *Science* **226,** 694-696

WHO (1997) Consultations and Workshops. Prevention and Control of Enterohaemorrhagic *Escherichia coli* (EHEC) Infections. *Report of a WHO Consultation,* Geneva, Switzerland. 8 April-1May.

APPENDIX A

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1. CULTURE MEDIA FOR THE ISOLATION OF *E. COLI* O157:H7 AND STX-CONVERTING PHAGES

1.1 Sorbitol MacConkey agar

Agar	51.5	g
Distilled water	1 000	ml

Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 min.

1.2 Rainbow agar O157

Agar	60	g
Distilled water	1 000	ml

Autoclave at 121°C for 10 min (Medium is heat sensitive).

1.3 CHROMagar O157

Agar	1	ampoule
Distilled water	250	ml

Bring to the boil to dissolve completely. Do not autoclave.

1.4 Luria-Bertani (LB) - media

Bacto-tryptone	10	g
Bacto-yeast extract	5	g
NaCl	10	g
Distilled water	1 000	ml

Adjust pH to 7.5 with NaOH
Sterilize by autoclaving
Add aseptically:

MgCl (1 M)	10	ml
CaCl2 (1 M)	10	ml

For LB solid agar add:

Bacto agar	15	g

For LB semi-solid agar add:

Bacto agar	8	g

1.5 Modified Scholten's - media

Peptone	10	g
Yeast extract	3	g
NaCl	3	g
Meat extract	12	g
Na_2CO_3 (150 $g.l^{-1}$)	5	ml
Mg solution (100 g $MgCl_2.6H_2O$/ 50 ml H_2O)	0.3	ml
Distilled water	1 000	ml

Sterilize by autoclaving
For MS solid agar add:

Bacto agar	15	g

For MS semi-solid agar add:

Bacto agar	8	g

1.6 Peptone buffered water

Peptone	10	g
NaCl	5	g
di-Sodium hydrogen phosphate	3.5	g
Potassium dihydrogen phosphate	1.5	g
Distilled water	1 000	ml

Adjust pH to 7.0
Autoclave at 121°C for 15 min

2. SMAC MEDIA CEFIXIME-TELLURITE (CT) SUPPLEMENT (PRODUCT # 740.01)

Preparation of stock solutions of Cefixime and Potassium Tellurite:

2.1. Cefixime Stock solution (final concentration 1 $mg.ml^{-1}$) in absolute ethanol

2.1.1 Add 1 ml of absolute ethanol to the vial of Cefixime.
2.1.2 Dissolve completely
2.1.3 Gently vortex to ensure complete mixing.

The prepared stock Cefixime is stable for 14 days under refrigeration (4°C).

2.2. Potassium Tellurite Stock solution (final concentration 50 $mg.ml^{-1}$)

2.2.1 Add 1 ml of sterile distilled water to the vial containing Potassium Tellurite.
2.2.2 Dissolve completely.
2.2.3 Gently vortex to insure comple mixing.
2.2.4 Store at room temperature.

3. SEQUENCING REACTION OF STX2 POSITIVE PCR PRODUCTS

3.1 Enzymatic pre-treatment of PCR product

3.1.1 Add the following in the specific order:

PCR amplification mixture	5 µl
Exonuclease 1 (10.0 $U.\mu l^{-1}$)	1 µl
Shrimp alkaline phosphatase (2 $U.\mu l^{-1}$)	1 µl

3.1.2 Mix and incubate at 37°C for 15 min

3.1.3 Inactivate exonuclease 1 and phosphatase at 80°C for 15 min

3.1.4 Label , fill and cap tubes with 2.5 µl of each termination mixture (deaza-G, -A, -T, -C).

3.1.5 Keep covered at room temperature for step 3.4

3.2 Annealing template and primer

3.2.1 Add the following in the specific order:

Treated PCR product DNA	7 µl
Primer (5-10 pmol. μl^{-1})	1 µl
H_2O	2 µl
DMSO	1 µl
	11 µl

3.2.2 Incubate 2-3 min at 100°C, then cool rapidly on ice for 5 min.
3.2.3 Prewarm termination tubes at 37°C for 1 min.

3.3 Labeling reaction (add in specific order)

3.3.1 Add the following in the specific order:

Annealed DNA mixture (ice cold)	11 µl
T7 Sequenase reaction buffer (5X)	2 µl
1:5 diluted labeling mix	2 µl
[33P] or [35S] dATP	0.5 µl
Polymerase	2 µl
	17.5 µl

3.3.2 Mix, incubate at room temperature for 2-5 min
(300 bp for 1 min, 4-500 bp for 2 – 3 min)

3.4 Termination reactions

3.4.1 Transfer 3.5 µl of labeling reaction to each termination tube
(G,A,T,C in step 3.1.3)
3.4.2 Mix and continue incubation of the termination reactions at 37°C
(Hybridisation oven)

3.5 Stop reaction by addition of 4 µl stop solution

3.6 Heat samples before loading onto sequencing gel: 75°C for 2 min

3.7 Load 2-3 µl of each reaction per lane, in the order **GATC**

APPENDIX B

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1. The nucleotide sequence of *E. coli* O157:H7 shiga toxin type 2 subunit A (stx2A) and shiga toxin type 2 subunit B (stx2B) as described by Chen *et al.* (2000) in GenBank (9858182) are shown below:

1	tacttcagcc	aaaaggaaca	ccttgtatat	gaagcgtata	ttatttaaat	gggtactgtg
61	cctgttactg	ggcttttctt	cggtatccta	ttcccgggaa	tttacgatag	acttttcgac
121	tcaacaaagt	tatgtatctt	cgttaaatag	tatacggaca	gagatatcga	cccctcttga
181	acatatatct	caggggacca	catcggtgtc	tgttatta**ac**	**cacaccccac**	**cgggcagtta**
241	**ttttgctgtg**	**gatatacgag**	**ggcttgatgt**	**ctatcaggcg**	**cgttttgacc**	**atcttcgtct**
301	**gattatt gag**	**caaaataatt**	**tatatgtggc**	**cgggttcgtt**	**aatacggcaa**	**caaatacttt**
361	**ctaccgtttt**	**tcagatttta**	**cacatatatc**	**agtgcccggt**	**gtgacaacgg**	**tttccatgac**
421	**aacggacagc**	**agttatacca**	**ctctgcaacg**	**tgtcgcacgc**	**ctggaacgtt**	**ccggaatgca**
481	**aatcagtcgt**	**cactcactgg**	**tttcatcata**	**tctggcgtta**	**atggagttca**	**gtggtaatac**
541	**aatgaccaga**	**gatgcatcca**	**gag**cagttct	gcgttttgtc	actgtcacag	cagaagcctt
601	acgcttcagg	cagatacaga	gagaatttcg	tcaggcactg	tctgaaactg	ctcctgtgta
661	tacgatgacg	ccgggagacg	tggacctcac	tctgaactgg	gggcgaatca	gcaatgtgct
721	tccggagtat	cggggagagg	atggtgtcag	agtggggaga	atatccttta	ataatatatc
781	ggcgatactg	ggcactgtgg	ccgttatact	gaattgtcat	catcaggggg	cgcgttctgt
841	tcgcgccgtg	aatgaagata	gtcaaccaga	atgtcagata	actggcgaca	ggcccgttat
901	aaaaataaac	aatacattat	gggaaagtaa	tacagctgca	gcgtttctga	acagaaagtc
961	acagttttta	tatacaacgg	gtaaataaag	gagttaagta	tgaagaagat	gtttatggcg
1021	gttttatttg	cattagtttc	tgttaatgca	atggcggcgg	attgcggctaa	aggtaaaatt
1081	gagttttcca	agtataatga	gaatgataca	ttcccagtaa	aagtggccgg	aaaagagtac
1141	tggaccagtc	gctggaatct	gcaaccgtta	ctgcaaagtg	ctcagttgac	aggaatgact
1201	gtcacaatca	aatccagtac	ctgtgaatca	ggctccggat	ttgctgaagt	gcagtttaat
1261	aatgactgag	gcat				

The highlighted region illustrates the shiga toxin-2-nucleotide sequence fragment investigated in this study.

Reference

Chen L., Pan T., and Sue Y. (2000) Identification and Characterization of Enterohaemorrhagic *escherichia coli* O157:H7 by Using Multiplex PCR Assays for hlyA, eaeA, stx1, stx2, fliC, and rfbO157. GenBank (9858182).