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Characteristion of virulence genes (Shigatoxins 1 and 2, and intimin) in Shiga toxin producing Escherichia coli (STEC) of ovine origin and an assessment of the role of these STEC in human pathogenesis

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Characterisation of virulence genes (Shiga toxins 1 and 2, and intimin) in Shiga toxin-producing *Escherichia coli* (STEC) of ovine origin and an assessment of the role of these STEC in human pathogenesis

A thesis submitted in partial fulfilment of the requirements for the award of the degree

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by

Vidiya Ramachandran, M.Sc.

Department of Biological Sciences

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DECLARATION

I, Vidiya Ramachandran, declare that this thesis, submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy, in the Department of Biological Sciences, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Vidiya Ramachandran

30th August 2002

Every great work, every big accomplishment, has been bought into being through holding to the vision.
And often, just before the big achievement, comes apparent failure and disappointment.
For the greatest accomplishment is not in never falling, but in rising again after your fall.

Mohammad Ali

Sometimes the greatest pleasures in life come from actually doing what people say is impossible.

David Bowden

Adapted from TIME, August 3rd 1998

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Publications & Manuscripts

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Bettelheim, K. A., M. Whipp, S. P. Djordjevic, and V. Ramachandran. 2002. First isolation outside Europe of sorbitol-fermenting verocytotoxigenic *Escherichia coli* (VTEC) belonging to O group O157. J. Med. Microbiol. **51:**1-2.

Brett, K. N., V. Ramachandran, M. A. Hornitzky, K. A. Bettelheim, M. J. Walker, and S. P. Djordjevic. (2003). stx_{1c} is the most common Shiga toxin 1 subtype in Shiga toxin-producing *Escherichia coli* isolates from sheep but not among isolates from cattle. J. Clin. Microbiol. **41:**926-936.

Ramachandran, V., K. N. Brett, M. A. Hornitzky, K. A. Bettelheim, M. Dowton, M. J. Walker, and S. P. Djordjevic. (Manuscript submitted). Development of a universal intimin-typing scheme for *Escherichia coli* and phylogenetic analysis of three new intimin variants.

Conference Presentations

Ramachandran, V., M. A. Hornitzky, K. A. Bettelheim, M. J. Walker, and S. P. Djordjevic. 2000. The detection of Shiga toxin-producing *Escherichia coli* in sheep using vancomycin-cefixime-cefsulodin agar and PCR. Proceedings: The Australian Society for Microbiology Annual Conference. July 2000, Cairns, Queensland, Australia, pA144.

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Mercieca, K. N., V. Ramachandran, M. A. Hornitzky, K. A. Bettelheim, M. J. Walker, and S. P. Djordjevic. 2002. *Stx*$_{1OX3}$ is the most common Shiga toxin 1 subtype in Shiga toxin producing *Escherichia coli* excreted by sheep, but not cattle. Proceedings: The Australian Society for Microbiology Annual Conference. September 2002, Melbourne, Victoria, Australia, PP27.2.

ABBREVIATIONS

A/E	Attaching and effacing
ANGIS	Australian National Genomic Information Service
bp	base pair
°C	Degrees Celsius
E. coli	*Escherichia coli*
ERIC	Enterobacterial repetitive intragenic consensus
EhxA	Enterohaemolysin
EPEC	Enteropathogenic *Escherichia coli*
Gb3	Globotriosyl ceramide
HC	Haemorrhagic colitis
HUS	Haemolytic uraemic syndrome
IEC	Intestinal epithelial cell
kb	kilo base
LEE	Locus of Enterocyte Effacement
min	minute(s)
µl	microliter
OMP	Outer membrane protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PMN	Polymorphonuclear leukocyte
pmole	picomoles
RE	Restriction enzyme
REP	Repetitive extragenic palindromic
RFLP	Restriction fragment length polymorphism
STEC	Shiga toxin-producing *Escherichia coli*
s	second(s)
Stx	Shiga toxin
TE	Tris-EDTA buffer
Tir	Translocated intimin receptor
VTEC	Verocytotoxin-producing *Escherichia coli*

ABSTRACT

Enterohaemorrhagic *Escherichia coli* (EHEC), represent a subset of Shiga toxin-producing *Escherichia coli* (STEC), which cause diarrhoea, haemorrhagic colitis (HC) and haemorrhagic uraemic syndrome (HUS) in humans worldwide. STEC are part of the normal gastro-intestinal flora of ruminants, especially cattle and sheep, and commonly enter the food chain by the faecal contamination of carcasses at slaughter. Most studies of STEC in ruminants have focused on the bovine reservoir. This study examines the genetic characteristics of key virulence genes (*stx*$_1$, *stx*$_2$ and *eae*) in STEC of ovine origin and a subset of STEC of human origin that possess same serotypes as those commonly recovered from sheep but not cattle.

Shiga toxins 1 and 2 are essential virulence attributes of STEC required for the induction of HUS and HC, and may play an important role in infections leading to milder gastro-intestinal diseases such as diarrhoea. In this study a PCR restriction fragment length polymorphism (PCR-RFLP) assay was developed that differentiates *stx*$_{1c}$ from other common *stx*$_1$ subtypes. The *stx*$_{1c}$ was the most common subtype identified (133 of 203; 65.5%) in STEC of ovine origin and was associated with 40 serotypes. Some serotypes, particularly O75:H8 (14 of 21 isolates) were shown to simultaneously possess both common *stx*$_1$ and *stx*$_{1c}$ subtypes. Furthermore, STEC isolates of serotypes commonly found in sheep and recovered from both symptomatic and healthy humans also contained *stx*$_{1c}$ (12 of 34; 35.3%). These data suggest that these 12 isolates from humans may have had an ovine origin. The predominance of *stx*$_{1c}$ among STEC isolated from ovine faeces suggests that the bacteriophage encoding this subtype preferentially inhabits the gastro-intestinal

tract of sheep and/or shows a host range restricted to *E. coli* serotypes that colonise sheep but not cattle.

For the genetic characterisation of stx_2 variants several previously published PCR-RFLP assays were used. The stx_{2d} ($stx_{2d\text{-}Ount/O111/OX3a}$) subtypes, representing 13 serotypes, were almost exclusively identified (141 of 146; 96.6%) in STEC recovered from ovine faeces. These subtypes were predominantly associated with serotypes (O5:H-, O91:H-, O123:H- and O128:H2) commonly recovered from healthy sheep and rarely from healthy cattle. Furthermore, STEC isolates with serotypes predominantly associated with sheep and recovered from both symptomatic and healthy humans also contained these stx_{2d} (11 of 21; 52.4%) subtypes suggesting that they probably had an ovine origin. A single isolate of serotype O91:H21 recovered from a human with diarrhoea simultaneously possessed two stx_2 variants (stx_2 and stx_{2vhb}).

Ovine STEC possessing stx_{1c} and stx_{2d} subtypes were never associated with the typical EHEC serogroups O26, O103 and O157. Recent clinical studies have demonstrated that stx_{1c} and stx_{2d} subtypes are rarely associated with STEC recovered from patients with HUS or HC. However, examples of human STEC isolates of serotypes O5:H- and OX3:H8 associated with HUS that do possess this combination of virulence factor subtypes were identified in this study.

Intimin (Int), encoded by the *eae* gene, is a well characterised outer membrane protein adhesin involved in the intimate adherence of STEC to the host epithelial membrane leading to the formation of the characteristic attaching and effacing lesions. A PCR-RFLP

subtyping assay capable of simultaneously differentiating all 10 recognised intimin subtypes is reported for the first time in this study. This assay was also used to identify and type two previously unreported intimin subtypes identified as Int-ε2 and Int-ι2. Int-β was the most commonly identified intimin subtype (58 of 153; 13.7%) and was associated with the greatest number of serotypes (n=16), followed by Int-ζ (21 of 153; 13.7%; 7 serotypes), Int-ε1 (18 of 153; 11.7%; 5 serotypes), Int-γ (13 of 153; 8.5%; 6 serotypes) and Int-θ (6 of 153; 4%; 5 serotypes). Intimin subtypes α1, α2, λ, δ and ι1 were infrequently identified. None of the 153 *eae*-containing isolates simultaneously harboured more than one intimin subtype. However, intimin genes from 19 of 153 (12.4%) ovine *E. coli* isolates representing 16 different serotypes were untypeable suggesting an even greater variety of intimin subtypes in STEC derived from ovine faeces. Phylogenetic analyses of the C-terminal 280 amino acids (Int_{280}) using the Phylip package confirmed the previous division of the family of intimin proteins into the six distinct clusters represented by subtypes α, β, γ, δ, ε and θ. In addition, the intimin subtypes ζ, ι and λ submitted to GenBank, also resolved as distinct groups but their relationship to other intimin subtypes remain unclear.

The clonal relationships of ovine and human STEC isolates of serotypes (O5:H-, O91:H- and O128:H2) were assessed by pulsed-field gel electrophoresis (PFGE) and repetitive sequence based PCR (REP and ERIC PCR). PFGE differentiated 11, 13 and 13 groups among the sixteen O5:H-, seventeen O91:H- and eighteen O128:H2 strains respectively suggesting that STEC with these serotypes represent genetically heterogenous groups. There were no matches observed in the PFGE profiles between strains of STEC isolated from sheep and those isolated from humans. However, one O91:H- isolate obtained from

mettwurst sausage and an O91:H- isolate from a patient with diarrhoea produced identical PFGE patterns and both the isolates were associated with a food poisoning outbreak in South Australia. This suggests that PFGE may be a useful epidemiological tool for tracing non-O157 STEC infections. Genetic fingerprinting using REP and ERIC PCR showed a low discriminative ability for these isolates and appears unsuitable for this purpose.

Serotypes O5:H-, O91:H-, O103:H2, O123:H-, O128:H2, O157:H-/H7 and OX3:H8 have been isolated from sheep faeces and have been recovered from patients with HC and HUS. With the exception of O157:H-/H7, the other serotypes have never been associated with outbreaks of these diseases and are only rarely recovered from sporadic cases of HC and HUS. The data obtained in this study show that sheep are a reservoir of STEC that possess Shiga toxin subtypes (stx_{1c} and stx_{2d}) that are rarely associated with HC and HUS. While EHEC serogroups such as O26, O103, O111 and O157 are important causal serogroups in HUS, the role of other less common non-O157 serogroups remains to be clearly defined but is probably underestimated. The subtyping assays developed in this study will play an important role in the future characterisation of STEC and will be useful tools to clarify the role of non-O157 STEC in human disease.

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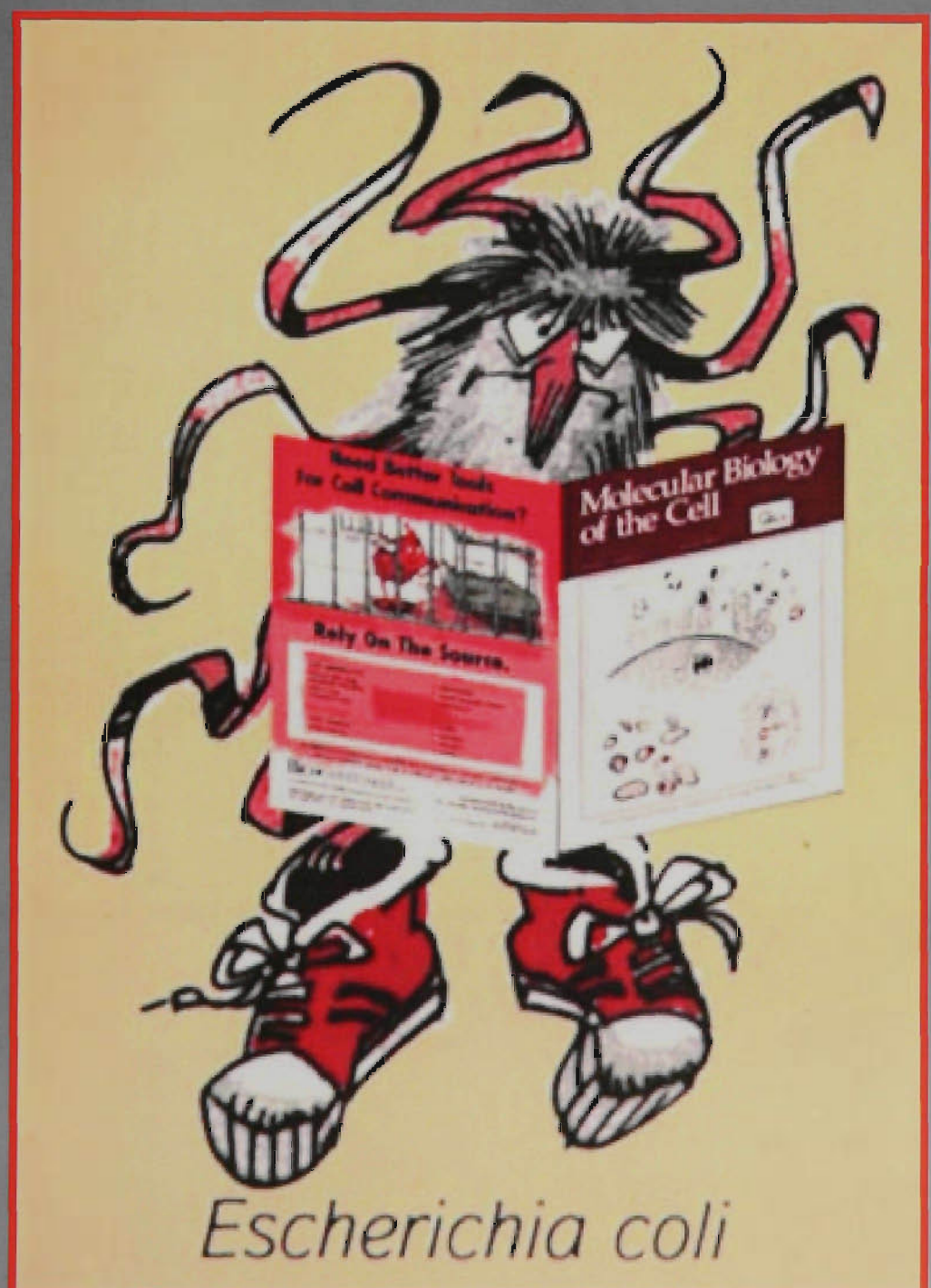
***E. coli* uses its knowledge of cell biology to maintain the flow of diarrhoea**



Adapted from www.biotech.ubc.ca/faculty/finlay/EPEC.HTM

Chapter 1

Shiga toxin-producing *Escherichia coli* – An overview

1 – Shiga toxin-producing *Escherichia coli* – An overview

1.1 *Escherichia coli*

Escherichia coli (*E. coli*) is a gram-negative bacillus and the predominant facultative anaerobe found in the gastro-intestinal tract of humans and animals. This organism colonises the infant gastro-intestinal tract within hours of birth and usually remains harmless within the host exhibiting a mutual benefit relationship (Drasar *et al.*, 1974; Bettelheim and Lennox-King, 1976). However, highly adapted *E. coli* clones have evolved which cause a broad spectrum of human diseases. Three general clinical syndromes result from infection with such *E. coli* strains: (i) enteric/diarrhoeal disease, (ii) urinary tract infection and (iii) sepsis/meningitis.

Diarrhoea causing strains of *E. coli* are classified into five groups based on the behaviour of the bacterial cells in the presence of mucosal cells of the host gastro-intestinal tract. This classification is based on the adherence pattern, invasiveness and the ability to produce one or more toxins (Table 1.1). For the purpose of this thesis, Shiga toxin-producing *Escherichia coli* (STEC) will be reviewed in detail.

1.2 Classification of *E. coli*

1.2.1 Taxonomical classification

E. coli are members of the *Enterobacteriaceae* family, and are usually identified via characteristic biochemical reactions. The ability to ferment glucose with the production of acid and gas is a basic characteristic of *E. coli*. Further, *E. coli* are citrate negative (99%), methyl red positive (99%), Voges-Proskauer negative (100%), produce β-galactosidase

(95%) and produce indole (98%) but do not form hydrogen sulphide (99%) or hydrolyse urea (99%) (Farmer *et al.*, 1985).

Table 1.1 Characteristics of the five major classes of diarrhoeagenic *E. coli*.

Pathotype	Virulence Determinants	Adhesion and Invasion Characteristics	Disease Association
Enterotoxigenic *E. coli* (ETEC)	Heat-labile (LT) and/or Heat-stable (ST) entero toxins.	Adhere uniformly but do not invade.	Traveller's diarrhoea; Infantile diarrhoea.
Enteroinvasive *E. coli* (EIEC)	Plasmid encoded outer membrane invasins and toxins.	Invade cells of colon and spreads laterally to adjacent cells.	*Shigella*-like dysentery.
Enteroaggregative *E. coli* (EAEC)	Enteroaggregative heat-stable enterotoxin (EAST); Aggregative adherence fimbriae.	Adhere in clumps but do not invade.	Mucoid diarrhoea; Watery diarrhoea.
Enteropathogenic *E. coli* (EPEC)	Bundle forming pili; Products of chromosomal pathogenicity island (LEE).	Adhere in clumps. Produce attaching and effacing lesions.	Infantile diarrhoea in less developed countries.
Shiga toxin-producing *E. coli* (STEC) Sub-class Enterohaemorrhagic *E. coli* (EHEC)	Shiga cytotoxins; Products of the LEE pathogenecity islands.	Adhere tightly. Attach and efface host cells, but do not invade.	Bloody diarrhoea; Haemorrhagic colitis (HC); Haemolytic uraemic syndrome; (HUS).

(Adapted and modified from Nataro and Kaper, 1998; Robins-Browne, 2000).

1.2.2 Serological classification

Serotyping is a well established and widely used scheme for identifying and classifying *E. coli* associated with disease, because both pathogenic and non-pathogenic *E. coli* shares similar biochemical reactions. Serotyping is based on the antigenic differences found in surface structures. *E. coli* are serotyped on the basis of four fundamental surface antigen profiles: (i) O (somatic), (ii) H (flagellar) (iii) K (capsular) (Lior *et al.*, 1996) and (iv) F (fimbrial) (Orskov *et al.*, 1982) antigen. (Figure 1.2)

The somatic (O) antigens are composed of lipopolysaccharide complexes, which are part of the cell wall structure of smooth (S) *E. coli* strains. It is the immunogenicity of the polysaccharide-repeating units, which gives the O antigens their specificity. Some strains lack these repeating side chains and become auto-agglutinatable, are considered as rough (R) and designated OR. More than 170 different O antigens, each defining a serogroup, are recognized (www.microbionet.com.au).

The antigenic diversity of flagellar (H) antigen is based on the different types of flagellin present as part of the flagellar structure. Many *E. coli* on primary isolation are either only sluggishly motile or non-motile. However, well-flagellated cells can be obtained by passage of the organism through semi-solid agar. Those strains not developing motility are designated non-motile (NM) or H- (Chandler and Bettelheim, 1974).

The capsular (K) antigen is composed of acidic polysaccharides and is independent of the O specific lipopolysaccharide (Lior *et al.*, 1996). The fimbrial (F) antigens were initially identified as K antigens before their proteinaceous molecular structure was known and

consequently removed from the K antigen profile and given their own profile (Orskov *et al.*, 1982).

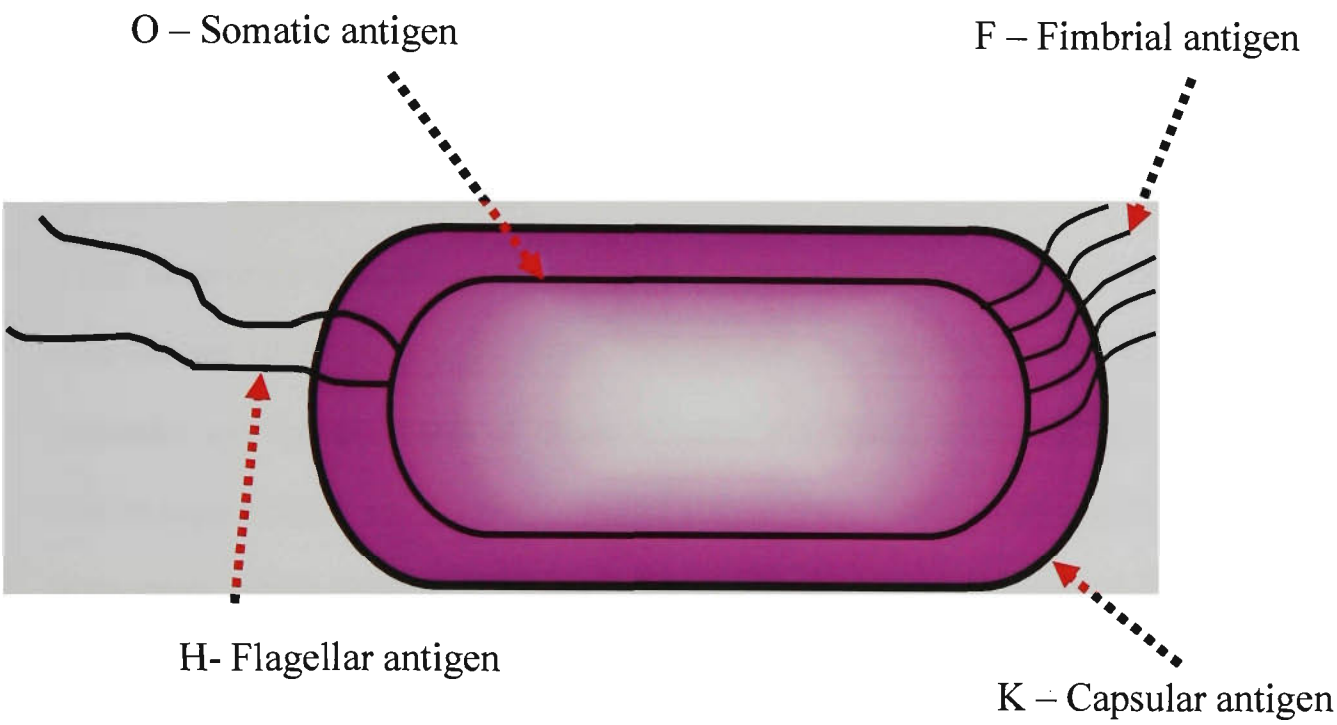


Figure 1.1 Schematic representation of the relationship between structure and antigens of *E. coli*.

1.3 Nomenclature of STEC

Certain *E. coli* strains produce a potent cytotoxin called Verocytotoxin (VT), which produces a cytotoxic effect on Vero (green monkey kidney) cells (Konowalchuk *et al.*, 1977). This group of bacteria was collectively termed Verocytotoxin-producing *E. coli* or VTEC. More recently these bacteria have also been referred to as STEC because one of the VT's, now known as VT1 (see below) was shown to be very similar to Shiga toxin (Sht), produced by strains of *Shigella dysenteriae* type 1, in terms of biological properties, physical characteristics and antigenicity (O'Brien *et al.*, 1982; O'Brien and LaVeck, 1983).

The term enterohaemorrhagic *E. coli* (EHEC) has also been coined to describe strains of STEC, which cause infections in humans resulting in bloody diarrhoea, haemorrhagic colitis (HC) and haemorrhagic uraemic syndrome (HUS) (Paton and Paton, 1998).

1.4 Epidemiology of STEC

1.4.1 STEC serotypes in humans

STEC of serotype O157:H7 is the classical serotype that was first associated from patients with HC and HUS (Riley *et al.*, 1983). This serotype has been responsible for numerous outbreaks and sporadic cases of illness (Griffin and Tauxe, 1991). Molecular genetics studies suggest that *E. coli* O157:H7 evolved from a strain of EPEC that acquired the Shiga toxin genes, which resulted in the development of a more virulent organism (Whittam *et al.*, 1993). However, over 100 different STEC serotypes other than O157:H7 have now been associated with severe human illness (Table 1.2) and some estimates suggest that, 20 to 25% of HUS cases are caused by non-O157 STEC in the USA (Johnson *et al.*, 1996). Since the 1980's non-O157 STEC have caused 10 to 30% of sporadic cases of HUS in various European countries (Caprioli *et al.*, 1997; Bonnet *et al.*, 1998). The most common non-O157 serotypes associated with human disease include O26:H11, O103:H2, O111:H-O128:H2 and O113:H21 (Griffin and Tauxe, 1991). The potential etiological significance of non-O157 STEC serotypes is underestimated in STEC related outbreaks involving STEC O157:H7 due to the current focus on the latter serotype. In many outbreaks attributed to STEC O157:H7/H-, these strains were isolated only from relatively few individuals, and this indicates that non-O157 STEC may have a role in these outbreaks (Goldwater and

Table 1.2 Serotypes of non-O157 STEC isolated from humans, cattle, sheep and other sources.

O1.H- [a]	O6.H28	O17.H-	O26.H16	O52.H19
O1.H1	O6.H34	O18.H-	O26.H21	O52.H25
O1:H7	O6.H49	O18.H11	O26.H?	O53.H2
O1.H18 [b]	O6.H?	O18.H12	O27.H21	O54.H2
O1.H20 [c]	O7.H-	O18.H15	O27.H30	O54.H21
O2.H-	O7.H4	O18.H?	O28.H4	O55.H-
O2.H1	O7.H8	O20.H-	O28.H35	O55.H6
O2.H5	O8.H-	O20.H7	O29.H34	O55.H7
O2.H6	O8.H2	O20.H16	O30.H12	O55.H10
O2.H7	O8:H8	O20.H19	O35.H21	O55.H17
O2.H25	O8.H9	O20.K38.H19	O38.H16	O55.H?
O2.H27	O8.H11	O20.H?	O38.H21	O56.H56
O2.H29	O8.H14	O21.K5.H14	O38.H?	O65.H9
O2.H32 [d]	O8.H16	O21.H21	O39.H7	O65.H14
O2.H39	O8.H19	O22.H-	O39.H-	O65.H16
O2.H45	O8.H21	O22.H1	O39.H8	O65.H48
O2.H? [e]	O8.H25	O22.H2	O39.H21	O68.H-
O4.H-	O8.H30	O22.H5	O39.H48	O68.H14
O4.H4	O8.H35	O22.H8	O39.H49	O69.H-
O4.H5	O8:H49	O22.H16	O40.H8	O69.H8
O4.H10	O8.H?	O22.H21	O40.H21	O69.H11
O4.H21	O8.Hnt	O22.H40	O42.H25	O69.H28
O4.H25 [f]	O9.H-	O22.H54	O43.H2	O69.H-
O4.H40	O10.H-	O22.H-	O45.H-	O70.H8
O5.H- [g]	O11.H-	022.H?	O45.H2	O70.H11
O5.H10	O11.H2	O23.H7	O45.H8	O71.H12
O5.H11	O11.H8	O23.H15	O46.H-	O71.HR
O5.H27	O12.H-	O23.H16	O46.H2	O73.H34
O5.H?	O14.H-	O25.H-	O46.H31	O73.H?
O6.H- [h]	O15.H-	O25.H2	O46.H38	O74.H-
O6.K13.H1	O15.H11	O25.H14	O46.H?	O74.H19
O6.H2	O15.H16	O25.H21	O48.H21	O74.H28
O6.H3	O15.H27	O26.H-	O49.H-	O74.H29
O6.H4	O15.H45	O26.H2	O50.H-	O74.H42
O6.K53.H7	O16.H2	O26.H11	O50.H7	O74.H?

Table 1.2 Serotypes of non-O157 STEC isolated from humans, cattle, sheep and other sources.

O6.H10	O16.H21	O26.H12	O51.H42	O75.H-
O75.H5	O88.H8	O103.H2	O113.H5	O121.H10
O75.H8	O88.H21	O103.H6	O113.H19	O121.H11
O75.H40	O88.H25	O103.H7	O113.H7	O121.H19
O75.H?	O88.H49	O103.H21	O113.H21	O123.H-
O76.H7	O89.H-	O103.H14	O113.H32	O123.H?
O76.H19	O90.H-	O103.H25	O113.H53	O125.H-
O76.H21	O90.H21	O103.H38	O113.H?	O125.H14
O76.H25	O90.H24	O103.H42	O113.HR	O125.H26
O76.H?	O91.H-	O103.H?	O114.H4	O126.H8
O77.H4	O91.H2	O104.H-	O115.H10	O126.H21
O77.H7	O91.H7	O104.H2	O115.H18	O126.H27
O77.H18	O91.H10	O104.H2	O116.H-	O128.H-
O78.H-	O91.H14	O104.H7	O116.H10	O128.H2
O79.H-	O91.H21	O104.H21	O116.H21	O128.H7
O79.H7	O91.H28	O105.H18	O116.H28	O128.H8
O79.H19	O91.H40	O106.H42	O117.H-	O128.H10
O80.H-	O91.H49	O107.H27	O117.H4	O128.H35
O81.H-	O91.H?	O109.H-	O117.H7	O128.H45
O81.H26	O92.H-	O109.H16	O117.H8	O129.H-
O82.H2	O93.H-	O110.H2	O118.H-	O130.H11
O82.H8	O93.H19	O110.H16	O118.H2	O130.H38
O83.H1	O95.H-	O110.H19	O118.H12	O130.H43
O84.H-	O96.H-	O111.H-	O118.H16	O131.H2
O84.H2	O96.H19	O111.H2	O118.H30	O132.H-
O84.H8	O98.H-	O111.H7	O118.H?	O132.H2
O84.H16	O98.H25	O111.H8	O119.H-	O132.H18
O84.H28	O100.H-	O111.H11	O119.H5	O133.H53
O84.H?	O100.H25	O111.H21	O119.H6	O134.H-
O85.H-	O100.H32	O111.H30	O119.H8	O134.H25
O85.H49	O101.H-	O111.H45	O119.H25	O136.H-
O86.H-	O101.H4	O111.H?	O120.H-	O136.H1
O86.H26	O101.H9	O112.H2	O120.H2	O136.H12
O87.H8	O101.H14	O112.H21	O120.H18	O136.H16
O87.H16	O102.H6	O113.H-	O120.H42	O136.H19
O87.H31	O102.H21	O113.H2	O121.H-	O136.H20

Table 1.2 Serotypes of non-O157 STEC isolated from humans, cattle, sheep and other sources.

O88.H-	O103.H-	O113.H4	O121.H7	O136.H40
O137.H41	O149.H?	O165.H25	OX3.H-	OR.H49
O138.H-	O150.H-	O166.H12	OX3.H2	O?.H-
O138.H2	O150.H8	O166.H15	OX3.H8	OR.H?
O138.H8	O150.H10	O166.H49	OX3.H21	O?.H1
O138.H14	O151.H12	O168.H-	OX3.H43	O?.H2
O139.H1	O152.H-	O168.H8	OX3.H49	O?.H4
O139.H19	O152.H4	O169.H-	OX3.HR	O?.H7
O140.H32	O153.H-	O169.H?	OX7.H16	O?.H8
O141.H-	O153.H2	O170.H8	OX177.H-	O?.H9
O141.H4	O153.H8	O171.H2	OX178.H7	O?.H10
O141.H7	O153.H9	O171.H25	OX178.H19	O?.H11
O141.H8	O153.H12	O172.H-	OR.H-	O?.H12
O142.H38	O153.H19	O172.H16	OR.H2	O?.H13
O143.H-	O153.H21	O172.H21	OR.H4	O?.H16
O144.H2	O153.H25	O173.H2	OR.H7	O?.H18
O145.H-	O153.H30	O174:H2	OR.H8	O?.H19
O145.H8	O153.H31	O174:H8	OR.H9	O?.H21
O145.H16	O153.H?	O174.H21	OR.H10	O?.H24
O145.H25	O156.H-	O175:H16	OR.H11	O?.H25
O145.H28	O156.H7	O28ac.H21	OR.H12	O?.H28
O145.H?	O156.H8	O28ac.H25	OR.H14	O?.H29
O146.H-	O156.H21	O105ac.H18	OR.H16	O?.H32
O146.H1	O156.H25	O111ac.H-	OR.H18	O?.H33
O146.H8	O156.H27	O111ac.H8	OR.H19	O?.H34
O146.H28	O156.H46	O112ab.H2	OR.H20	O?.H38
O146.H21	O156.H?	O112ab.H21	OR.H21	O?.H42
O147.H11	O159.H28	O112ac.H19	OR.H25	O?.H45
O147.H29	O163:H-	O125ac.H-	OR.H32	O?.H47
O148.H28	O162.H7	O128ab.H-	OR.H34	O?.H49
O149.H45	O163.H2	O128ab.H2	OR.H38	O?.HR
O149.H10	O163.H19	O128ab.H45	OR.H42	O?.H?
O149.H19	O165.H-	O128ab.H8	OR.H47	

[a] isolated from humans; [b] isolated from cattle and/or beef; [c] isolated from humans and cattle; [d] isolated from other animals and other food sources; [e] isolated from sheep and/or mutton; [f] isolated from sheep and cattle; [g] isolated from humans, cattle and sheep; [h] isolated from humans and sheep (Adapted from http://www.microbionet.com.au/frames/feature/vtec/brief01.html).

Bettelheim, 1998). In the USA, STEC of serotype O157:H7 have been declared a food adulterant. Non-O157 STEC serotypes are not included as a food adulterant and therefore not normally screened for, which would have major implications in terms of human health since STEC other than O157:H7 cause disease, which are similar to that, associated with STEC O157:H7 (Johnson *et al.*, 1996).

1.4.1.1 Geographical distribution of STEC in humans

STEC are a problem mainly in developed countries (Johnson *et al.*, 1996; Elliot *et al.*, 2001). The non-sorbitol-fermenting, β-glucuronidase-negative O157:H7 is the STEC that is most frequently isolated from HC and HUS patients in the United States, Canada, Japan, and the United Kingdom. Significant numbers of O157:H- EHEC strains have been recovered from samples from continental Europe and Australia (Karch *et al.*, 1993; Robins-Browne *et al.*, 1998; Caprioli and Tozzi, 1998). However, in countries such as Chile, Argentina and Australia, non-O157 STEC serotypes account for the majority of HUS cases (Ojeda *et al.*, 1995; Lopez *et al.*, 1989; Robins-Browne *et al.*, 1998; Elliot *et al.*, 2001). Investigation of an outbreak of HUS and national surveillance of sporadic HUS cases in Australia revealed that among STEC serotypes, O111:H- is currently the most frequent cause of serious human disease (Cameron *et al.*, 1995; Henning *et al.*, 1998; Elliot *et al.*, 2001). STEC of serotypes O26:H-, O113:H21, O130:H11, OR:H9, O157:H-, Ont:H7 and Ont:H- were also isolated from sporadic cases in the 4-year aetiological study of HUS in Australia (Elliot *et al.*, 2001).

1.4.2 STEC in ruminants

The usual habitat of STEC is the intestinal tract of ruminants, especially cattle and sheep (Beutin *et al.*, 1993; Kudva *et al.*, 1996; Beutin *et al.*, 1997; Kudva *et al.*, 1997). Foods that become contaminated with faeces from these animals serve as the primary source of human infection. Meat may become contaminated in the slaughterhouse by the transfer of faeces or gut contents and the process of grinding meat may transfer the pathogens from the surface of the meat to the interior (Brooks *et al.*, 2001). Inadequate cooking may enable the bacteria to survive and be ingested, thus leading to human illness. Figure 1.2 explains the possible routes by which STEC can enter the food supply from ruminant reservoirs. There is increasing evidence that STEC serotypes commonly inhabiting the gastro-intestinal tract of one ruminant species are rarely isolated from other ruminant hosts (Beutin *et al.*, 1993; Beutin *et al.*, 1995; Beutin *et al.*, 1997; Djordjevic *et al.*, 2001). While STEC O157:H7 causes most human diseases, the isolation rates of non-O157 STEC from animal faeces and food are higher that those of STEC O157:H7 (Willshaw and Smith, 1993; Johnson *et al.*, 1996; Djordjevic *et al.*, 2001). Therefore humans are exposed to non-O157 STEC more often than STEC O157:H7 from food and animal sources.

1.4.2.1 STEC serotypes in cattle

Epidemiological investigations in addition to numerous surveys have demonstrated that cattle (both beef and dairy) are a principal reservoir of STEC of serotype O157:H7 and a variety of non-O157 STEC serotypes (Table 1.2) (Griffin and Tauxe, 1991; Hancock *et al.*, 1994; Zhao *et al.*, 1995; Chart, 1998). The most common bovine STEC serotypes are O26:H-, O26:H11, O91:H21, O103:H2, O111:H- and O111:H8, which have all been reported as causing disease in humans (Butler and Clarke, 1994). Reported estimates of the

prevalence of O157 STEC in North American and European cattle range from 0 to 10% (Armstrong *et al.*, 1996). However, in a recent study Hallaran and Sumner (2001) reported that O157 serogroup was rarely detected in dairy cattle (1 in 505 faecal samples) presented for slaughter in Victoria, Australia. In an extensive study involving 1796 healthy slaughter age cattle from eastern Australia, 69 STEC serotypes were recovered, and out of these 38 had not previously been reported in cattle. Furthermore, only two animals excreted STEC O157:H7/H- (Hornitzky *et al.*, submitted), which highlights the high prevalence of non-O157 STEC in cattle.

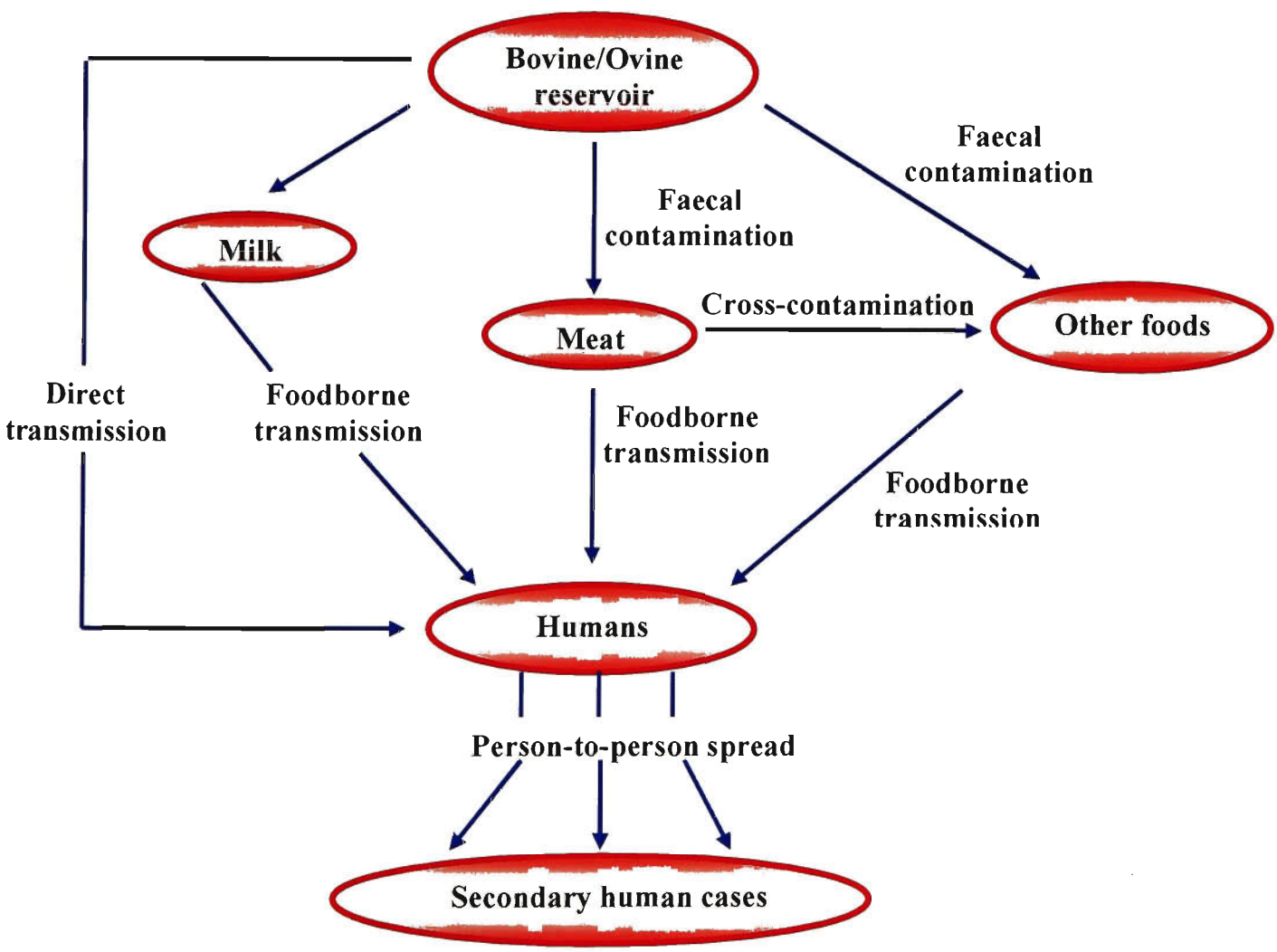


Figure 1.2 Flowchart outlining the mode of transmission of STEC into the human food chain from ruminant reservoirs. (Adapted from Armstrong *et al.*, 1996).

1.4.2.2 STEC serotypes in sheep

Sheep, the second most commonly reared species of ruminant food animal, also harbour STEC at high rates (Beutin *et al.*, 1997; Djordjevic *et al.*, 2001) and like cattle can also carry virulent strains of STEC O157:H7 in their intestinal flora (Kudva *et al.*, 1996). STEC O157:H7 has also been isolated from unpasteurised sheep milk and cheese (Rubini *et al.*, 1999). Kudva *et al.* (1997) investigated the presence of *E. coli* O157:H7 in a single flock over a 16-month period and described the presence of non-O157 STEC isolates of serotypes O5:H-, O6:H49, O88:H-, O91:H- and O128:H- . Limited studies have been carried out to determine the STEC population of sheep in Australia. In a study involving 101 healthy Australian sheep, Bettelheim *et al.* (2000) reported the presence of STEC serotypes O5:H-, O91:H- and O163:H19, which were previously reported to be associated with severe human illness (www.microbionet.com.au). In a recent study of sheep and lambs from 14 farms primarily located in southern Queensland, 117 STEC isolates (45% of 144 sheep faeces and 36% of 72 lamb faeces) were identified highlighting the importance of sheep as a reservoir of STEC (Fegan and Desmarchelier, 1999). More recently, a study screening faecal samples for STEC from 20 mutton sheep and 20 prime lamb flocks (25 sheep per flock) from geographically distinct properties across New South Wales, Australia resulted in the detection of 90 STEC isolates (Djordjevic *et al.*, 2001). The predominant serotypes identified in this study were O5:H-, O91:H-, O123:H- and O128:H2, which are the common serotypes associated with sheep (Djordjevic *et al*, 2001) and have been recovered from human patients with clinical disease (www.microbionet.com.au). These data depicts the role of sheep as a reservoir for pathogenic STEC strains. Table 1.2 summarises the various non-O157 STEC serotypes isolated from sheep.

1.4.3 STEC in other animals

The incidence of STEC strains appears to be lower in pigs and predominantly belong to serotypes O138:K81, O139:K82 and O141:K85, which are implicated in porcine oedema disease (Garabal *et al.*, 1995; Parma *et al.*, 2000). These strains are generally considered to be pathogenic only to pigs and non-pathogenic to humans and produce a characteristic Stx2 subtype, Stx2e which is only associated with porcine serotypes (Gannon *et al.*, 1990). However, Stx2e-producing *E. coli* has also sporadically been isolated from patients with diarrhoea and HUS. These isolates belong to serogroups O101 and O9 that have not been reported as STEC strains associated with oedema disease (Franke *et al.*, 1995). STEC have also been isolated from other animals, such as dogs, horses, pigeons and cats (Beutin *et al.*, 1993; Trevena *et al.*, 1996; Schmidt *et al.*, 2000). However, it is not prevalent or as persistent in these animals as it is in ruminants.

1.5 Virulence associated factors of STEC

STEC possess numerous potential virulence determinants (Table 1.4). Virulence of STEC is not reliant on a single gene or gene product but is believed to be a multi-factorial process (Law, 2000). These pathogens are finely adapted and can survive in water and a wide range of food including acidic foods and thereafter remain viable during the passage from mouth to the intestine via the acidic environment of the stomach. Colonisation of the bowel, mediated by one or more potential adhesins including intimin (an outer membrane protein) and the production of certain Shiga toxin subtypes are likely to be the two factors required for pathogenesis of STEC (Boerlin *et al.*, 1999). However, intimin negative STEC strains are known to cause disease in humans (Paton *et al.*, 1999). Further, it is not clearly

understood why many STEC serogroups producing Stx and expressing intimin have not been reported to cause disease or cause infection at a lower scale when compared to STEC O157:H7.

Table 1.3 Virulence associated factors of STEC.

Protein (Gene)	Carried on	Predicted or detected function of gene product
Shiga toxin (*Stx*)	Chromosome	Inhibition of protein synthesis (Obrig *et al.*, 1987)
Intimin (*eae*)	Chromosome	Adhesion (McKee *et al.*, 1996)
Enterohaemolysin (*ehxA*)	Plasmid	Lyse erythrocytes to release haem and haemoglobin (Law and Kelly, 1995)
Enteroaggregative heat-stable toxin (*astA*)	Chromosome	Stimulates and/or inhibits sodium chloride absorption (Savarino *et al.*, 1991)
Catalase/peroxidase (*katP*)	Plasmid	Detoxify cytotoxic oxidants produced by macrophages and neutrophils (Brunder *et al.*, 1996)
Extracellular serine protease (*espP*)	Plasmid	Cleavage of human coagulation factor V (Brunder *et al.*, 1997)
Clostridium difficile-like toxin	Plasmid	Modifies proteins involved in cell architecture (Burland *et al.*, 1998)
EHEC factor for adherence (*efa*1)	Chromosome	Adhesion (Nicholls *et al.*, 2000)
STEC auto agglutinating adhesin (*saa*)	Chromosome	Adhesion (Paton *et al.*, 2001)
Urease (*ure*)	Chromosome	Neutralise acidic pH (Heimer *et al.*, 2002)
Type IV pilus (*pil*)	Plasmid	Adhesion and bacterial conjugation (Srimanote *et al.*, 2002)

The question of which factors make STEC O157 more virulent compared to other non-O157 STEC strains is unanswered. The genome of *E. coli* O157 is approximately 1Mb larger than the conventional laboratory *E. coli* K-12 genome (Kaper *et al.*, 1998b). This is due to the presence of additional DNA such as Stx phage genes, the locus of enterocyte effacement (LEE) and the pO157 plasmid. The pO157 plasmid in *E. coli* O157:H7 comprises approximately 100 genes (Burland *et al.*, 1998) including a type 2 protein secretion system, a novel haemolysin known as enterohaemolysin, *Clostridium difficile* like toxin and Catalase/peroxidase (Figure 1.3). The relative importance of the above mentioned pO157 factors in pathogenesis are not fully understood. The pO157 plasmid can serve as a marker for pathogenic STEC strains, because of the high prevalence of this plasmid in disease-associated strains (Beutin *et al.*, 1994). Further, there may be unidentified genes in the O157 genome, which could be associated with virulence and severity of disease caused by these strains. These additional potential virulence factors may contribute towards the increased virulence of STEC O157. Figure 1.3 schematically illustrates the characteristic virulence associated markers of a typical O157:H7 STEC strain.

Possession of pO157 plasmid has also been observed in non-O157 STEC serotypes and the prevalent plasmid marker being the enterohaemolysin, whereas the frequency of other plasmid markers is low when compared to STEC O157:H7 (Karch *et al.*, 1998). However, further studies need to be carried out to investigate these plasmids in non-O157 STEC and to evaluate the prevalence of other potential plasmid and chromosomally encoded virulence markers to elucidate the role of non-O157 STEC in pathogenesis. Further, limited information is available on virulence factors especially Stx and intimin present in ruminant

STEC serotypes, particularly in STEC recovered from sheep. Characterisation of these virulence markers would present substantial insight in determining the association of these strains in human infections.

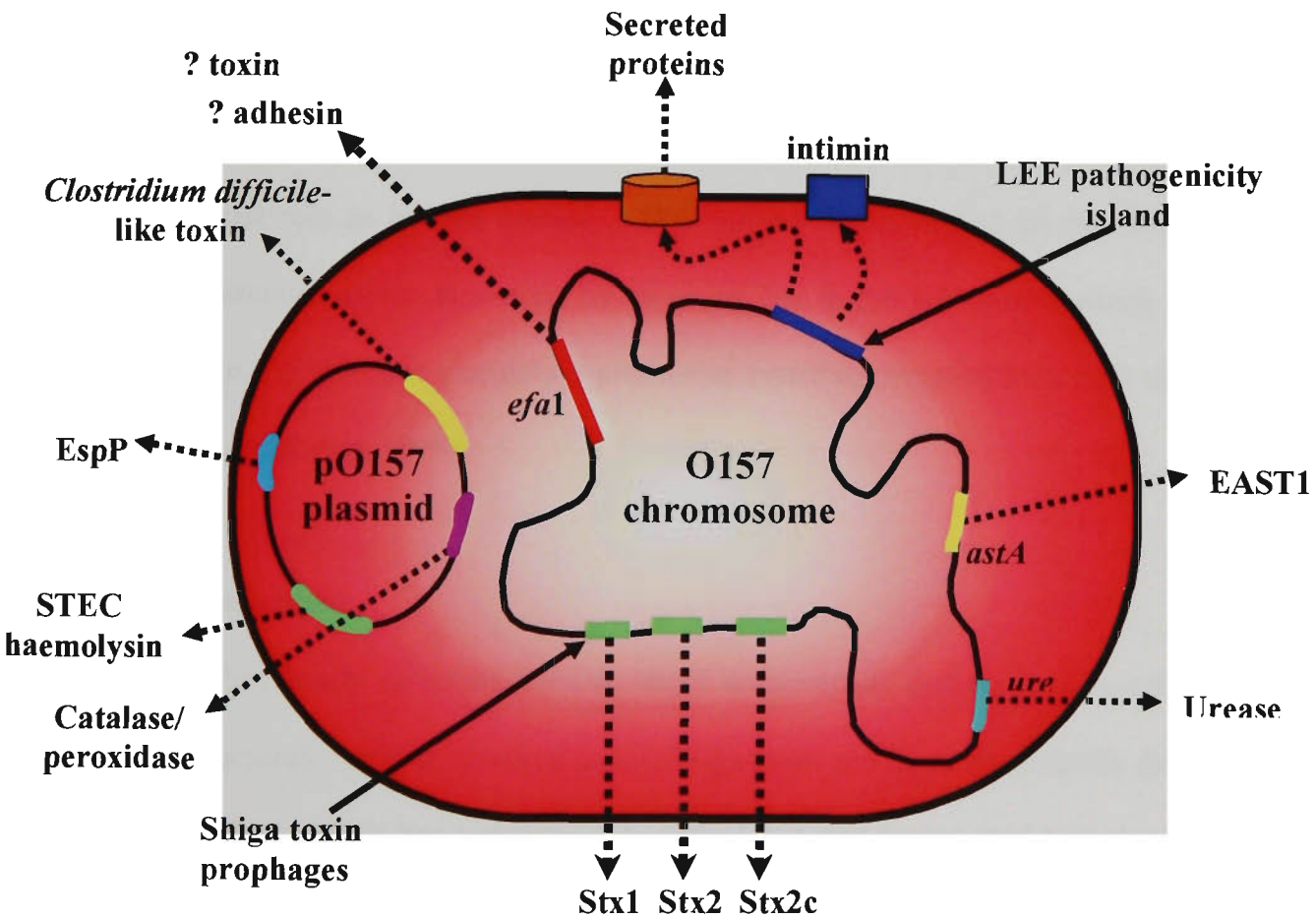


Figure 1.3 Schematic representation of a typical Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 strain illustrating the characteristic virulence-associated markers. (Adapted and modified from Robins-Browne, 2000).

There are several major drawbacks in confirming the role of potential virulence factors and mechanisms of STEC in human infection. These include the lack of suitable animal models, lack of volunteer studies in understanding the pathogenesis, lack of knowledge regarding relative contribution of virulence factors for infection and poor characterisation of STEC other than O157:H7 which leads to decreased understanding of virulence properties associated with these strains (Law, 2000).

1.5.1 Shiga toxins

Shiga toxin (Stx) not only distinguishes STEC from other diarrhoeagenic *E. coli* but also plays a crucial role in human disease. STEC are non-invasive and do not infiltrate the intestinal epithelial tissue. However, by secreting Stx in the gut lumen, which then gets absorbed into the systemic circulation producing systemic complications such as HC and HUS (Paton and Paton, 1998).

1.5.1.1 Stx structure

The *E. coli* Stx family of toxins has two major antigenically distinct groups, called Stx1, which is structurally almost identical to the Shiga toxin produced by *Shigella dysenteriae* type 1, and Stx2, which has approximately 60% DNA and amino acid homology to Stx1 (O'Brien and Holmes, 1987). The basic A and B subunit structure is conserved across all members of the Stx family. The holotoxin comprises one A subunit and five B subunits and are structurally similar to other toxins such as cholera toxin expressed by toxigenic *Vibrio cholerae*, heat labile toxin of enterotoxigenic *E. coli* and the plant toxin ricin (O'Brien *et al.*, 1992). The A subunit (32 kDa) is the active component of the toxin and is composed of two fragments A1 (28 kDa) and A2 (4 kDa) which are linked by a disulphide bond. These

two peptides are proteolytically cleaved at the disulphide bond in the endoplasmic reticulum (ER) and cytosol (Garred *et al.*, 1995). The A1 peptide contains the enzyme activity and the A2 peptide serves to non-covalently bind the A1 polypeptide to the pentameric B subunit (7.7 kDa monomers) (Austin *et al.*, 1994). The B subunit serves the important function of binding the toxin to specific glycolipid receptors on the surface of the target cells (O'Brien and Tesh, 1992). Individual B subunits are composed of antiparallel β sheets and an α helix. Molecular modelling, site-directed mutagenesis and X-ray crystallographic analysis of the B subunit demonstrated three potential carbohydrate-binding sites on each B subunit within the clefts formed by the interaction between the neighbouring β sheets (Nyholm *et al.*, 1995; Nyholm *et al.*, 1996; Ling.,1998; Bast *et al.*, 1999). Further, the B subunits form a doughnut shaped pentamer, which is penetrated by the A2 fragment (Richardson *et al.*, 1997).

1.5.1.2 Stx receptors

Membrane glycolipids containing the carbohydrate sequence galactose α1-4 galactose β1-4 glucose ceramide were identified as the recognised receptor for Stx, which includes globotriosyl ceramide (Gb3), globotetraosyl ceramide (Gb4) and P_1 (a blood group glycolipid antigen present in red blood cell membranes that is structurally related to Gb3) (Jacewicz *et al.*, 1986; Lindberg *et al.*, 1987; Lingwood *et al.*, 1987; Bitzan *et al.*, 1994). Individuals with high expression of P blood glycolipids containing Gb3 are at a lower risk of developing HUS after STEC infection because the red cells can bind the systemic toxin and prevent access for the toxin to more critical renal endothelial cells (Taylor *et al.*, 1990). These various glycolipid receptors are composed of short carbohydrate chains attached to a

ceramide backbone carrying fatty acids of variable lengths. The length of both the Gb3 fatty acid chain and the chain of the phospholipid bilayer that may contain Gb3 has been shown to affect binding of the toxin to the globotriose oligosaccharide moiety within Gb3 (Boyd *et al.*, 1994; Kiarash *et al.*, 1994; Arab and Lingwood, 1996).

The distribution of Stx receptors among various cell types has a major impact on the pathogenesis of disease, both in humans and in various animal models (Lingwood, 1996). Endothelial cells are the primary target of Stx (Paton and Paton, 1998). Nevertheless, microvascular endothelial cells such as human renal and intestinal endothelial cells are much more sensitive to Stx than are large-vessel endothelial cells, such as saphenous vein or human umbilical vein (Tesh *et al.*, 1991; Obrig *et al.*, 1993; Louise and Obrig, 1995; Jacewicz *et al.*, 1999). The difference in the level of expression of Stx receptors by the latter cell lines may be the reason for lower sensitivity to the toxin (Obrig *et al.*, 1993). Gb3 is also found on human intestinal epithelial cells and renal epithelial cells. High levels of Gb3 expression are found in the human renal tissue, in both cortex and medulla with equivalent levels of toxin binding (Boyd and Lingwood, 1989).

Recently studies with Gb3-deficient Vero cells established a binding pattern of Stx and identified a putative membrane protein receptor (Devenish *et al.*, 1998). However, the exact role of this protein receptor is not clearly understood. Other possible candidates for binding and transporting Stx are platelets and lipoproteins (Chatterjee and Kwiterovich, 1984; Cooling *et al.*, 1998). Recently, it was shown that Stx binds to human polymorphonuclear leukocytes (PMNs) in whole blood and the receptor responsible for binding was different from Gb3 found on endothelial cells. It is not yet known if the receptor on PMNs represents

a glycolipid since the binding was sensitive to trypsin treatment. Furthermore, unlike Gb3 the receptor on PMNs does not internalise Stx but allows the transfer of Stx from PMNs to endothelial cells directly due to the lower K_d values (te Loo *et al.*, 2000). Stx receptors have also been identified recently in small sensory neurons of dorsal root ganglia in humans, rabbits and rodents (Ren *et al.*, 1999) and astrocytoma cells (Arab *et al.*, 1998).

1.5.1.3 Stx internalisation and mode of action

Once bound to the receptor expressed on the target cell membrane, Stx molecules are internalised by Clathrin-coated pits, which subsequently pinches off from the cell membrane to form sealed coated vesicles with toxin bound to the internal surface (Sandvig and vanDeurs, 1996). In cells that are insensitive to Stx the vesicles undergo fusion with cellular lysosomes, resulting in toxin degradation (Sandvig *et al.*, 1992). In cells, which are sensitive to Stx the vesicle containing the toxin-receptor complexes undergo retrograde transport to the endoplasmic reticulum via the *trans*-Golgi network (TGN) and Golgi apparatus before being translocated to the cytosol (Figure 1.4) (Sandvig and van Deurs, 1996).

At some stage in this process the A subunit is nicked by a membrane bound protein furin, producing a catalytically active 27 kDa N-terminal A1 peptide and a 4 kDa C-terminal A2 peptide (Garred *et al.*, 1995). At this point both A1 and A2 peptide remain linked by a disulfide bond, which subsequently gets nicked at a trypsin-sensitive site in the C-terminal end of the A1 peptide resulting in the release of the active A1 component into the cytosol (Sandvig *et al.*, 1996).

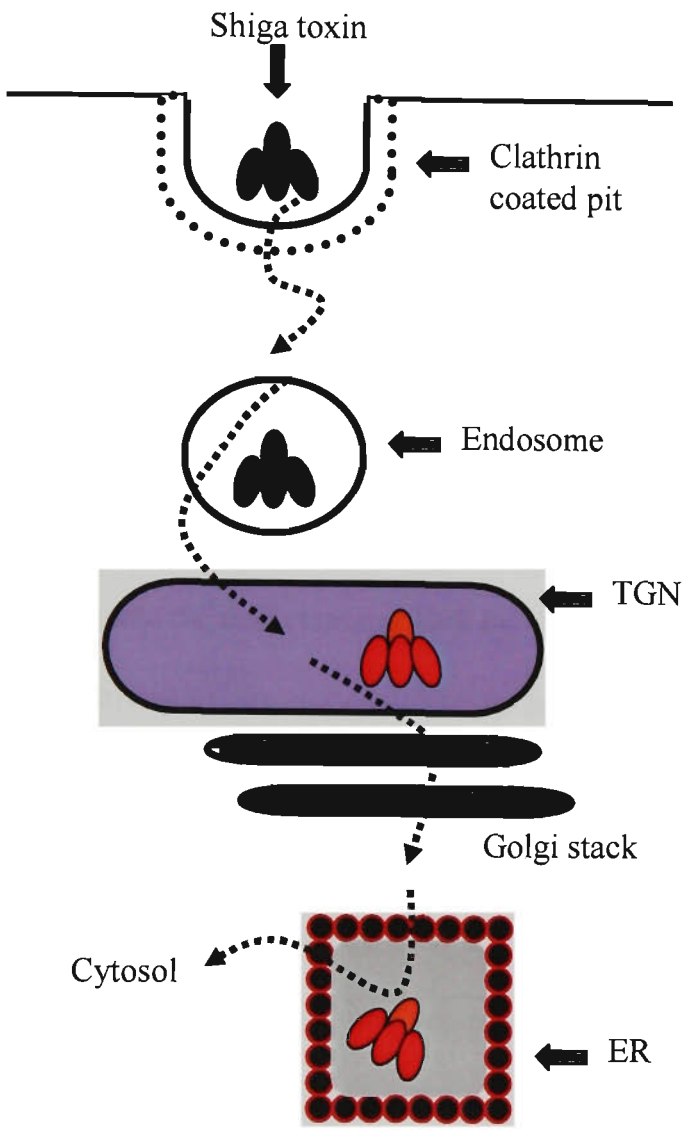


Figure 1.4 Receptor-mediated uptake and retrograde transport of Shiga toxin from the apical membrane to the Cytosol. TGN, *trans*-Golgi network; ER, endoplasmic reticulum. (Adapted and modified from O'Loughlin and Robins-Browne, 2001).

The A1 polypeptide contains the *N*-glycosidase activity that cleaves a single adenine residue from the 28s rRNA component of 60s ribosome, which is ubiquitous in eukaryotic cells (Endo *et al.*, 1988; Skinner and Jackson, 1997). This cleavage prevents the binding of the amino acyl-tRNA to the acceptor site on the 60s ribosomal subunit, thereby inhibiting

the peptide chain elongation step of protein synthesis and eventually causing cell death (Brown *et al.*, 1986; Obrig *et al.*, 1987).

1.5.1.4 Stx and immune response

Purified Stx has been shown to induce the expression of proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin (IL)-1β by macrophages *in vitro* (van Setten *et al.*, 1996; Ramegowda and Tesh, 1996). Using radiolabeled toxin-binding studies and thin layer chromatography techniques van der Kar *et al.* (1992) showed that these inflammatory factors upregulate the expression of Gb3 by vascular endothelial cells *in vitro* resulting in increased sensitivity to Stx toxicity. TNF-β and bacterial lipopolysaccharide (LPS) are also shown to induce the expression of Gb3 and increase the binding of Stx to human endothelial cell (van der Kar *et al.*, 1992). These data suggest that the host cytokine response to the toxin may play an important role in HUS pathogenesis by rendering vascular endothelial cells in intestine, kidney and central nervous system more sensitive to the cytotoxic nature of the toxins.

Stx are also potent inducers of chemokine expression such as interleukin-8 (IL-8) by epithelial cells and can trigger PMN infiltration into the lamina propria and kidneys (Thorpe *et al.*, 1999). IL-8 is a powerful selective activator and a chemo-attractant of PMN and this correlates to the observation of increased PMN counts in HUS patients (Walter *et al.*, 1989). It has been shown that neutrophil transmigration across polarized intestinal epithelial cell (IEC) monolayers diminishes the barrier functions of IEC *in vitro* (Nash *et al.*, 1987).

Acheson *et al.* (1996) demonstrated that intact Stx translocates in an apical-to-basolateral manner across polarized IEC monolayers without sustaining damage from the toxin. In a recent study Hurley *et al.* (2001) demonstrated that the neutrophil transmigration across polarized IEC in a basolateral-to-apical direction drastically increased the translocation of Stx in an apical to basolateral direction by the paracellular pathway (Figure 1.5). This led the authors to speculate that the degree of inflammation in the intestine may contribute to the amount of Stx gaining access to underlying tissue. It has also been hypothesised that Stx mediated immune cell activation in the gut, particularly T-cell activation, could be an important event for the succession of disease from a diarrhoeal phase to HUS (Heyderman *et al.*, 2001). This hypothesis is based on the migration of activated T cells from the gut into the circulation via lymphatic system, from where it can gain access to the glomeruli of the kidney and interact with $CD40^+$ endothelial cells which in turn leads to the upregulation of pro-coagulant factors and adhesion molecules and elevated expression of GB3 receptors (Heyderman *et al.*, 2001). Moreover, the cytotoxic effect of Stx in cultured human cerebral endothelial cells is also increased by co-incubating the cells with inflammatory mediators such as TNF-α and IL-1β thus implicating their role in central nervous system (CNS) damage (Ramegowda *et al.*, 1999) Overall, the cytotoxicity of Stx is enhanced by the release of toxin induced inflammatory mediators and chemo- attractants, which subsequently increases tissue inflammation, receptor expression and sensitivity towards Stx toxicity (Figure 1.5).

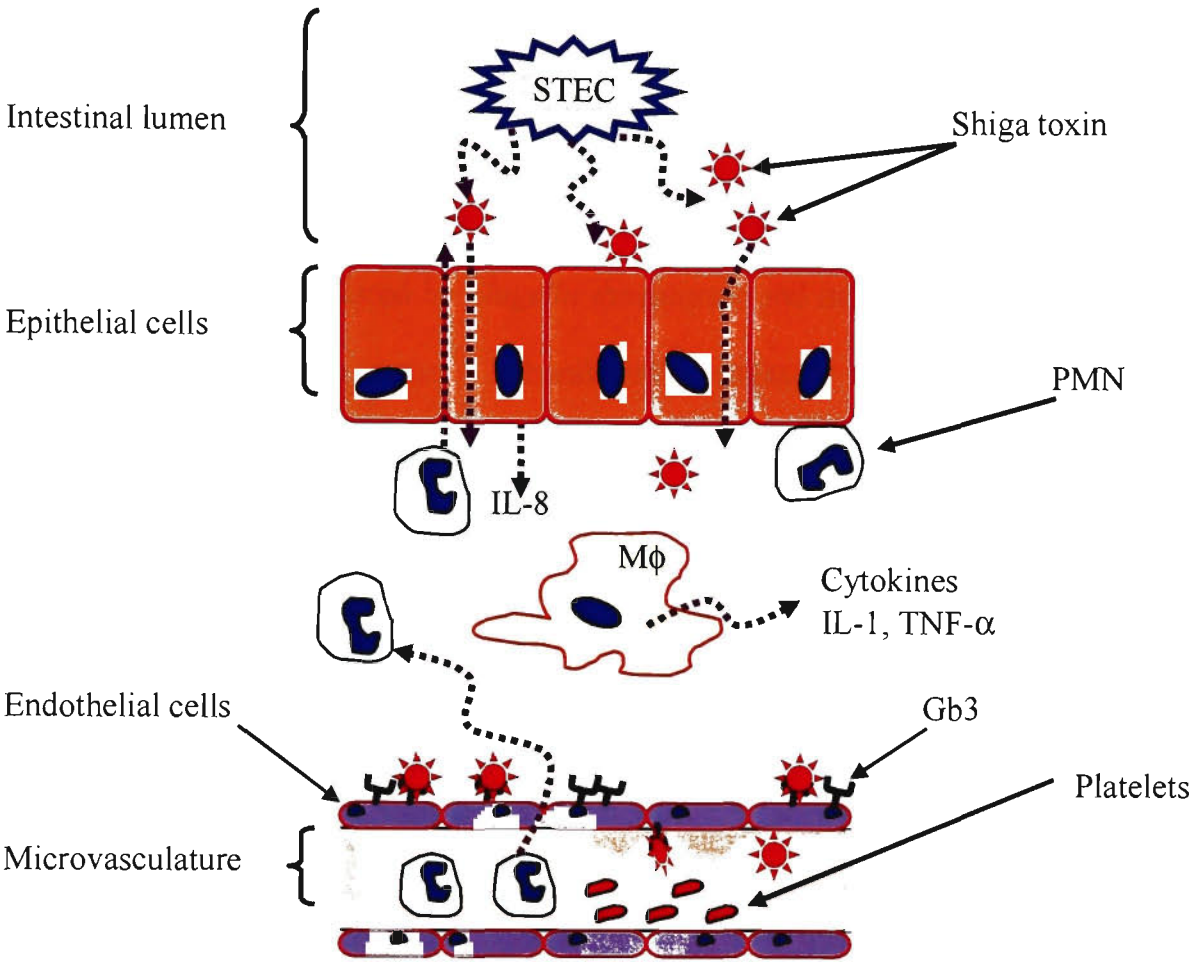


Figure 1.5 Schematic representation of Shiga toxin-mediated inflammatory response. Ingestion of STEC leads to the release of Stx in the gut. Stx translocates across polarized epithelial cells. The translocated Stx may then act on submucosal immunocytes, which release inflammatory mediators, thereby increasing tissue inflammation and increasing receptor expression. Stx may also bind to receptors on underlying endothelial cells and possibly platelets in the microvasculature leading to microvascular thrombosis, which occurs largely in GIT, kidney and brain. Neutrophils transmigrate across polarized epithelial cells in a basolateral to apical direction increasing translocation of Stx in an apical to basolateral manner. Stx induces production of IL8 by epithelial cells and proinflammatory cytokines by macrophages. Gb3, globotriaosylceramide; IL, interleukin, Mϕ, macrophage; PMN, polymorphonuclear leukocyte; TNF, tumour necrosis factor. (Adapted and modified from O'Loughlin and Robins-Browne, 2001).

1.5.1.5 Subtypes of Stx1 and Stx2

1.5.1.5.1 Stx1

Analysis of the genes encoding Stx1 and Stx2 revealed the existence of various stx_1 and stx_2 subtypes, which show differences in their nucleotide and amino acid sequences. stx_1 is closely related to *sht* produced by *Shigella dysenteriae* and its nucleotide sequence differs by only three nucleotides in the A subunit which results in a single amino acid change whilst the B subunit genes are identical (Strockbine *et al.*, 1988; Kozlov *et al.*, 1988). Paton *et al.* (1993; 1995) have described four variants of the stx_1 gene in STEC strains of serotypes O48:H21 (stx_{1O48}), O111:H- (stx_{1CB}, stx_{1PH}) and OX3:H8 (stx_{1OX3}) isolated from humans and animals. These variants were more closely related to *sht* than to the previously published *E. coli* phage-encoded stx_1 gene $stx_{1933\text{-}J}$ (Paton *et al.*, 1993; Paton *et al.*, 1995). Among the four stx_1 variants, stx_{1OX3} showed greatest deviation in amino acid sequence with a total of 43 nucleotide difference resulting in 9 and 3 amino acid changes in the A and B subunits respectively. The stx_{1OX3} was identified in a STEC OX3:H8 strain isolated from sheep in Germany (Paton *et al.*, 1995). Recently Koch *et al.* (2001) reported the presence of stx_{1OX3} among 38 of 48 (79.2%) STEC strains isolated from sheep belonging to serotypes O5:H-, O125:H-, O128:H2, O146:H21 and OX3:H8 but was not detected in ovine isolates with serotype O91:H-. Most of these serotypes are commonly isolated from sheep and not from cattle (Beutin *et al.*, 1993; Beutin *et al.*, 1995; Beutin *et al.*, 1997; Kudva *et al.*, 1997; Djordjevic *et al.*, 2001). Koch *et al.* (2001) also showed that STEC isolated from humans with the same serotypes as STEC recovered from sheep possessed the stx_{1OX3} gene. These data lead to the hypothesis that sheep may have been the source of human infection (Koch *et al.*, 2001). Recently stx_{1OX3} has been renamed as stx_{1c} and was shown to be present in 36 of 212 (17%) STEC strains isolated from asymptomatic humans and from patients with

uncomplicated diarrhoea (Zhang *et al.*, 2002). In addition, two STEC strains isolated from healthy sheep were included in this study and were shown to harbour the stx_{1c} variant. Apart from the reports by Koch *et al.* (2001) and Zhang *et al.* (2002) little is known about the prevalence of stx_1 subtypes in STEC recovered from sheep, particularly in Australia. Understanding the distribution of stx_1 subtypes among the sheep STEC population and comparing them with stx_1 subtypes recovered from both bovine and human STEC can provide information to determine if there is an association with particular stx_1 subtype with host and serotype. Further, comparing the ovine stx_1 subtypes with human stx_1 subtypes can strengthen the hypothesis made by Koch *et al.* (2001).

1.5.1.6.2 Stx2

Unlike stx_1, considerable sequence variations exist among stx_2 on the basis of sequence homology and immunological cross-reactivity. To date, 14 different stx_2 variants have been described from STEC strains isolated from patients with HUS, abdominal cramps, sudden infant death syndrome or diarrhoea, and from healthy or diseased animals (Table 1.4). The most prevalent Stx2 variants are stx_{2c}, stx_{2d} and stx_{2e} (Pierard *et al.*, 1998; Schmitt *et al.*, 1991; Weinstein *et al.*, 1988). stx_{2e} was the first stx_2 variant to be described and is primarily associated with oedema disease in swine (Weinstein *et al.*, 1988) and rarely recovered from humans (Pierard *et al.*, 1991; Thomas *et al.*, 1994). Currently the characterisation of stx_2 subtypes among STEC isolates recovered from sheep has not been reported.

Table 1.4 *stx*$_2$ gene family variants, their origin and associated disease.

Variant	Serotype/Serogroup	Associated syndrome or origin	Reference
Stx2	O157:H7	HUS	Datz *et al.*, 1996
Stx2c	O157:H-	HUS	Schmitt *et al.*, 1991
Stx2-O113	O113:H21	HUS	Paton *et al.*, 1999
Stx2-O48	O48:H21	HUS	Paton *et al.*, 1995
Stx2vh-a	O91:H21	HUS	Ito *et al.*, 1990
Stx2vh-b	O91:H21	HUS	Ito *et al.*, 1990
Stx2d-OX3a	OX3:H21	Sudden infant death	Paton *et al.*, 1992
Stx2d-OX3b	OX3:H21	Sudden infant death	Paton *et al.*, 1993
Stx2d-O111	O111:H-	HUS	Paton *et al.*, 1993
Stx2d-Ount	Ount:H21	Abdominal cramps	Pierard *et al.*, 1998
Stx2e	O139:H1	Porcine oedema disease	Weinstein *et al.*, 1988
Stx2ev	O128:B12	Diarrhoea	Gannon *et al.*, 1990
Stx2f	O128:H2	Feral Pigeon	Schmidt *et al.*, 2000
Stx2-NV206	O6:H10	Healthy cow	Bertin *et al.*, 2001

1.5.1.6 Toxicity of Stx1 and Stx2

Although Stx1 and Stx2 have similar structures and mode of action their toxicities appear to be discrete. Epidemiological studies have indicated that STEC strains producing Stx2 are more likely to be associated with serious human disease such as HUS, than those producing Stx1 or Stx1 and Stx2 (Ostroff *et al.*, 1989; Kleanthous *et al.*, 1990; Boerlin *et al.*, 1999). Challenging mice with a strain of *E. coli* K-12 carrying cloned *stx*$_2$ caused death, but *E. coli* K-12 expressing Stx1 did not have a fatal effect on the mice (Wadolkowski *et al.*, 1990). Furthermore, purified Stx2 had an approximately 400-fold lower LD_{50} for mice than Stx1 by both intravenous and intraperitoneal routes (Tesh *et al.*, 1993).

Human renal microvascular endothelial cells were approximately 1000 times more sensitive to the cytotoxic effect of Stx2 than Stx1 (Louise and Obrig, 1995). Jacewicz *et al.* (1999) showed that Stx2 was more toxic than Stx1 to transformed human intestinal microvascular endothelial cells. Some Stx2 variants (stx_{2c}) can be activated by the proteolytic enzyme elastase found in the intestinal mucus, which may lead to greater toxicity (Melton-Celsa *et al.*, 1996). However, STEC strains producing only Stx1 have been recovered from patients with HUS (Willshaw *et al.*, 1992; Gyles *et al.*, 1998).

Some STEC strains are capable of carrying more than one Stx2 variant (Ito *et al.*, 1990; Schmitt *et al.*, 1991) therefore determining the relative virulence contribution of each variant is difficult. Furthermore, several Stx2 variants differ from one another by only a few amino acid changes in either the A or B subunit (Paton and Paton, 1998), which can affect their enzymatic activity or receptor binding capability.

1.5.1.7 Stx-converting bacteriophages

The structural genes encoding Shiga toxins (stx_1 and stx_2) are located in the genome of lambdoid phages and play an important role in the dissemination of *stx* genes in *E. coli* (Schmidt, 2001). In contrast, Sht in *Shigella dysenteriae* is believed to be chromosomally encoded. However, insertion elements and genes homologous to lambdoid phage genomes have been detected upstream of *shtA* in *S. dysenteriae* type 1, suggesting involvement of bacteriophage (Unkmeir and Schmidt, 2000). The complete nucleotide sequences of Stx1-converting phage H-19B (isolated from STEC O26:H11), Stx2-converting phage 933W (isolated from STEC O157:H7) (Plunkett *et al.*, 1999), phage VT2-Sakai (isolated from STEC O157:H7, responsible for an outbreak in Sakai City) (Makino *et al.*, 1999), phage

VT1-sakai (isolated from STEC O157:H7 from the Sakai outbreak) (Makino *et al.*, 2000) and Stx2e-converting phage ϕP27 (isolated from STEC Ont:H-) (Recktenwald and Schmidt, 2002) have been determined. These studies have shown that the bacteriophages have similar morphology, consisting of hexagonal heads, tails and are related to bacteriophage λ.

Bacteriophages carrying the *stx* genes can horizontally transfer these virulence genes within *E. coli* populations (Cheetham and Katz, 1995), therefore *E. coli* of any serotype may acquire the toxin genes, although the barriers (if any) of transmission have not been intensively investigated. Recently Muniesa and Jofre (1998) reported Stx2 carrying bacteriophages in sewage. Therefore the abundance of Stx encoding bacteriophages in nature can attribute to the presence of *stx* genes in genetically diverse STEC populations. Recently, a novel type IV pilus (*pil*) was identified on the mega plasmid of an O113:H21 STEC strain responsible for an outbreak of HUS and homologues of *pil* were detected in several other STEC strains (Srimanote *et al.*, 2002). This led the authors to hypothesise that the presence of the *pil* locus can enhance the chances of lysogeny by Stx-converting phages and may play a role in contributing to the diversity of *E. coli* strains carrying *stx* genes. Furthermore, the interactions between bacteriophages can promote the acquisition of other virulence genes along with *stx* and horizontal transfer of these genes into *E. coli* can lead to the emergence of highly virulent pathogenic strains.

Recently, it was shown that bacteriophages, in addition to serving as vectors for propagating *stx* genes, may also play a role in the expression of Stx (Neely and Friedman,

1998; Wagner *et al.*, 2001). *stx* genes are located downstream and in the same transcriptional orientation as a λ Q homolog that serves as a transcriptional activator of late phage genes (Neely and Friedman, 1998; Plunkett *et al.*, 1999) (Figure 1.6). Functional studies have shown that Q mediated transcription from P_R through the terminator T_R and through to the lysis gene S, is the mechanism of production and release of toxin (Figure 1.6) (Neely and Friedman, 1998; Wagner *et al.*, 2001). These observations suggest that Stx-encoding phages play a role in regulating Stx expression and spread of Shiga toxin genes in *E. coli*.

1.5.2 Intimin

Although Stx is essential for STEC virulence, several other factors may contribute to the pathogenecity of STEC (Table 1.4). One is intimin, a 94 - 97 kDa outer membrane protein (OMP) (McDaniel *et al.*, 1995). It has been shown that intimin is essential for full virulence both in human volunteers (Donnenberg *et al.*, 1993) and in animal models (Donnenberg *et al.*, 1993; Schauer and Falkow, 1993; McKee *et al.*, 1995; Dean-Nystorm *et al.*, 1998). However, most but not all STEC recovered from humans with HUS possess intimin. Furthermore, non-O157 STEC recovered from animals express intimin less frequently when compared to human STEC isolates (Barrett *et al.*, 1992; Beutin *et al.*, 1995; Johnson *et al.*, 1996).

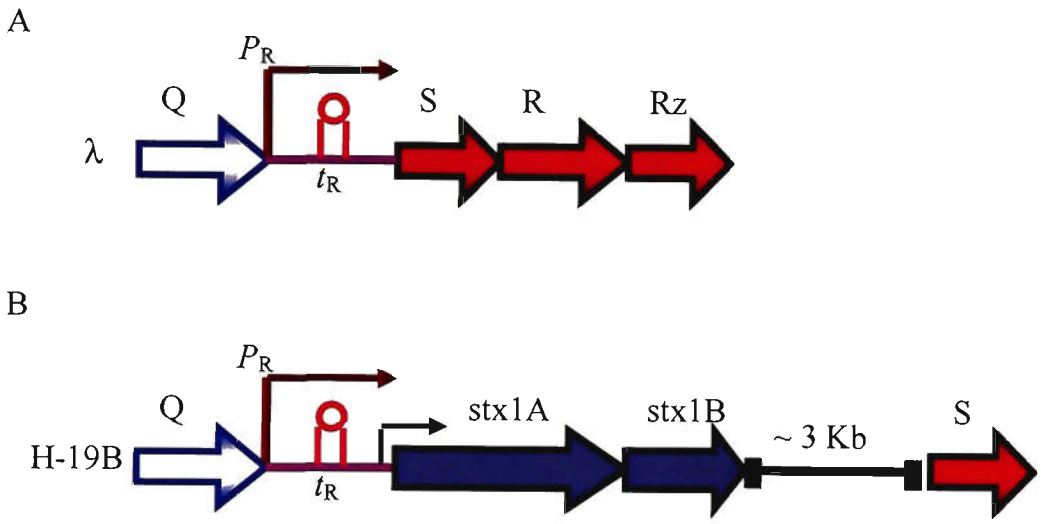


Figure 1.6 DNA sequence of (A) phage λ (B) phage H-19B. The genes encoding Stx1 in the H-19B genome are located downstream of an H-19B homolog of the λ late gene anti terminator Q. Similar to the λ genome, the late promoter P_R and the terminator T_R immediately follows the H-19B Q homolog. Also shown in the figure, is the previously mapped *stxAB* promoter. Following a ~3 Kb open reading frames of unknown function, is the homolog of the λ *S* lysis gene. Two additional lysis genes, namely *R* and *Rz* follows the *S* gene in phage λ (Adapted and modified from Walder, 1998).

Encoded by the *eae* gene, intimin is involved in the intimate adherence of STEC to the host epithelial membrane leading to the formation of characteristic attaching and effacing (A/E) lesions (Paton and Paton, 1998). A/E lesions result from effacement of the intestinal epithelial cell microvilli and intimate adherence between the bacterium and host membrane. Marked cytoskeletal reorganisation occurs directly beneath the intimately adherent bacteria including actin filaments and several other cytoskeletal components such as, α-actinin, ezrin, cortactin, talin, fimbrin and villin (Finlay *et al.*, 1992; Knutton *et al.*, 1992; Kalman *et al.*, 1999; Cantarelli *et al.*, 2000; Goosney *et al.*, 2000). Pedestals can extend up to 10 μm

in length and can bend and undulate while remaining attached in place to the cell surface as well as intimately cupping individual bacteria (Figure 1.7) (Rosenshine *et al.*, 1996, Kalman *et al.*, 1999). However, little is known about the cytoskeletal composition of STEC pedestals other than that it contains cortactin, α-actin and actin (Ismaili *et al.*, 1995; Cantarelli and Akeda, 2000). Apart from STEC, several other pathogens such as EPEC (Jerse and Yu, 1990), *Citrobacter rodentium* (Schauer and Falkow, 1993) and *Hafnia alvei* (Albert *et al.*, 1992), are also known to induce A/E lesion formation upon infection. However, A/E lesions produced by EPEC have been well studied and serve as the paradigm for this process.

1.5.2.1 Locus of enterocyte effacement

The *eae* gene is part of a 35 kb pathogenicity island designated the locus of enterocyte effacement (LEE) (Jerse *et al.*, 1990; McDaniel *et al.*, 1995). Genes within LEE are transcribed as five polycistronic operons known as *LEE*1, *LEE*2, *LEE*3, *tir* and *LEE*4 (Mellies *et al.*, 1999). *LEE*1, *LEE*2 and *LEE*3 encode components of a type III secretion system (Esc and Sep). The *tir* operon encodes intimin, the translocated intimin receptor (Tir) and the Tir chaperone CesT. *LEE*4 encodes proteins secreted by the type III secretion system (Esp) (Figure 1.8). LEE also includes a number of open reading frames of uncertain function (Perna *et al.*, 1998). The secreted proteins of LEE4 include EspA, EspB and EspD, which are involved in signal transduction in host cells and A/E lesion formation (Frankel *et al.*, 1998a; Elliot *et al.*, 1998). EspA constitutes the major component of a translocation apparatus (Knutton *et al.*, 1998; Ebel *et al.*, 1998) through which Tir (Kenny *et al.*, 1997) and EspB (Knutton *et al.*, 1998) are translocated into the host cell. In EPEC, the regulation

of LEE genes involves a plasmid-encoded regulator (Per), which activates transcription of a LEE gene namely, LEE encoded regulator (Ler) which then upregulates expression of multiple LEE-located genes (Elliott *et al.*, 2000). A homologue for Per has not yet been described in strains of STEC and therefore it is believed that STEC LEE genes are activated only by Ler (Elliott *et al.*, 2000). However, the mechanism by which Ler regulates the expression of LEE genes in STEC is yet to be determined. The origin of intimin and LEE is unknown.

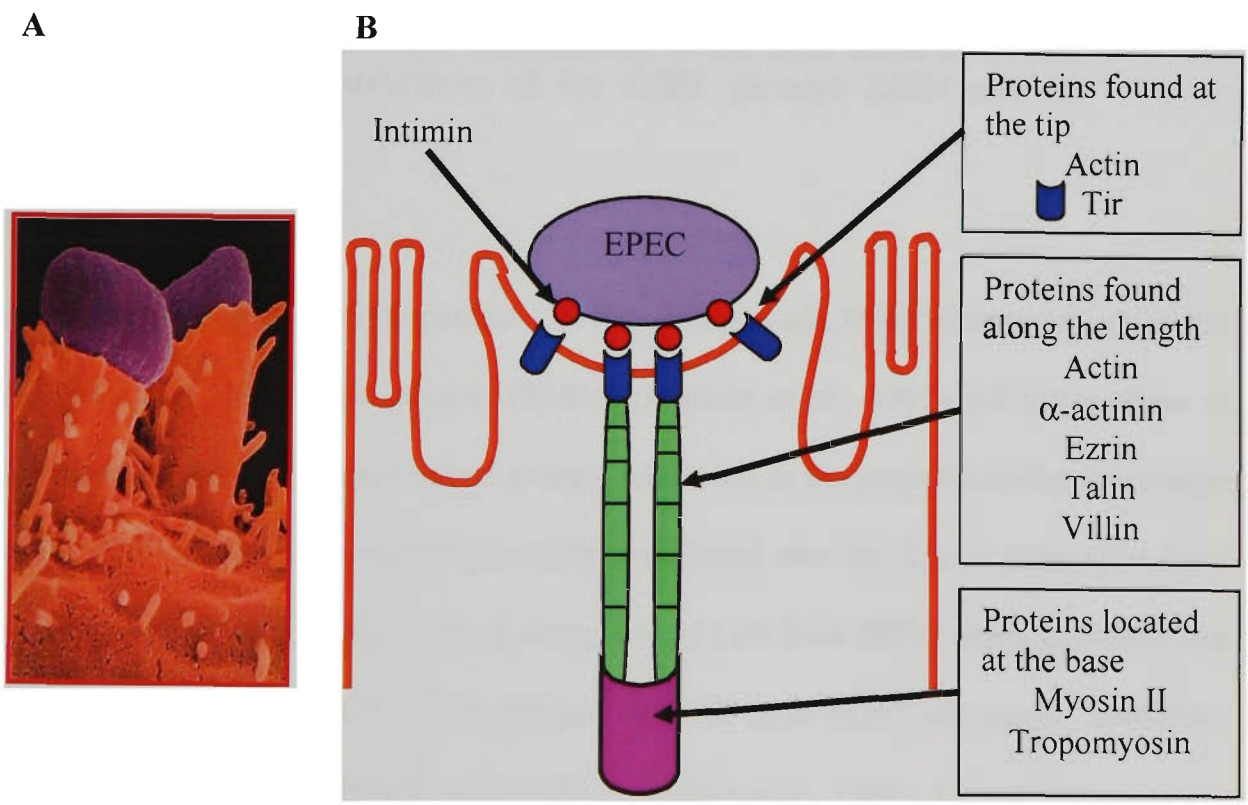


Figure 1.7 Pedestal formations by enteropathogenic *Escherichia coli* (EPEC). (A) Scanning electron micrograph of EPEC (purple) induced pedestal formation on cultured epithelial cell surface (orange). (B) Schematic representation of the formation of pedestal and the cytoskeletal components within the pedestal. Tir, Translocated intimin receptor (Adapted and modified from http://www.biotech.ubc.ca/faculty/finlay/EPEC.HTM).

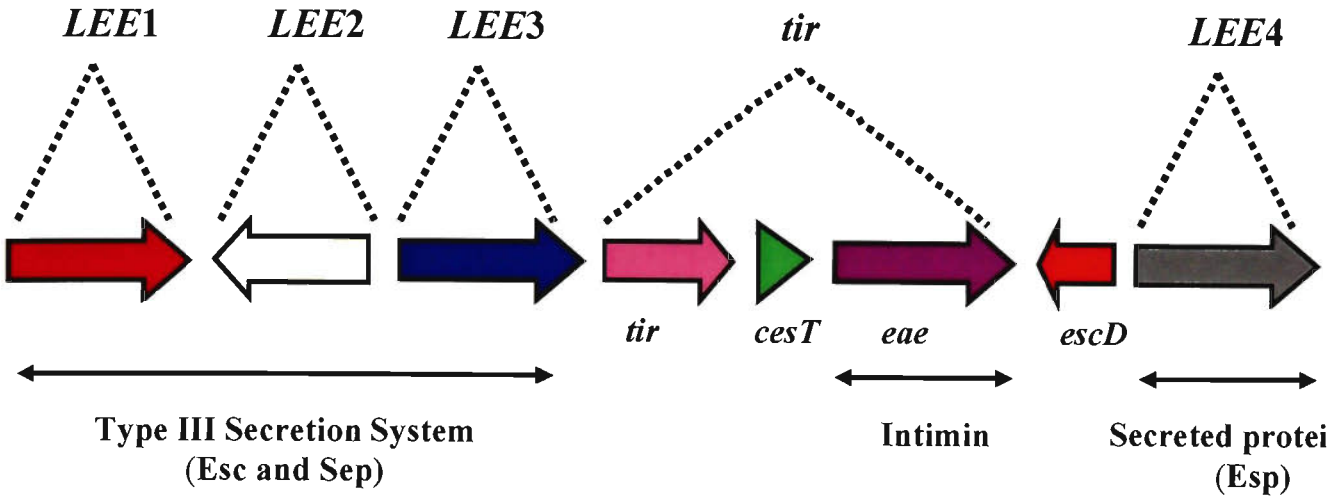


Figure 1.8 Schematic organization of the *LEE* locus of STEC, showing the structures of the *LEE1* through *LEE4* and *tir* operons.

Genes of LEE have a low G+C content (38.4%) (Elliot *et al.*, 1998) when compared to the G+C content of the *E. coli* genome (50.8%) (Blattner *et al.*, 1997). LEE also occur at different locations in divergent clonal groups, which led to the proposition that these large blocks of DNA may have been horizontally transferred into the *E. coli* population from foreign species (Wieler *et al.*, 1997). Comparison of LEE from EPEC strain E2348/69 and STEC O157:H7 strain EDL933 revealed that the LEE from STEC was larger, containing 43,359 bp compared with 35,624 bp from EPEC (Elliot *et al.*, 1998). This difference is due to the presence of a 7.5 kb putative prophage designated 933L, which is inserted at the distant end of STEC LEE (Elliot *et al.*, 1998). The remaining genes are present exactly in the same order and number in both LEEs. The LEE of EPEC E2348/69 and STEC O157:H7 is inserted at 82 min in the *E. coli* chromosome just downstream of the gene encoding the tRNA for selenocysteine (*selC*) (McDaniel *et al.*, 1995). Sperandio *et al.*

(1998) reported that LEE in both EPEC and STEC was either inserted in *selC* or *pheU* sites. However, EPEC strains of serotype O111ab: H25 did not fit either category suggesting a third chromosomal insertion site for the LEE (Sperandio *et al.*, 1998). Recently it was shown that *E. coli* K-12 containing a cloned LEE region of STEC O157:H7 was unable to induce A/E lesions or secrete Esps, whereas EPEC LEE results in A/E activity when cloned into *E. coli* K-12, suggesting complex functional and regulatory differences between EPEC and STEC LEE regions (Elliot *et al.*, 1999).

1.5.2.2 Intimin variants

Intimin has an amino-terminal bacterial membrane anchor and carboxy-terminal domains needed for receptor binding activity (Luo *et al.*, 1999). Receptor binding activity of intimin resides within the carboxy terminal 280 amino acids (Int_{280}) of the polypeptide (Frankel *et al.*, 1994). Analysis of the amino acid sequences of different intimins show highly conserved N-terminal regions whereas the C-termini have considerable sequence divergence. Based on the variable Int_{280} region six distinct intimin types designated α, β, γ, δ, ε and θ have been identified (Adu-Bobie *et al.*, 1998; Oswald *et al.*, 2000; Tarr and Whittam, 2002). Recently Oswald *et al.* (2000) divided Int-α into 2 subgroups α1 and α2 due to the different RFLP profiles produced by the strains investigated. Further, 5 intimin types designated Int-ζ (accession no. AJ298279) (J. Jores, K. Zehmke, L. Roumer and L. Wieler, unpublished data), Int-ι (accession no. AJ308551) (W. L. Zhang, H. Karch and H. Schmidt, unpublished data), Int-λ (accession no. AF439538) (J. Pan, unpublished data), Int-η (accession no. AJ271407) (H. Schmidt, H. Karch, E. Oswald) and Int-κ (accession no. AJ308552) (W. L. Zhang, H. Karch and H. Schmidt, unpublished data) have been

submitted to the GenBank database. However, comparison of the C terminal Int_{280} region of Int-κ with Int-δ reveal 99.6% amino acid sequence similarity and therefore is referred to as Int-δ in this thesis. Further, comparison of the Int_{280} region of Int-η with Int-ζ reveal 100% amino acid sequence homology and therefore is cited as Int-ζ in this thesis.

Twenty three distinctive *E. coli* clones known as the diarrhoeagenic *E. coli* have previously been identified by analysis of 20 housekeeping genes using multilocus enzyme electrophoresis (Whittam *et al.*, 1993). Distinct multilocus enzyme genotypes and the conservation of flagella antigens distinguished two groups of EPEC and EHEC known as EPEC 1 and EPEC 2 and EHEC 1 and EHEC 2 (Whittam *et al.*, 1993). Distinct serotypes and intimin subtypes were initially shown to associate within these groups. Serotypes O55:H6 and O127:H6 (Int-α) and O111:H2/H-, O128:H2 and O45:H2 (Int-β) are representative of EPEC1 and EPEC2 groups respectively and of the LEE insertion in these clonal lineages disrupts the chromosomal gene *selC* (Wieler *et al.*, 1997). Similarly, in serotype O157:H7 (Int-γ), representative of EHEC 1, and serotypes O111:H8/H11/H- and O26:H11/H- (Int-β), representative of EHEC 2, the LEE is inserted in the *PheU* site (Whittam *et al.*, 1993; Wieler *et al.*, 1997; Sperandio *et al.*, 1998). Int-δ is usually expressed by EPEC strains belonging to serotype O86:H34 (Adu-Bobie *et al.*, 1998) and Int-ε is found among a range of human STEC serotypes (Oswald *et al.*, 2000). Some EHEC strains with serogroup O111 express a subset of Int-γ known as γ2 (Oswald *et al.*, 2000), whereas others have reported the presence of Int-β in serogroup O111 (Abu-Bobie *et al.*, 1998). Studies by Tarr and Whittam (2002) have shown that strains with an O111:H8 serotype showed a mosaic of divergent segments that alternatively clustered with intimin

subtypes α, β, and γ and displayed enough sequence divergence to warrant a new intimin subtype designation (identified as Int-θ) and *eae* from strains with serotype O111:H9 was more related to Int-ζ (GenBank accession no. AJ298279.1) (J. Jores, K. Zehmke, L. Roumer, and L. Wieler, unpublished data). The sequence for intimin θ is similar to the *eae* sequence published for human EHEC isolate O111:H- strain 95NR1(Voss *et al.*, 1998) that was classified as Int-γ2 by Oswald *et al.* (2000).

Intimin types may also contribute towards the pattern of colonisation and tissue tropism in the host (Tzipori *et al.*, 1995; Phillips and Frankel, 2000; Reece *et al.*, 2001). Recent studies using infected *in vitro* organ cultures (IVOC), revealed that EPEC colonised and induced A/E lesions on many regions of the small intestinal paediatric mucosa whereas EHEC adhesion and A/E lesion formation was limited to the follicle-associated epithelium (FAE) of the Peyer's patch (Phillip *et al.*, 2000). These observations have prompted studies to construct *E. coli* strains expressing atypical intimin types. Intimin replacement studies showed that replacing Int-α with Int-γ in EPEC affected tissue tropism resulting with EPEC expressing Int-γ adhering to FAE using human intestinal organ cultures. Similarly replacing Int-γ with Int-α in EHEC resulted in the strain expressing Int-α and adhering and infecting many small intestinal regions (Phillips and Frankel, 2000; Phillips *et al.*, 2000).

Recently, Deibel *et al.* (2001) reported that the N-terminal region of intimin may play a role in controlling the binding capacity of the C-terminal domain. This report is in agreement with the observation made by Frankel *et al.* (1995) who showed that a maltose binding protein (MBP) fusion of Int-α_{680} expressed weaker binding with Hep-2 cells compared to

MBP-Int-α_{280}. This controlled binding activity might be important for EPEC/STEC to avoid unproductive binding of intimin.

The structure of Int_{280} complexed with Tir was determined recently by multi-dimensional nuclear magnetic resonance (NMR) and X-ray crystallography studies (Batchelor *et al.*, 2000; Luo *et al.*, 2000). These studies showed that intimin contains four distinct domains (D1, D2, D3 and D4) within the carboxy terminus that protrudes from the bacterial surface. D1, D2 and D3 are immunoglobulin-like and extend to link the C-type-lectin like domain D4. C-type lectins are a family of proteins responsible for cell surface carbohydrate recognition (Weis and Drickamer, 1996). D4 is involved in the interaction between the intimin-binding domain of Tir and the carboxy terminus of intimin.

Determining the type of intimin expressed by STEC is important since the type of intimin influences tissue targeting specificities of STEC (Phillips and Frankel, 2000). There is a paucity of information describing intimin types from *E. coli* recovered from cattle and sheep, two meat producing animals that represent major reservoirs of *E. coli* that enter the human food chain.

1.5.2.3 Intimin receptors

During EPEC infection of epithelial cells, a 90-kDa tyrosine phosphorylated eukaryotic membrane protein (Hp90) was shown to serve as a receptor for intimin (Rosenshine *et al.*, 1996). However, it was later shown that Hp90 was not a host cell protein but a bacterial protein, which is translocated into the plasma membrane of the host cell by a bacterial type

III secretion system and acts as a receptor for intimin (Kenny *et al.*, 1997). As a result Hp90 was renamed translocated intimin receptor (Tir). Tir and its chaperone, CesT (Abe *et al.*, 1999; Elliot *et al.*, 1999) are located within the *tir* operon immediately upstream of the *eae* gene (McDaniel *et al.*, 1995). Tir is produced in the bacterial cell as a 78 kDa unphosphorylated protein, which after being translocated to the host cell becomes phosphorylated and its molecular mass increases to 90 kDa (Kenny *et al.*, 1997). This tyrosine phosphorylation of Tir is observed in human EPEC and rabbit EPEC strains but not in Tir from *E. coli* O157:H7 strains. However, Deibel *et al.* (1998) reported that STEC O26:H- produced a homologue of Tir (designated EspE), which is phosphorylated during translocation into infected host cells. It has been shown that Tir phosphorylation is not necessary for *in vitro* intimin binding and both EHEC and EPEC intimins can bind to the unphosphorylated forms of EHEC and EPEC Tir respectively (DeVinney *et al.*, 1999). A probable explanation for the lack of tyrosine phosphorylation by EHEC may be associated with the deficiency of some regulatory factors that are present in EPEC and not in EHEC and also the synthesis and secretion of Tir may be regulated differently in these two pathogens. Further, a single tyrosine to serine substitution observed between EPEC and EHEC Tir molecules at position 474, may be associated with the variation in phosphorylation patterns (Kenny, 1999). Therefore the differences in synthesis and secretion of Tir between EPEC and EHEC may reflect differences in adhesion mechanisms and pedestal formations.

Tir consists of at least three functional regions, an extracellular domain that interacts with intimin, a transmembrane domain and a cytoplasmic domain that is involved in the pedestal-like structure formation beneath the intimately attached bacterium. Membrane

topology studies have revealed that Tir is inserted into the host cell membrane in a hairpin manner with the extracellular domain exposed at the host cell surface for interaction with intimin (de Grado *et al.*, 1999; Hartland *et al.*, 1999). The intimin-binding domain of Tir (Tir-M) has been localized to a central 107 amino acid extra cellular domain and both the amino and carboxy termini are located within the host cell (Hartland *et al.*, 1999). The Tir-M region forms a coiled structure composed of two helices connected by a β-hairpin turn at the tip of the structure and this motif is primarily responsible for the formation of intimin-Tir complex by making contact with an analogous region within intimin D4 (Luo *et al.*, 2000).

Hartland *et al.* (1999) showed that the formation of the disulfide loop within intimin was not essential for the interaction with Tir, as previously envisaged (Hicks *et al.*, 1998; Frankel *et al*, 1998b). This lead to the suggestion that intimin can bind to host cells in a Tir independent manner. Hartland *et al.* (1999), using immunogold labelling microscopy, showed that intimin was capable of binding to host cells even in the absence of Tir utilising a lectin like component on the C-terminal of intimin. Figure 1.9 schematically represents the Tir-intimin interaction and possible host cell intimin receptor (Hir)-intimin interaction.

Purified cell binding domain of intimin also exhibits *in vitro* binding activities towards β1 integrins (Frankel *et al.*, 1996) and this may be due to the similarity of intimin to invasin, a protein secreted by *Yersinia* species mediating adherence and invasion into epithelial cells (Isberg *et al.*, 1987). The 78 amino acid disulfide loop is necessary for this interaction (Frankel *et al.*, 1996). However, polarized intestinal epithelial cells are believed not to

express β1 integrins on their apical surface and β1 integrins are not essential for the formation of A/E lesions (Liu *et al.*, 1999). Therefore this mode of interaction and its contribution towards colonisation is not fully understood.

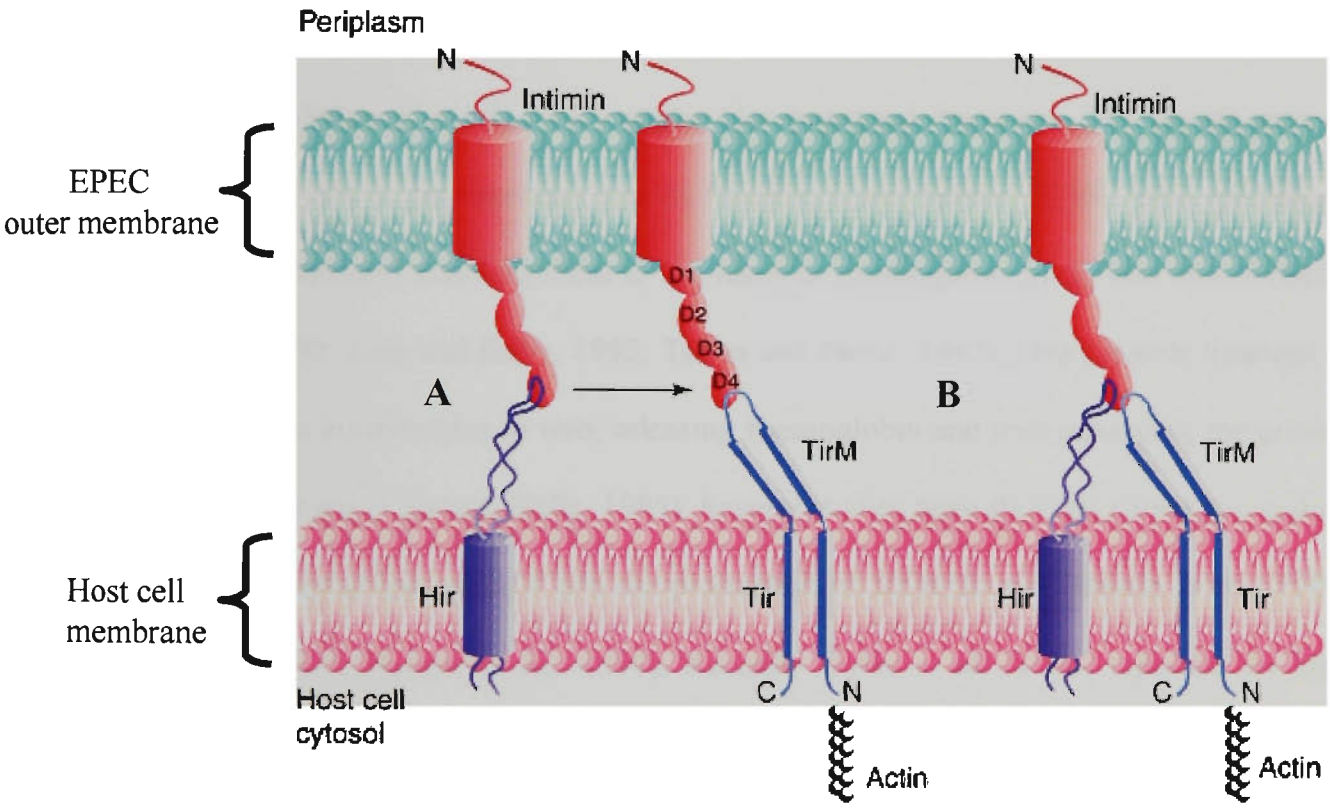


Figure 1.9 Schematic representation of a model EPEC - host cell adhesion mechanisms. Two possible mechanisms of A/E pathogens and host cell interactions are demonstrated. (A) The initial binding takes place between intimin and a possible host cell intimin receptor (Hir) which is followed by translocation of Tir to the host cell membrane, to which intimin binds via the terminal D4 domain (C-type lectin cell binding domain) to TirM (intimin binding domain of translocated intimin receptor (Tir). This interaction produces the characteristic A/E lesion formation. The model shows a monomeric intimin-Tir interaction, however X-ray crystallography has revealed a possible dimeric interaction. (B) This mechanism proposes a simultaneous interaction between intimin, Hir and Tir. (Adapted and modified from Frankel *et al.*, 2001).

1.5.3 Potential accessory virulence factors

1.5.3.1 EHEC-haemolysin

STEC of serotypes O157:H7 and most non-O157 serotypes isolated from humans produce a plasmid-encoded haemolysin termed EHEC-haemolysin (EhxA) (Beutin *et al.*, 1989; Bettelheim, 1995). EhxA belongs to the RTX toxin family (Bauer and Welch, 1996) and shows 60% relatedness to α-haemolysin produced by uropathogenic *E. coli* (Bauer and Welch, 1996; Schmidt *et al.*, 1995) and is also a general cytotoxin. The significance of EHEC-haemolysin in pathogenesis and its function is not known. STEC have specialised iron transport systems enabling them to use haem or haemoglobin as an iron source (Mills and Payne, 1995; Law and Kelly, 1995; Torres and Payne, 1997). One possible function of EhxA is to lyse erythrocytes *in vivo*, releasing haemoglobin and thus enhancing the growth of STEC in the gut (Law and Kelly, 1995). Several studies have shown a strong association between the expression of *ehxA* and the production of *stx* in *E. coli* (Beutin *et al.*,, 1989; Bettelheim, 1995; Boerlin *et al.*, 1999). Recently Gyles *et al.* (1998) reported the high frequency of EhxA expression in serotypes frequently implicated in disease compared to a lesser extent in serotypes that are less frequently involved in disease in humans.

1.5.3.2 Enteroaggregative heat-stable toxin

Enteroaggregative heat-stable toxin (EAST1) encoded by the *astA* gene is an enterotoxin that was first identified in enteroaggregative *E. coli* (EAEC) (Savarino *et al.*, 1991). In a study by Savarino *et al.* (1996), the *astA* gene was present in all 75 O157:H7 STEC strains examined, as well as 8 of 9 O26:H11 and 12 of 23 non-O157/O26 STEC strains. The significance of EAST1 in STEC pathogenesis is unknown, however, the high prevalence of

astA gene in STEC O157:H7 suggests that this toxin may contribute towards the overall virulence of STEC O157:H7. Furthermore, it might play a part in the watery diarrhoea symptoms often seen in patients infected with STEC strains due to its resemblance to heat stable toxins, which stimulates and/or inhibits sodium chloride absorption, resulting in net intestinal fluid secretion (Crane *et al.*, 1992; Sears and Kaper, 1996).

1.5.3.3 Catalase/peroxidase

A protein possessing both catalase and peroxidase activities (encoded by *katP*) has been identified on the virulence plasmid of STEC O157:H7 (Brunder *et al.*, 1996). This protein shows similarities to several bacterial catalase/peroxidase such as PerA of *Bacillus stearothermophilus*, M185 of *Mycobacterium intracellulare* and HPI catalase KatG of *E. coli* (Brunder *et al.*, 1996). *katP* is not observed in all STEC plasmids. Brunder *et al.* (1999) reported that only 66% of STEC O157:H7 isolates and 38% of non-O157 STEC possessed *katP*. The role of KatP in pathogenesis is unclear, however it is believed that it assists in evading host defence mechanisms by detoxifying cytotoxic oxidants produced by certain leukocytes such as macrophages and neutrophils during the oxidative burst (Brunder *et al.*, 1996).

1.5.3.4 Extracellular serine protease

Another plasmid encoded putative virulence factor is a protein secreted by *E. coli* O157:H7 known as extracellular secreted protease (EspP) (Brunder *et al.*, 1997). EspP shares homology with EspC secreted by EPEC and SepA secreted by *Shigella flexneri* (Brunder *et al.*, 1997). STEC O26:H- is also known to secrete a protein homologous to EspP (Djafari *et al.*, 1997). EspP exhibits proteolytic activity against human coagulation factor V

(Brunder *et al.*, 1997). The cleavage of factor V could lead to prolonged bleeding due to decreased coagulation reactions, thus exacerbating haemorrhagic disease (Karch *et al.*, 1998). EspP is also known to be cytotoxic towards Vero cells (Djafari *et al.*, 1997). Detection of antibodies towards EspP in children suffering STEC infection substantiates expression of protein *in vivo* and a possible role in pathogenesis (Brunder *et al.*, 1997). However, observations made by Schmidt *et al.* (1999) revealed that EspP is not uniformly expressed by all STEC harbouring the pO157 plasmid. The significance of EspP in disease is uncertain since no correlation was established with the expression of EspP in major STEC serotypes and disease in humans (Boerlin *et al.*, 1999).

1.5.3.5 *Clostridium difficile*-like toxin and Efa1

The pO157 plasmid also carries a novel open reading frame, which resembles the genes for clostridial cytotoxins, such as ToxA and ToxB of *Clostridium difficile* (Burland *et al.*, 1998). These toxins consist of a C-terminal that functions in toxin entry into the cell and an N-terminal glucosyl-transferase, which modifies proteins regulating cell architecture. A homologue of this protein, termed Efa1, was identified on the chromosome of STEC and EPEC strains producing A/E lesions (Nicholls *et al.*, 2000). This gene is believed to contribute towards adhesiveness (Nicholls *et al.*, 2000). Recently, Tatsuno *et al.* (2001) reported that *toxB* gene on pO157 of EHEC contributes to adherence by inducing the production and secretion of EspA, EspB and Tir which are required for bacterial attachment. This was demonstrated by constructing a mini pO157 plasmid composed of the *toxB* and *ori* regions and introducing it into an EHEC O157:H7 cured of pO157 (O157Cu). This restored full adherence capacity to O157Cu, and stimulated the production and/or secretion of type III secreted proteins (Tatsuno *et al.*, 2001).

1.5.3.6 STEC auto agglutinating adhesin (Saa)

Recently, a novel outer membrane adhesin designated Saa, was identified in a LEE negative STEC strain of serotype O113:H21, which was responsible for an outbreak of HUS in South Australia (Paton *et al.*, 2001). Saa is carried on the large plasmid of certain LEE negative but not LEE positive STEC isolates. *E. coli* K-12 containing cloned Saa exhibited a 9.7 fold increase in adherence to Hep-2 cells. Homologues of Saa were also identified in LEE negative STEC strains of serotypes O48:H21 and O91:H21, which were also isolated from HUS patients (Paton *et al.*, 2001). Saa contains 4 copies of a 37-amino acid repeat unit in the C-terminal region, which contains a motif presumed to form coiled coils, as does a region directly downstream of the repeat domain. Such coiled-coil motifs are often associated in protein-protein interaction. Saa could be a possible marker for LEE negative STEC strains that are capable of causing human infections (Paton *et al.*, 2001).

1.5.3.7 Urease

Two urease gene (*ure*) clusters situated at non-adjacent loci have been identified in the chromosome of STEC O157:H7 strain EDL933 (Perna *et al.*, 2001). These gene clusters are believed to encode the structural proteins and accessory polypeptides necessary for the assembly of urease. Urease is a complex nickel metalloenzyme, which catalyses the hydrolysis of urea into ammonia and carbon dioxide (Mobley *et al.*, 1995). It has been hypothesised that urease may contribute to the tolerance exhibited by STEC O157:H7 towards the acidic environment in the human intestinal tract (Heimer *et al.*, 2002). This could be achieved by modifying surrounding anion concentration by accumulating ammonium ions in the proximal surroundings of the bacterium (Mobley *et al.*, 1995). Heimer *et al.* (2002) demonstrated that STEC O157:H7 strain EDL933 carried functional

urease genes, which could be expressed in an *E. coli* K-12 background. However, urease activity was not detected in the native strain. Several Fur-like (ferric uptake regulator) boxes were identified upstream of *ure* and was shown to regulate the expression of cloned EHEC *ure* clusters in *fur* mutant *E. coli* strain MC3100 (Heimer *et al.*, 2002). Further, a secondary unidentified *trans*-acting factor was also shown to be involved in the regulation of cloned *ure*. Recently, a urease operon was also identified in STEC of serogroups O26 and O111 (Nakano *et al.*, 2001). Further *ure* was not detected in any of the non-STEC isolates examined (Nakano *et al.*, 2001). However, further studies need to be carried out in determining the role of urease in pathogenesis and the prevalence of this gene in other non-O157 STEC isolates.

1.5.3.8 Type IV pilus

A novel type IV pilus locus (*pil*) mediating expression of long thin pili was recently identified on the mega plasmid (pO113) from an LEE negative O113:H21 STEC strain (98NK2) (Srimanote *et al.*, 2002). Several other LEE negative STEC strains were also shown to harbour homologues of *pil* locus. However, LEE positive serogroups that are known to cause serious disease in humans such as O26, O111 and O157 did not possess this locus. This type IV pilus biosynthesis locus comprises of 11 closely associated genes (*pil*L to *pil*V) and an upstream gene (*pil*I), which is transcribed independently from the other 11 genes. Type IV pilus have been shown to be involved in the adherence of a number of pathogenic bacteria such as EPEC (Bieber *et al.*, 1998; Stone *et al.*, 1996), *Vibrio cholerae* (Manning, 1997) and *Salmonella enterica* serovar Typhi (Zhang *et al.*, 2000) to host epithelial cells. However, the type IV pili expressed by 98NK2 did not appear to mediate adherence to Hep-2 or Hct-8 cells *in vitro*. The authors also report the conjugal

transfer of p0113. It is believed that *pil* locus may assist in conjugation. The authors also hypothesise that the presence of the *pil* locus may enhance the chances of lysogeny by Stx-converting phages. However, further studies need to be carried out in order to determine the exact role of *pil* loci in adherence, conjugation and virulence.

1.6 Pathogenesis of STEC

1.6.1 STEC associated diseases in humans

STEC infections in humans can be asymptomatic or produce a wide variety of clinical presentations, including watery diarrhoea, bloody diarrhoea, HUS, thrombotic thrombocytopenic purpura (TTP) and death (Griffin *et al.*, 1988). The incubation period for STEC induced diarrhoea is usually 3-4 days, in which time patients usually develop watery diarrhoea, which is preceded by abdominal cramps (Riley, 1987; Griffin *et al.*, 1988). In 25-75% of patients, the diarrhoea resolves without progression to severe illness. However, in some patients the watery diarrhoea progresses to bloody diarrhoea and HC within 1-2 days with blood in stools ranging from streaks, to stools that are all blood (Riley *et al.*, 1983; Riley, 1987; Griffin *et al.*, 1988). The illness may resolve after a week with no apparent sequelae, but in 5-10% of cases usually in children and in elderly patients, the illness will progress to HUS. HUS is defined as a triad of haemolytic anaemia, thrombocytopenia and acute renal failure. Most patients will recover with treatment, however, 3-5% of HUS affected children will die. Approximately 12-30% will have severe sequelae including renal impairment, hypertension and central nervous system manifestations, such as lethargy, seizures, coma or hemiparesis, requiring long term therapy (Pickering *et al.*, 1994). STEC infection can also result in a variant form of HUS called

TTP. TTP resembles HUS histopathologically and clinically, but differs in that it occurs mainly in adults and there is often an absence of prodromal diarrhoea (Morrison *et al.*, 1985). In TTP neurological signs and fever are usually present and renal impairment is less severe (Morrison *et al.*, 1986). Currently treatment of HUS is largely limited to supportive care.

1.6.2 STEC as a cause of disease in animals

1.6.2.1 STEC in cattle

In contrast to humans, *E. coli* O157:H7 can colonise the bovine intestine without causing disease (Cray and Moon, 1995; Brown *et al.*, 1997). However, STEC O157:H7 and other bovine STEC strains are pathogenic in very young calves (Dean-Nystrom *et al.*, 1997). Following inoculation with a STEC O157:H7 strain, neonatal calves develop diarrhoea and have O157:H7 associated A/E lesions in the large and small intestine (Dean-Nystrom *et al.*, 1997). In contrast to neonatal calves, experimental inoculation of cattle older than 3 weeks with STEC O157:H7 did not cause histological lesions and the cattle remained free of disease (Cray and Moon, 1995; Brown *et al.*, 1997). Severe cases of infection of susceptible calves with bovine virulent STEC may result in atrophy of the villi, epithelial cell damage, diffuse infiltration of neutrophils into the lamina propria and intestinal lumen, and the formation of a pseudomembrane containing blood, fibrin, cellular debris, and neutrophils (Hall *et al.*, 1985; Pearson *et al.*, 1999). Infection of neonatal colostrum-deprived calves with *E. coli* O157:H7 may result in enterocolitis similar to that seen in humans (Dean-Nystrom *et al.*, 1997).

Intimin is required for colonisation of the gastro-intestinal tract and the induction of diarrhoea in neonatal calves by *E. coli* O157:H7, which was demonstrated by infecting newborn calves with intimin-positive or intimin-negative STEC O157:H7 strains (Dean-Nystorm *et al.*, 1998). The role of Stx in cattle and its effects on bovine intestinal mucosa is not fully understood. In a recent study it was shown that cattle lack the Stx receptor Gb3 in the gastrointestinal tract but Gb3 was detected in kidney and brain tissues (Pruimboom-Brees *et al.*, 2000). Six gastro-intestinal and five extra-intestinal organs were examined in this study and Stx did not bind to blood vessels in any of these organs (Pruimboom-Brees *et al.*, 2000). The lack of vascular receptors for Stx may be the reason why cattle are resistant to Stx and remain an asymptomatic reservoir for STEC O157:H7 and a variety of non-O157 STEC. However, a recent study by Hoey *et al.* (2002) identified the expression of Stx receptor in the bovine intestinal tract, which contradicts the results published by Pruimboom-Brees *et al.* (2000). Stx binding was observed in the epithelial cells present in the crypt regions adjacent to the submucosa of jejunum, ileum, caecum and colon (Hoey *et al.*, 2002). However, binding of Stx1 to capillaries in jejunum, ileum, caecum, colon or kidney was not observed which was similar to the observations made by Pruimboom-Brees and colleagues (2000) (Hoey *et al.*, 2002). Furthermore Stx1 also bound to submucosal lymphoid tissue suggesting the potential of Stx to target the host immune system (Hoey *et al.*, 2002). The absence of receptors on vasculature and the lower binding of Stx to blood vessels and kidney glomeruli may contribute to the resistance of cattle to Stx toxicity and to STEC pathogenicity (Hoey *et al.*, 2002).

1.6.2.2 STEC in sheep

Like cattle, sheep are natural reservoir for *E. coli* O157:H7 and many non-O157 STEC strains (Kudva *et al.*, 1996; Bettelheim *et al.*, 2000; Djordjevic *et al.*, 2001). However, little is known about the pathogenesis of these STEC strains to sheep. Recently it was shown that intimin facilitates colonisation by STEC O157:H7 in the alimentary tract of asymptomatic sheep (Cornick *et al.*, 2002). However, it is not known if A/E lesions are formed in the intestinal tract due to the expression of intimin. Further studies need to be carried out to determine the effect of shiga toxins and other STEC virulence genes on sheep.

1.6.2.3 STEC in pig

STEC of serotypes O138:H14, O139:H1 and O141:H4 are known to cause oedema disease or bowel disease in pigs especially after weaning (Gannon *et al.*, 1990). This disease is characterised by sudden death and the development of oedema and nervous signs. Diarrhoea is only rarely observed in oedema disease. Outbreaks are sudden in onset and are short lived, averaging about 8 days (Cornick *et al.*, 2000). Oedema causing strains of STEC produce a characteristic Stx2 variant Stx2e that binds to the specific glycolipid receptor Gb4 and plays a central role in the pathogenesis of the disease (Weinstein *et al.*, 1988; Gyles *et al.*, 1988). The toxin produces vascular lesions in the intestine, subcutis and the brain (Bertschinger and Pohlenz, 1983; Waddell *et al.*, 1996).

1.7 Diagnosis of STEC infection

Meat producing animals, notably cattle and sheep represent the major reservoir of STEC. Foods that become contaminated with faeces from these animals serve as the principal

source of human infection. Isolating and genetically characterising virulence factors of STEC recovered from ruminant faeces helps in determining the nature of STEC that can contaminate carcasses during slaughter. Also, isolation and characterisation of STEC from stool specimens presented by patients with diarrhoea will facilitate early diagnosis of the serotype involved in the infection and to determine the virulence potential of these strains.

1.7.1 Culture-based detection

The agar medium most commonly used for the isolation of STEC O157:H7 is sorbitol-MacConkey-agar culture (SMAC) (Farmer and Davis, 1985; March and Ratnam, 1986). This medium exploits the observation that most STEC O157 are unable to ferment sorbitol, distinguishing them from majority of other faecal *E. coli* of different serotypes (March and Ratnam, 1986). There are various modifications of SMAC agar, which improve sensitivity and selectivity for STEC O157 from other organisms on the plate. These include CT-SMAC (inclusion of cefixime and potassium tellurite), which prevents growth of other *E. coli* strains (Zadik *et al.*, 1993) and CR-SMAC (inclusion of cefixime and rhamnose), which differentiates STEC O157:H7 from other non-sorbitol fermenting strains, since STEC O157:H7 does not ferment rhamnose on agar plates (Chapman *et al.*, 1991). STEC O157:H7 appears as clear, non-sorbitol fermenting colonies, which can be tested for the O157 antigen by agglutination tests. Addition of β-D-glucuronidase in SMAC can also aid in the detection of STEC O157, since these strains also fail to produce β-D-glucuronidase (Okrend *et al.*, 1990). However, sorbitol fermenting, β-D-glucuronidase positive STEC O157:H7 and O157:H- strains have been identified in continental Europe and Australia

(Feng *et al.*, 1998; Gunzer *et al.*, 1992; Karch and Heesemann, 1993; Bettelheim *et al.*, 2002 in press).

The above mentioned detection method cannot be applied for the detection of non-O157 STEC serotypes and these serotypes do not have any distinct biochemical properties to distinguish them from other commensal faecal *E. coli* strains. However, nearly all O157 and most non-O157 STEC strains produce enterohaemolysin and can be distinguished phenotypically from α haemolytic strains using washed sheep blood agar supplemented with calcium (WSBA) (Beutin *et al.*, 1989). α haemolysis is characterised by a clear, broad zone of haemolysis surrounding the colonies after 4 hours of incubation at 37°C on WSBA, whereas the enterohaemolytic phenotype is characterised by a restricted turbid zone of haemolysis after incubation for 18 to 24 hours at 37°C on WSBA (Beutin *et al.*, 1989). Few strains of STEC produce α haemolysin but none produce both haemolysins (Beutin *et al.*, 1989; Bettelheim, 1995). In a recent study, Lehmacher *et al.* (1998) reported that blood agar supplemented with vancomycin, cefixime and cefsulodin (BVCCA) considerably facilitated the identification of STEC compared to WSBA and this may be due to vancomycin increasing the permeability of the *E. coli* cell, which facilitates the secretion of haemolysin. The enterohaemolytic phenotype produced by STEC colonies on BVCCA is similar to the appearance on WSBA (Figure 1.10). However, BVCCA positive colonies not containing *ehxA* have been reported, which may be due to low expression of α haemolysin or vancomycin enhancing expression of haemolysin by non-STEC (Hornitzky *et al.*, 2001). However, these anomalies do not undermine the utility of this agar medium for the isolation of STEC from ruminants (Hornitzky *et al.*, 2001; Djordjevic *et al.*, 2001).

1.7.2 PCR-based detection

A variety of PCR amplification methods have been developed for detecting and characterising STEC virulence genes. The PCR detection technique is one of the most sensitive and rapid methods. PCR assays have been developed to detect the presence of shiga toxin genes (*stx*$_1$ and *stx*$_2$) or simultaneous identification (multiplex PCR) of *stx*$_1$, *stx*$_2$, *eae* and *ehxA* from cultures of faeces and foodstuffs (Gannon *et al.*, 1992; Begum *et al.*, 1993; Cebula *et al.*, 1995; Fratamico *et al.*, 1995; Paton and Paton, 1998; Fagan, *et al.*, 1999). However, if STEC virulence factors are detected in faecal broths, it has to be cultured in order to isolate the *E. coli* to confirm these factors and its serotype.

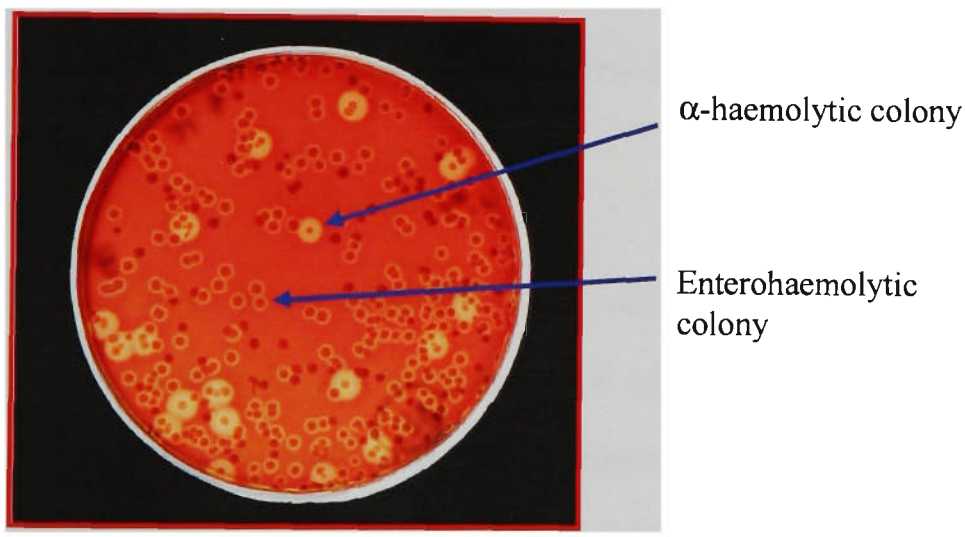


Figure 1.10 BVCCA plate showing *E. coli* colonies expressing alpha and enterohaemolytic phenotypes.

Recently Paton and Paton. (2002) have developed a pentavalent PCR assay for the simultaneous detection of *stx*$_1$, *stx*$_2$, *eae*, *ehxA* and *saa*. Primers for the detection of various virulence markers are usually designed from a conserved region of the gene, since some markers may have many variants, which can be widely divergent. PCR assays have been

developed for discriminating among the variant forms of *stx*$_2$ (Bastian *et al.*, 1998; Johnson *et al.*, 1990; Tyler *et al.*, 1991) and *eae* (Adu-Bobie *et al.*, 1998; Reid *et al.*, 1999; Oswald *et al.*, 2000). PCR detection methods have proved to be invaluable in the detection of STEC associated virulence genes from samples such as faeces, foodstuffs and samples containing nonviable bacteria, which are otherwise more complex to screen with current microbiological techniques.

1.8 Thesis Objectives

STEC are recognised as an important group of bacterial enteropathogens and ruminants, particularly cattle and sheep serves as natural reservoirs. Previous studies have mainly focused on STEC recovered from cattle and relatively few studies have focused on STEC isolated from sheep.

The overall aim of this thesis was to characterise STEC virulence factors particularly Stx1 and Stx2 in STEC and intimin in *eae*-containing *E. coli* isolates (STEC and non-STEC) recovered from healthy sheep primarily in Australia. Furthermore, this thesis also examines and compares these virulence factors with STEC of human origin that possess same serotypes as those commonly recovered from sheep. These observations may contribute in determining to what extent sheep may represent a source of STEC involved in human infections.

Chapter 2 and 3 describes the use of PCR, restriction fragment length polymorphism (RFLP) and DNA sequencing techniques to characterise *stx*$_1$ and *stx*$_2$ genes respectively in

STEC isolates recovered from healthy sheep and humans with clinical and non-clinical infections. Chapter 4 describes the development of a universal intimin typing system and the characterisation of intimin in a collection of STEC and non-STEC isolates recovered from healthy sheep and from symptomatic and asymptomatic humans. This is the first system reported that could identify all reported intimin subtypes as well as the subtypes submitted to the GenBank database. Also described in this chapter is the identification of 2 previously unreported intimin subtypes detected in *eae*-containing *E. coli* strains isolated from sheep. This chapter also describes the phylogenetic analysis of the new subtypes with those described previously. Chapter 5 describes the genomic DNA fingerprint analysis of non-O157 STEC serotypes (O5:H-, O91:H- and O128:H2) commonly recovered from sheep by pulsed-field gel electrophoresis and explores the clonal relationship between human and sheep STEC isolates of same serotypes. Chapter 6 provides a general discussion of the importance and implications of these virulence factor characterisation studies for human health and makes an assessment of the role of STEC of ovine origin in human infections.

Chapter 2

Development of a *stx*$_1$ subtyping PCR-RFLP system and characterisation of ovine *stx*$_1$ subtypes

2 – Development of a stx_1 subtyping PCR-RFLP system and characterisation of ovine stx_1 subtypes

2.1 INTRODUCTION

Shiga toxins play a major role in inducing vascular injury in the intestinal microcirculation and have been shown to directly affect the intestinal epithelium although different responses have been reported in different hosts (O'Loughlin and Robins-Browne, 2001). More specifically, Stx perturb cytokine expression patterns as a consequence of their interaction with epithelial cells (Acheson *et al.*, 1996; Thorpe *et al.*, 1999; Yamasaki *et al.*, 1999). Rabbit models have been used to demonstrate the ability of stx_1-positive strains to induce more severe diarrhoea and mucosal injury (Sjogren *et al.*, 1994), increased inflammatory changes and elevated mucosal IL-1 activity (Bertin *et al.*, 2001) compared with rabbits infected with isogenic strains lacking stx_1. The ability of purified stx_1 to induce similar inflammatory responses when inoculated intragastrically in rabbits reinforces these observations (Pai *et al.*, 1986).

Shiga toxin genes (*stx*) are encoded in the genome of lambdoid phages (Schmidt *et al.*, 1999). Bacteriophage transmission represents the major vehicle in the spread of *stx* among serologically diverse populations of *E. coli* (Schmidt *et al.*, 1999; Koch *et al.*, 2001) and contributes significantly to the emergence of new STEC clones (Schmidt *et al.*, 1999). The location of *stx* downstream of phage lysis genes suggests that phage promoters (Unkmeir *et al.*, 2000; Wagner *et al.*, 2001) control the expression of Shiga toxin. Bacteriophages survive better in water than their bacterial hosts and are reported to be more resistant to chlorination and pasteurisation (Muniesa *et al.*, 1998; Muniesa *et al.*, 1999). Monitoring *stx*

subtypes within ruminant and environmental populations of STEC should lead to a better understanding of the movement of bacteriophage within these environments.

In marked contrast to the *stx*$_2$ family, which comprise of many variants, *stx*$_1$ appears to be more homogenous. *stx*$_1$ sequences derived from STEC strains O111:H- and O48:H21 and from three bacteriophages (H19B, 933J and H30) have been reported to be very similar to the sequence derived from *Shigella dysenteriae* (Paton *et al.*, 1995) and contain a limited number of amino acid substitutions. Unlike these common *stx*$_1$ subtypes, *stx*$_1$ from a STEC strain of serotype OX3:H8 isolated from sheep referred to as *stx*$_{1OX3}$ possesses 43 nucleotide mismatches compared to *stx*$_{1933\text{-}J}$ resulting in 12 amino acid changes (Paton *et al.*, 1995). Recently, *stx*$_{1OX3}$ has been renamed as *stx*$_{1c}$ (Zhang *et al.*, 2002) and this new nomenclature will be used in this thesis. The biological significance of these different *stx*$_1$ subtypes and its association with the activity of the toxin is not known at present.

Although ruminants represents one of the largest reservoirs of STEC (Beutin *et al.*, 1993; Beutin *et al.*, 1995; Gyles *et al.*, 1998; Djordjevic *et al.*, 2001), limited studies have been carried out in determining the type(s) of *stx* present in these animals. Further, there are no reports characterising the *stx*$_1$ in STEC recovered from Australian sheep. Recently, Koch *et al.* (2001) examined the presence of *stx*$_{1c}$ among 148 *stx*$_1$-containing *E. coli* derived from human and animal sources from different locations in Germany. The *stx*$_{1c}$ gene was shown to be present in 38 of 48 (79.2%) sheep-derived STEC belonging to serotypes O5:H-, O125:H-, O128:H2, O146:H21 and OX3:H8 but was not present in isolates with serotype O91:H-. These serotypes are commonly recovered from ovine but rarely from bovine sources (Beutin *et al.*, 1995; Beutin *et al.*, 1997; Kudva *et al.*, 1999; Djordjevic *et al.*,

2001). Koch *et al.* (2001) also showed that stx_{1c} carrying strains were recovered from humans with diarrhoea and that a proportion of these isolates possessed serotypes commonly associated with STEC from ovine sources. In a recent study, stx_{1c} was identified in 36 of 212 (17%) STEC strains isolated from sporadic cases of human infections (Zhang *et al.*, 2002). Also included in this study were 2 STEC isolates of serotype O128:H- recovered from healthy sheep in the Czech Republic which were reported to possess the stx_{1c} subtype (Zhang *et al.*, 2002). These preliminary observations suggest that the association of the stx_{1c} with ovine STEC strains from other geographical locations require further investigation to determine the true prevalence of stx_{1c} subtype in sheep.

It has been reported that STEC that commonly inhabit the gastrointestinal tract of healthy sheep and cattle represent serologically distinct populations and particular STEC serotypes preferably inhabit different ruminant species (Beutin *et al.*, 1995; Beutin *et al.*, 1997; Djordjevic *et al.*, 2001; Hornitzky *et al.*, submitted). It was considered important to subtype stx_1 genes in a serologically diverse collection of STEC derived from ovine sources in Australia to determine their stx_1 subtype(s) and to establish whether specific stx_1 subtype(s) associate with particular serotypes and host. In this study a PCR-RFLP assay was developed which differentiates stx_{1c} from the common stx_1-related sequences. The assay was used to characterize 203 stx_1-containing STEC derived from ovine source. This study also examined stx_1 subtypes in 34 human STEC strains mostly derived from patients with gastro-intestinal and systemic diseases.

2.2 MATERIALS AND METHODS

2.2.1 STEC Isolates

Two hundred and thirty seven *stx*$_1$-containing STEC isolates were used in this study (Table 2.1) of which 197 were derived from 197 healthy slaughter age sheep, 6 were from newborn lambs and 34 were of human origin. The ovine STEC were obtained from the Elizabeth Macarthur Agricultural Institute (EMAI), New South Wales, Australia and were isolated using methods described by Djordjevic *et al.* (2001). In brief, faeces were collected by rectal palpation and placed into sterile 50 ml plastic containers and stored at 4°C during transit from the property to the laboratory. Twenty-seven *stx*$_1$-containing isolates from humans sourced from the Microbiological Diagnostic Unit (MDU), Victoria, Australia and eight isolates from the National Reference Laboratory for Foodborne Diseases, Bern, Switzerland (Table 2.1) were also included. The Swiss isolates were serotyped by Kim Ziebel and Roger Johnson from the Guelph Laboratory, Health Canada, Guelph, Ontario, Canada. Dr. Karl Bettelheim from MDU, Victoria, Australia, serotyped all other isolates.

2.2.2 DNA extraction

DNA extraction was performed as outlined by Fagan *et al.* (1999). A single colony of *E. coli* was mixed in 1 ml of sterile water in a micro centrifuge tube. Bacteria were pelleted by centrifugation at 11,000 rpm for 1 min in a Biofuge *pico* (Heraeus, Hanau, Germany). The supernatant was subsequently discarded and 200 µl of InstaGene matrix (Bio-Rad, Richmond, CA) was added to the pellet and the mixture was incubated at 56°C for 30 min. After incubation, the mixture was vortexed for 10 sec and then incubated at 100°C for 8

Table 2.1 Virulence factor profiles and *stx*$_1$ subtypes among STEC of ovine and human origin.

Source[a]	Clinical condition	Serotype	No. of isolates	Virulence factor profile				No. of isolates with *stx*$_1$ subtype		
				stx$_1$	*stx*$_2$	*eae*	*ehxA*	*stx*$_1$	*stx*$_{1c}$	*stx*$_1$/*stx*$_{1c}$
Ovine (E)	Healthy	O2:H29	1	+	-	-	+	1		
Ovine (E)	Healthy	O5:H-	14	+	+	-	+		14	
Ovine (E)	Healthy	O5:H-	6	+	-	-	+		6	
Ovine (E)	Healthy	O5:H-	1	+	+	-	-		1	
Human (V)	HUS	O5:H-	1	+	-	+	+		1	
Human (V)	HUS	O5:H-	2	+	-	-	+		2	
Human (V)	HUS	O5:H-	1	+	+	-	+		1	
Ovine (E)	Healthy	O5:HR	1	+	+	+	+			1
Ovine (E)	Healthy	O6:H-	1	+	+	-	+		1	
Ovine (E)	Healthy	O6:H-	1	+	-	-	+		1	
Ovine (E)	Healthy	O8:Hnt	1	+	+	-	+		1	
Human (S)	HUS	O8:H8	1	+	-	-	-	1		
Ovine (E)	Healthy	O21:H21	1	+	+	-	+		1	
Ovine (E)	Healthy	O26:H-	2	+	-	+	+	2		
Human (V)	Diarrhoea	O26:H-	3	+	-	+	+	3		
Ovine (E)	Healthy	O26:H11	4	+	-	+	+	4		
Human (V)	Diarrhoea	O26:H11	3	+	-	+	-	3		
Human (V)	Diarrhoea	O26:H11	4	+	-	+	+	4		
Human (V)	Bloody Diarrhoea	O26:H11	1	+	-	+	+	1		
Human (S)	Diarrhoea	O26:H11	1	+	-	+	+	1		
Human (S)	Diarrhoea	O26:H11	1	+	-	+	-	1		

Table 2.1 Virulence factor profiles and stx_1 subtypes among STEC of ovine and human origin.

Source[a]	Clinical condition	Serotype	No. of isolates	Virulence factor profile				No. of isolates with stx_1 subtype		
				stx$_1$	*stx*$_2$	*eae*	*ehxA*	*stx*$_1$	*stx*$_{1c}$	*stx*$_1$/*stx*$_{1c}$
Ovine (E)	Healthy	O55:H20	1	+	+	+	+			1
Ovine (E)	Healthy	O69:H8	1	+	-	-	-		1	
Ovine (E)	Healthy	O75:H-	1	+	+	-	-		1	
Ovine (E)	Healthy	O75:H8	3	+	+	-	-	1	1	1
Ovine (E)	Healthy	O75:H8	1	+	-	-	-	1		
Ovine (E)	Healthy	O75:H8	16	+	+	-	+	1	2	13
Ovine (E)	Healthy	O75:H8	1	+	-	-	+		1	
Ovine (E)	Healthy	O75:H40	1	+	-	-	+		1	
Ovine (E)	Healthy	O75:H40	1	+	+	-	+		1	
Ovine (E)	Healthy	O77:H4	1	+	-	-	+		1	
Ovine (E)	Healthy	O77:H-	1	+	-	-	+		1	
Ovine (E)	Healthy	O81:H26	1	+	-	-	+		1	
Ovine (E)	Healthy	O84:H-	1	+	-	+	+	1		
Ovine (E)	Healthy	O88:H8	1	+	+	+	-			1
Ovine (E)	Healthy	O91:H-	20	+	+	-	+	17	3	
Ovine (E)	Healthy	O91:H-	6	+	-	-	+	4	2	
Ovine (E)	Healthy	O91:H-	5	+	+	-	-	5		
Human (V)	Healthy	O91:H-	2	+	+	-	-	2		
Ovine (E)	Diagnostic	O103:H2	1	+	-	+	+	1		
Human (V)	Diarrhoea	O103:H2	1	+	-	+	+	1		
Ovine (E)	Healthy	O103:H38	1	+	+	-	+			1
Ovine (E)	Healthy	O106:H18	1	+	+	-	+		1	

Table 2.1 Virulence factor profiles and *stx*$_1$ subtypes among STEC of ovine and human origin.

Source[a]	Clinical condition	Serotype	No. of isolates	Virulence factor profile				No. of isolates with *stx*$_1$ subtype		
				stx$_1$	*stx*$_2$	*eae*	*ehxA*	*stx*$_1$	*stx*$_{1c}$	*stx*$_1$/*stx*$_{1c}$
Ovine (E)	Healthy	O106:HR	1	+	-	+	-		1	
Ovine (E)	Healthy	O112ab:H2	2	+	+	-	-	2		
Ovine (E)	Healthy	O112ab:H2	1	+	-	-	-		1	
Ovine (E)	Healthy	O112ab:H2	1	+	+	+	+	1		
Human (S)	Diarrhoea	O117:H7	2	+	-	-	-	2		
Human (S)	Diarrhoea	O118:H16	1	+	-	+	+	1		
Ovine (E)	Healthy	O121:H2	1	+	+	-	+		1	
Ovine (E)	Healthy	O123:H-	1	+	-	-	+		1	
Ovine (E)	Healthy	O123:H-	20	+	+	-	+		20	
Human (V)	Diarrhoea	O123:H-	1	+	+	-	+		1	
Ovine (E)	Healthy	O123:H11	1	+	-	-	+		1	
Ovine (E)	Healthy	O128:H2	19	+	+	-	+		19	
Ovine (E)	Healthy	O128:H2	5	+	-	-	+		5	
Ovine (E)	Healthy	O128:H2	2	+	+	-	-		2	
Ovine (E)	Healthy	O128:H2	1	+	-	-	-		1	
Human (V)	Diarrhoea	O128:H2	4	+	+	-	+		4	
Human (V)	Diarrhoea	O128:H2	2	+	+	-	-		2	
Ovine (E)	Healthy	O128:H-/H2	1	+	-	-	-		1	
Ovine (E)	Healthy	O128:H8/H2	1	+	+	-	+		1	
Ovine (E)	Healthy	O128:Hnt	1	+	+	-	+		1	
Ovine (E)	Healthy	O152:H21	1	+	+	-	+		1	
Ovine (E)	Healthy	O153:H-	1	+	-	-	-		1	

Table 2.1 Virulence factor profiles and *stx*$_1$ subtypes among STEC of ovine and human origin.

Source[a]	Clinical condition	Serotype	No. of isolates	Virulence factor profile				No. of isolates with *stx*$_1$ subtype		
				stx$_1$	*stx*$_2$	*eae*	*ehxA*	*stx*$_1$	*stx*$_{1c}$	*stx*$_1$/*stx*$_{1c}$
Ovine (E)	Healthy	O153:H8	1	+	+	-	-		1	
Ovine (E)	Healthy	O153:H25	3	+	-	-	+		3	
Ovine (E)	Healthy	O153:H25	1	+	+	-	+		1	
Ovine (E)	Healthy	O153:H25	1	+	-	-	-		1	
Ovine (E)	Healthy	O154:HR	1	+	-	-	+		1	
Ovine (E)	Healthy	O157:H-	2	+	+	+	+	2		
Ovine (E)	Healthy	O157:H-	2	+	+	-	+	2		
Ovine (E)	Healthy	O157:H21	1	+	+	+	+	1		
Ovine (E)	Healthy	O158:HR	1	+	-	+	+		1	
Ovine (E)	Healthy	O163:H19	1	+	-	-	-		1	
Ovine (E)	Healthy	O168:H21	1	+	-	-	-		1	
Ovine (E)	Healthy	Ont:H8	1	+	+	+	-			1
Ovine (E)	Healthy	Ont:H8	1	+	+	-	-		1	
Ovine (E)	Healthy	Ont:H19	1	+	+	-	-	1		
Ovine (E)	Healthy	Ont:H49	1	+	+	+	-	1		
Ovine (E)	Healthy	Ont:H-	1	+	-	-	-		1	
Ovine (E)	Healthy	Ont:H-	2	+	+	-	-	2		
Human (V)	Unknown	Ont:H-	1	+	-	+	+	1		
Ovine (E)	Healthy	Ont:H-	1	+	-	-	+		1	
Ovine (E)	Healthy	Ont:HR	7	+	-	-	+		7	
Ovine (E)	Healthy	Ont:Hnt	1	+	+	-	-	1		
Human (V)	Healthy	Ont:Hnt	1	+	-	+	+	1		

Table 2.1 Virulence factor profiles and *stx*$_1$ subtypes among STEC of ovine and human origin.

Source[a]	Clinical condition	Serotype	No. of isolates	Virulence factor profile				No. of isolates with *stx*$_1$ subtype		
				stx$_1$	*stx*$_2$	*eae*	*ehxA*	*stx*$_1$	*stx*$_{1c}$	*stx*$_1$/*stx*$_{1c}$
Ovine (E)	Healthy	OR:H2	2	+	+	-	+		2	
Ovine (E)	Healthy	OR:H2	1	+	-	-	+		1	
Ovine (E)	Healthy	OR:H4	2	+	+	-	+		2	
Ovine (E)	Healthy	OR:HR	1	+	-	-	+		1	
Ovine (E)	Healthy	OR:H-	2	+	+	-	+		2	
Ovine (E)	Healthy	OR:H-	1	+	+	-	-		1	
Ovine (E)	Healthy	OX3:H2	1	+	-	-	-		1	
Ovine (E)	Healthy	OX3:H8	1	+	+	-	-		1	
Human (S)	HUS	OX3:H8	1	+	+	-	-		1	
Ovine (E)	Healthy	OX3:HR	3	+	-	-	+		3	

[a] E, isolates obtained from Elizabeth Macarthur Agricultural Institute, New South Wales, Australia; V, isolates obtained from Microbiological Diagnostic Unit, Victoria, Australia; S, isolates obtained from National Reference Laboratory for Foodborne Diseases, Bern, Switzerland.

min, followed by vortexing and centrifugation at 11,000 rpm for 1 min prior to removal of the nucleic acid template for PCR.

2.2.3 Multiplex PCR analysis of STEC isolates

All isolates were prepared and subjected to multiplex PCR for the detection of STEC virulence factors *stx*$_1$, *stx*$_2$, *ehxA* and *eae* as described previously (Paton and Paton, 1998), with the following modification. For the DNA preparation, InstaGene matrix (Bio-Rad) was used as described by Fagan *et al.* (1999). Amplification was performed in a 50 µl reaction mixture, containing 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 200 µM of each dNTP, 2 mM $MgCl_2$, 1 U of Taq DNA polymerase, 400 nM of each primer and 2 µl of nucleic acid (extracted with InstaGene matrix). Amplified PCR products were then resolved by agarose gel electrophoresis (Sambrook *et al.*, 1989) using 2% w/v agarose and stained with ethidium bromide (5 µg/ml). Visualisation was achieved by UV illumination and the images were captured using a GelDoc 1000 image analysis station (Bio-Rad).

2.2.3 *stx*$_1$ subtyping

The Clustal W program was used to align *stx*$_1$ genes deposited in GenBank (Appendix A) and the mapplot program (www.angis.org.au) was used to identify restriction enzyme cleavage sites. For this chapter all non-*stx*$_{1c}$ sequences will be referred to as common *stx*$_1$ subtypes. To subtype *stx*$_1$ sequences, a 603 bp fragment of the gene was amplified using Gannon F and R primers (Sigma Genosys, St Louise, Mo) (Table 2.2). The restriction enzymes *Cfo*I and *Rsa*I were used to cut the 603 bp fragment because these enzymes were predicted to generate RFLP profiles that readily distinguish *stx*$_{1c}$ from the common *stx*$_1$

subtypes (Table 2.3). PCRs were carried out in a 50 µl total volume containing 5 µl of whole cell DNA template prepared using InstaGene matrix (Fagan *et al.*, 1999), 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 2 mM $MgCl_2$, 10 pmol of each primer, 200 µM of each dNTP and 1 U of Taq DNA.

Thermocycler steps included an initial denaturation step of 94°C for 180 sec, followed by 35 cycles of denaturation (94°C for 60 sec), annealing (60°C for 60 sec) and extension (72°C for 120 sec). A final extension step of 72°C for 300 sec completed the PCR. PCR product (3-5 µg) was separately digested with 5 U each of *Cfo*I and *Rsa*I in 1 × buffer L (Roche) and incubated at 37°C for a minimum of 4 hours. Agarose gel (2%) electrophoresis was used to separate the restricted fragments and subtypes were identified according to their restriction pattern.

Table 2.2 Primers used to amplify and sequence stx_1.

Primer name	Primer sequence	Size (bp)	Reference
stx_1 typing			
Gannon F	5'-ACACTTGATGATCTCAGTGG-3'	603	Gannon *et al.*, 1992
Gannon R	5'-CATAATGGAGGGGGATTCA-3'		
stx_1 sequencing			
Paton 1F	5'-TCGCATGAGATCTGACC-3'	1470	Paton *et al.*, 1995
Paton 1R	5'CTTCTCAATTCAGTCAGTT-3'		
Paton 2 F	5'-ATAAATCGCCATTCGTTGACTAC-3'	180	Paton *et al.*, 1998
Paton 2 R	5'-GATGATCTCAGTGGGCGTTCT-3'		
Gannon F	5'-ACACTTGATGATCTCAGTGG-3'	603	Gannon *et al.*, 1992
Gannon R	5'-CATAATGGAGGGGGATTCA-3'		
Paton 1F	5'-TCGCATGAGATCTGACC-3'	448	Paton *et al.*, 1995
Vidiya 1R	5'-AATAATCTACGGCTTATT-3'		This study

Table 2.3 Restriction fragment sizes used for analysis of stx_1.

Primers used to amplify fragment	Restriction enzyme	Expected fragment size for:	
		stx_1	stx_{1c}
Gannon F, Gannon R	*Cfo*I	322, 135, 83, 63	414, 189
	Rsa I	no cut site	322, 135, 83, 63

2.2.4 Statistical analysis

Data of proportion of sheep and cattle (Brett *et al.*, 2003) containing stx_{1c} were analysed using a generalised linear model (McCullagh and Nelder, 1989) with errors assumed to follow a binominal distribution. A deviance value contributed by the different animal species was calculated and compared against Chi square distribution with 1 degree freedom.

2.2.5 Sequence analysis of stx_1

stx_1 from an STEC isolate with serotype O5:H- (isolate 531) was sequenced. This isolate was selected because O5:H- are common STEC serotypes recovered from healthy sheep. Secondly, serotype O5:H- possesses an stx_1 RFLP profile that is indistinguishable from stx_{1c}. A 1470 bp fragment encoding both A and B subunits of all Shiga toxin 1 genes was amplified using primers Paton 1 F and Paton 1 R (Table 2.2) in a reaction volume of 50 µl. The PCR was carried out using 5 µl of whole cell DNA template prepared using InstaGene matrix (Fagan *et al.*, 1999) (refer section 2.2.2), 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 2 mM $MgCl_2$, 10 pmol of each primer, 200 µM of each dNTP and 1 U of Taq DNA polymerase. Thermocycler steps involved an initial denaturation step of 94°C for 180 sec,

followed by 35 cycles of denaturation (94°C for 30 sec), annealing (54°C for 30 sec) and extension (72°C for 60 sec). A final extension step of 72°C for 300 sec completed the PCR. Amplified PCR product was separated by agarose gel electrophoresis (2%) and purified for DNA sequencing using the QIA quick DNA purification kit (Qiagen, Hilden, Germany). Primers used for sequencing are listed in Table 2.2. Sequencing reactions were performed using the BigDye terminator cycle sequencing ready reaction DNA sequencing kit and electrophoresed on an ABI prism 377 DNA sequencer (Perkin-Elmer, Santa Clara, USA). Auto Assembler software (Perkin Elmer) was used to compile and analyse the DNA sequences. Nucleotide and amino acid homology analysis was performed using programs accessed via the Australian National Genomic Information Service (ANGIS) (www.angis.org.au). Sequences were compared with those deposited in public databases using the BlastN and Blast P algorithms (Altschul *et al.*, 1990).

2.2.6 Nucleotide sequence accession number

The sequence of stx_1 from the ovine O5:H- isolate (531) has been submitted to the GenBank database under the accession no. AY135685.

2.3 RESULTS

2.3.1 Detection of STEC virulence factors using multiplex PCR

Of 237 stx_1–containing isolates 203 and 34 were derived from ovine and human sources respectively. Of 203 ovine isolates, 108 (53.2%) contained stx_1, stx_2 and *ehxA*, 22 (10.8%) contained stx_1 and stx_2, 43 (21.2%) contained stx_1 and *ehxA*, 11 (5.4%) contained stx_1 alone, 9 (4.4%) contained stx_1, *eae* and *ehxA*, 6 (2.9%) contained all 4 factors, 3 (1.4%) possessed

stx$_1$, *stx*$_2$ and *eae*, and 1 (0.4%) possessed *stx*$_1$ and *eae*. Of 34 isolates from humans, 14 (41.1%) contained *stx*$_1$, *ehxA* and *eae*, 6 (17.6%) contained *stx*$_1$, *stx*$_2$ and *ehxA*, 5 (14.7%) contained *stx*$_1$ and *stx*$_2$, 4 (11.7%) contained *stx*$_1$ and *eae*, 3 (8.8%) contained *stx*$_1$ alone and 2 (5.8%) contained *stx*$_1$ and *ehxA*. STEC serotypes and their virulence factor profiles are listed in Table 2.1.

2.3.2 Development of a PCR-RFLP assay to distinguish *stx*$_{1c}$ from common *stx*$_1$ subtypes

RFLP patterns generated by digestion of a 603 bp fragment of *stx*$_1$ (Figure 2.1) separately with *Cfo*I and *Rsa*I are shown in Figures.2.2 A and B respectively. The 603 bp fragment amplified from STEC isolates possessing common *stx*$_1$ subtypes generated fragments of 322, 135, 83 and 63 bp with *Cfo*I and 603 bp with *Rsa*I (Figures 2.2 A and B, lanes 3 and 4 respectively). However, the 603 bp fragment amplified from STEC isolates possessing *stx*$_{1c}$ generated fragments of 414 and 189 bp with *Cfo*I and 386 and 217 bp with *Rsa*I respectively (Figures 2.2 A and B, lanes 1, 2 and 7-10 respectively). This assay was used to type *stx*$_1$ from 237 *stx*$_1$-containing STEC (Table 2.1).

2.3.3 *stx*$_1$ subtyping and association with serotype

Of 203 ovine isolates, 133 (65.5%) possessed *stx*$_{1c}$, 51 (25.1%) possessed a common *stx*$_1$ subtype and 19 (9.2%) concomitantly possessed both *stx*$_{1c}$ and a common *stx*$_1$ subtype. STEC that possessed both *stx*$_{1c}$ and *stx*$_1$ were all of ovine origin and belonged to serotypes O75:H8 (14 of 21 isolates), O103:H38, Ont:H8, O88:H8, O55:H20, O5:HR (each represented by a single isolate) (Table 2.1).

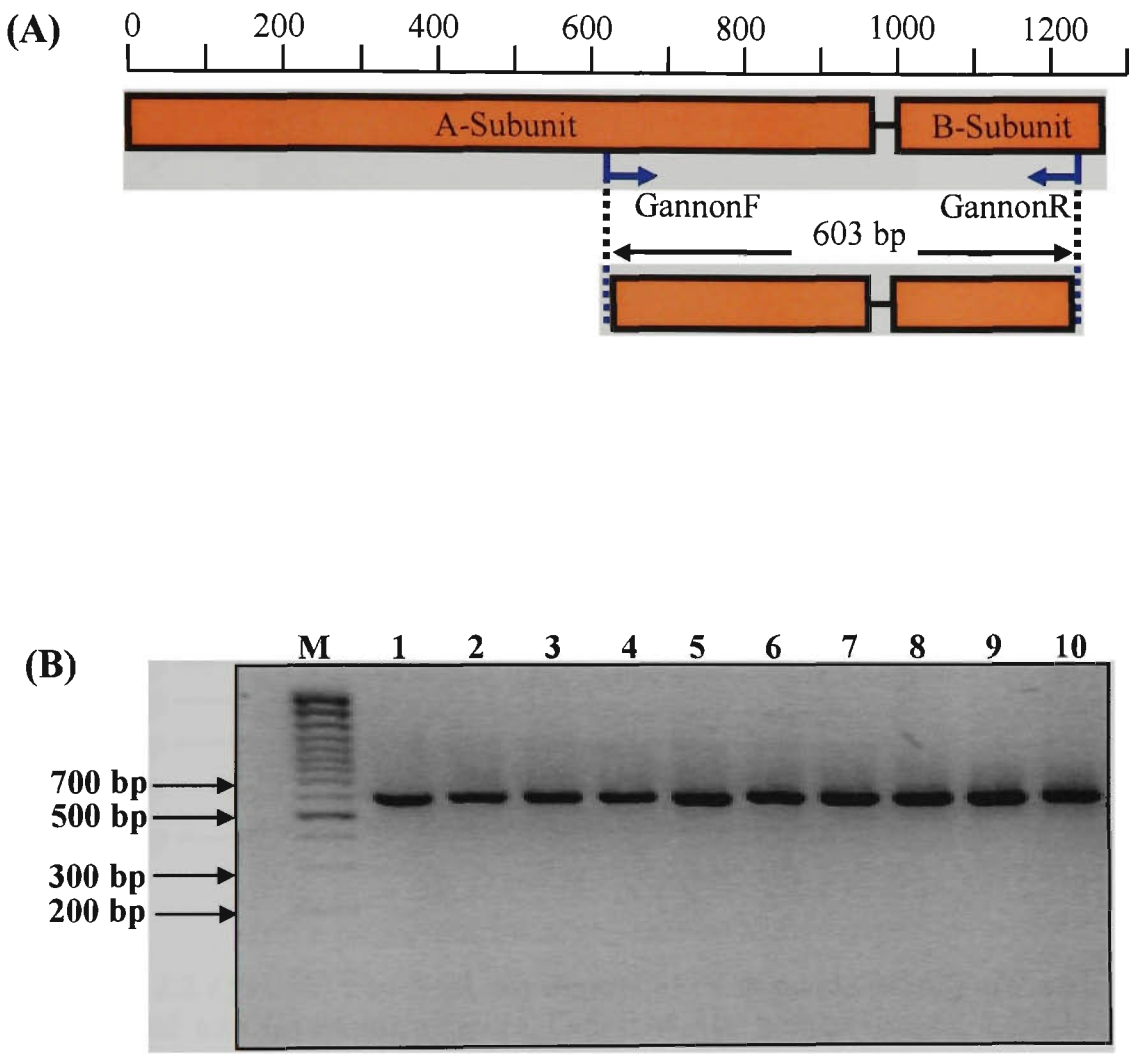


Figure 2.1 Amplification of *stx*$_1$ gene family by primers GannonF and GannonR described by Gannon *et al.* (1992). (A) Schematic representation of amplification .The blue arrows denotes the primer annealing positions and extension directions. (B) PCR products electrophoresed through 2% agarose gel.. Lanes: M, 100 bp plus marker; 1, O5:H- (ovine); 2, O123:H- (ovine); 3, O91:H- (ovine); 4, O157:H- (ovine); 5, O75:H8 (ovine); 6, O88:H8 (human); 7, O123:H- (human); 8, O128:H2 (human); 9, O5:H- (human); 10, OX3:H8 (human).

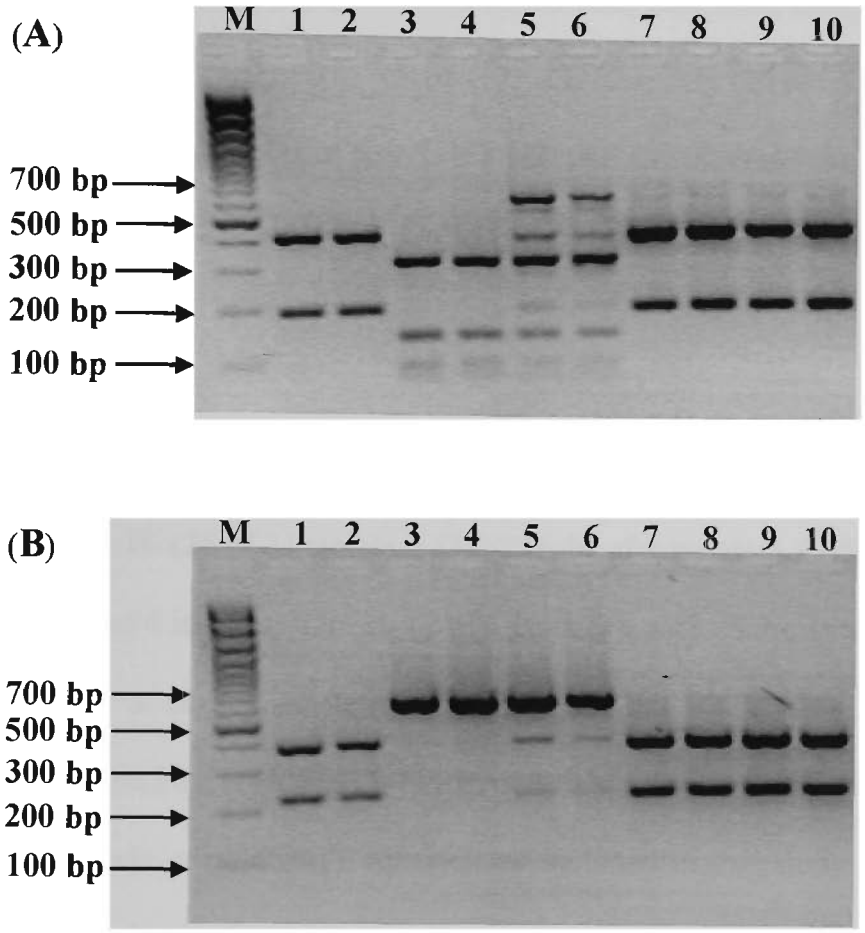


Figure 2.2 *Cfo*I (A) and *Rsa*I (B) digests of PCR product obtained with GannonF and GannonR primers. Lanes: M, 100 bp plus marker; 1, O5:H- (ovine); 2, O123:H- (ovine); 3, O91:H- (ovine); 4, O157:H- (ovine); 5, O75:H8 (ovine); 6, O88:H8 (ovine); 7, O123:H- (human); 8, O128:H2 (human); 9, O5:H- (human); 10, OX3:H8 (human). The band at 603 bp in lanes 5 and 6 in Figure 2.2 A is the uncut PCR product.

Ovine STEC positive for stx_{1c} alone comprised 40 serotypes including O128:H2 (all 28 isolates), O5:H- (all 21 isolates), O6:H- (both isolates), O91:H- (5 of 31 isolates), O75:H8 (4 of 21 isolates), O75:H40 (both isolates), O123:H- (all 21 isolates), O153:H25 (all 5 isolates), OR:H- (all 3 isolates), OR:H2 (all 3 isolates), OR:H4 (both isolates), OX3:HR (all

3 isolates) and Ont:HR (all 7 isolates) (Table 2.1). The remaining serotypes containing stx_{1c} were represented by a single isolate of O8:Hnt, O21:H21, O69:H8, O75:H-, O77:H4, O77:H-, O81:H26, O106:H18, O106:HR, O112ab:H2 (1 of 4 isolates), O121:H2, O123:H11, O128:H2/H8, O128 Hnt, O152:H21, O153:H8, O153:H-, O154:HR, O158:HR, O163:H19, O168:H21, Ont(A):H8, Ont(A):H-, Ont:H- (1 of 3 isolates), OR:HR, OX3:H2, and OX3:H8 (Table 2.1).

Ovine isolates that possessed a common stx_1 included O26:H- (both isolates), O26:H11 (all 4 isolates), O91:H- (26 of 31 isolates), O157:H- (4 of 4 isolates), O75:H8 (3 of 21 isolates), O112ab:H2 (3 of 4 isolates), Ont:H- (2 of 3 isolates); each of the remaining serotypes were represented by a single isolate of O2:H29, O84:H-, O157:H21, O103:H2, Ont:H19, Ont:H49 and Ont:Hnt (Table 2.1). Serogroup O26 is not commonly isolated from ovine faeces (Djordjevic *et al.*, 2001). Six isolates included in this study were recovered from newborn lambs during intensive sampling on a property that simultaneously grazed sheep and cattle and their stx_1 subtype was indistinguishable from stx_1 subtypes found in O26 isolates recovered from cattle (Brett *et al.*, 2003).

Of 34 isolates from humans, 30 were isolated from patients with diarrhoea (24 isolates, 7 serotypes) and HUS (6 isolates, 3 serotypes) (Table 2.1). The remaining four isolates were recovered from a asymptomatic patient (two isolates, both serotype O91:H-), a healthy individual (one isolate, serotype Ont:Hnt) and a patient of unknown aetiology (serotype Ont:H-) (Table 2.1). All 6 isolates from humans with serotype O128:H2 (diarrhoea), four O5:H- (HUS) isolates, and single isolates with serotypes O123:H- (diarrhoea) and OX3:H8 (HUS), all serotypes frequently isolated from sheep, possessed stx_{1c}. Ten isolates with

serotype O26:H11 and three with serotype O26:H- (all 13 isolates recovered from patients with diarrhoea), both serotypes commonly isolated from bovine faeces, possessed a common stx_1 subtype. Interestingly, two isolates with serotype O91:H- (recovered from asymptomatic patients), a common ovine serotype, contained an stx_1 subtype. Serotype O91:H- isolates are commonly recovered from ovine faeces and are atypical compared with other common ovine STEC serotypes in that they possess a common stx_1 subtype. Serotypes O117:H7 (2 isolates from patients with diarrhoea) and single isolates with serotypes O8:H8 (HUS), O103:H2 (diarrhoea), O118:H16 (diarrhoea), Ont:H- (unknown symptoms), and Ont:Hnt (healthy patient) all possessed the common stx_1 subtype. STEC isolates belonging to the classical EHEC serogroups (O26, O103 and O157) did not possess stx_{1c}; an observation that supports the findings of Koch *et al.* (2001).

Statistical comparison of the distribution of stx_{1c} between sheep and cattle (Brett *et al.*, 2003) reveal that sheep and cattle have different rate of possessing stx_{1c} (Chi square = 94.41; $p<0.001$) which is statistically significant. Ovine STEC isolates contained 65.5% (SE = 3.34) of stx_{1c} compared to bovine STEC isolates having 6.2% (SE = 2.67) of stx_{1c}.

2.3.4 stx_1 subtypes in STEC isolates containing the *eae* gene

Of 37 (19 ovine and 18 human) STEC isolates that contained *eae*, 30 (81%) possessed a common stx_1 subtype (Table 2.1). Of the remaining seven isolates (serotypes O5:H-, O5:HR, O37:H10, O55:H20, O88:H8, and Ont:H8), four contained both common stx_1 and stx_{1c} subtypes and three (serotypes O5:H-, O106:HR and O158:HR) contained the stx_{1c} subtype (Table 1). These data suggest that STEC isolates containing *eae* predominantly possess common stx_1 subtypes.

2.3.5 stx_1 sequence analysis

stx_1 from an ovine STEC of serotype O5:H- (isolate 531) predicted by RFLP analyses to possess stx_{1c} subtype in this study were examined by DNA sequence analysis. The derived nucleotide sequence (accession no. AY135685) showed 100% homology with the reported stx_{1c} gene sequence. (accession no. Z36901) (Paton *et al.*, 1995).

3.4 DISCUSSION

This study describes the development of a PCR-RFLP assay that differentiates stx_{1c} from other stx_1 subtypes and its application for subtyping stx_1 genes in 237 STEC isolates from ovine and human sources. The most striking result of this study was the predominance of stx_{1c} (133 of 203 isolates; 65.5%) among STEC of ovine origin and the infrequent identification of this subtype among STEC of bovine origin (5 of 81 isolates; 6.2%) (Brett *et al.*, 2003). Of the 203 ovine STEC, 70 (34.5%) belonged to common ovine serotypes O5:H-, O123:H- and O128:H2 (Beutin *et al.*, 1995; Beutin *et al.*, 1997; Kudva *et al.*, 1999; Djordjevic *et al.*, 2001) and were all positive for the stx_{1c} gene. Isolates with serotype O91:H-, another commonly reported ovine STEC serotype, predominantly possessed a common stx_1 subtype (26 of 31 isolates; 83.8%) although 5 isolates possessed stx_{1c}. In a similar study of ovine STEC, all ten O91:H- isolates from Germany were reported to possess a common stx_1 subtype (Koch *et al.*, 2001) suggesting that this serotype is rarely infected by lysogenic phage carrying stx_{1c} (see below).

In this study, the stx_{1c} gene was identified among a serologically diverse collection of STEC, the vast majority of which have previously been recovered from ovine faeces (Djordjevic *et al.*, 2001; Djordjevic *et al.*, unpublished results). In a study of sheep in

Germany, stx_{1c} was detected in 48 stx_1-containing STEC comprising serogroups O5, O125, O128, O146 and OX3 (Koch *et al.*, 2001). A lysogenic bacteriophage carrying the stx_{1c} gene was isolated from STEC derived from ovine faeces and the ability of the phage to integrate into the genomes of genetically heterogeneous *E. coli* types was established (Koch *et al.*, 2001). The authors suggested that the promiscuous nature of this bacteriophage may provide an explanation for the presence of the stx_{1c} gene among serologically diverse populations of STEC belonging to different clonal lineages. This study confirms and extends these preliminary observations by showing that the stx_{1c} gene is present among 45 STEC serotypes predominantly of ovine origin. The low prevalence of this gene in STEC recovered from bovine sources (5 of 81 isolates; 12.3%) (Brett *et al.*, 2003) suggests that phage carrying stx_{1c} are not prevalent in the gastrointestinal tract of cattle or that most serotypes that inhabit cattle are refractory to infection by this phage.

Of 203 ovine stx_1-containing STEC, 19 (9.3%) possessed *eae*. Of these, 12 contained a common stx_1 subtype, three contained stx_{1c}, and the remaining four contained both common stx_1 and stx_{1c} subtypes. Of the 154 isolates in this study that possessed the stx_{1c} gene, 122 (84.1%) were found to contain *ehxA*. STEC serotypes that are commonly recovered from patients with serious diseases typically carry the *eae* gene and also possess the EHEC plasmid that encodes *ehxA* and other potential virulence associated factors (Willshaw *et al.*, 1992; Gyles *et al.*, 1998; Boerlin *et al.*, 1999). The majority (83 of 133; 62.4%) of ovine STEC that possessed stx_{1c} also contained stx_2. Studies undertaken for this thesis have shown that STEC recovered from ovine faeces typically possess stx_{2d} subtypes (refer to Chapter 3), a subtype that is not commonly found in STEC isolated from patients with severe disease (Pierard *et al.*, 1998; Pierard *et al.*, 1999; Friedrich *et al.*, 2002). In a recent

study it was shown that STEC strains harbouring stx_{1c} were usually associated with asymptomatic infection or uncomplicated diarrhoea in humans (Zhang *et al.*, 2002). Collectively, these data lend weight to the hypothesis that STEC recovered from sheep faeces are uncommonly associated with HUS. However, it should be emphasised that in this study, five STEC (four O5:H- isolates and a single OX3:H8 isolate) from humans with HUS have been shown to possess stx_{1c}. In addition, 51 of 203 (25.1%) ovine isolates contained a common stx_1 subtype and 41 (80.4%) of these belonged to serogroups (O26, O91, O103, and O157), which have been associated with serious human illnesses in Australia and around the world. Further studies are required to elucidate the role that STEC derived from ovine sources contribute to milder human gastrointestinal conditions such as diarrhoea.

There is mounting evidence that STEC serotypes that commonly inhabit the gastrointestinal tract of one ruminant species are rarely isolated from other hosts (Beutin *et al.*, 1995; Beutin *et al.*, 1997; Djordjevic *et al.*, 2001; Hornitzky *et al.*, submitted). In addition to serotype, Shiga toxin gene subtypes also appear to associate with particular STEC serotypes and consequently ruminant hosts. For example, stx_2-containing STEC recovered from ovine faeces commonly possess stx_{2d} subtypes (refer to Chapter 3) whereas stx_2-containing STEC commonly recovered from cattle faeces typically possess stx_2, stx_{2vha} and stx_{2vhb} subtypes (Beutin *et al.*, 1993; Brett *et al.*, 2003). Similarly, stx_{2e} is typically isolated from porcine sources and has not been reported from ovine or bovine sources (Gannon *et al.*, 1990). These observations are consistent with hypotheses raised by Hoey *et al.* (2002), which suggest that the effects of Shiga toxins on bovine epithelial cells are likely to significantly affect the success of colonisation, dissemination and persistence of STEC in

cattle reservoirs. Furthermore, these authors also speculate that genetic heterogeneity among both Shiga toxin subtypes and other associated virulence factors, particularly serotype-dependent variation, may account for differences in pathogenicity of different STEC populations for cattle and hence the potential for distribution in humans. These hypotheses may also apply for STEC in sheep, although further studies need to be carried out to elucidate these hypotheses.

In contrast to these observations, STEC of serogroup O157 can be isolated from different animal species including humans, cattle, sheep and swine (Chapman *et al.*, 1993; Heuvelink *et al.*, 1999; Nakazawa and Akiba, 1999; Djordjevic *et al.*, 2001). Irrespective of host source, O157 isolates have never been shown to possess stx_{1c} or stx_{2d} genes and always possess either the common stx_1 and/or stx_2/stx_{2vh} subtypes. The current study also show that some serotypes, particularly O75:H8 (14 of 21 isolates) simultaneously possess both stx_1 and stx_{1c} subtypes. Similarly, some bovine STEC isolates have been reported to possess up to three different stx_2 subtypes (Beutin *et al.*, 1993). Shiga toxin genes are uniformly flanked by bacteriophage-linked sequences in serologically different STEC strains (Schmidt, 2001; Unkmeir *et al.*, 2000). Collectively, these observations support the contention that bacteriophage transmission plays a key role in the spread of Shiga toxin genes among *E. coli* and that serotype may influence the outcome of these interactions (Schmidt *et al.*, 1999; Koch *et al.*, 2001; Schmidt, 2001).

Chapter 3

Characterisation of ovine *stx*$_2$

3 – Characterisation of ovine stx_2 subtypes

3.1 INTRODUCTION

Stx2 is considered to be the most important virulence factor associated with human disease (Ostroff *et al.*, 1989; Boerlin *et al.*, 1999). In addition, Stx2 is about 400-fold more toxic for mice compared to Stx1 and has also been shown to induce fetoplacental resorption, intrauterine haematoma, fibrin deposition and neutrophil infiltration when injected intravenously into mice on day 5 of pregnancy (Tesh *et al.*, 1993; Yoshimura *et al.*, 2000). Unlike stx_1, considerable sequence variation among stx_2 has been reported (Ito *et al.*, 1990; Schmitt *et al.*, 1991; Pierard *et al.*, 1998; Weinstein *et al.*, 1988). More importantly, differences in the degree of pathogenicity of STEC have been associated with variations in stx_2 subtype (Lindgren *et al.*, 1993; Melton-Celsa *et al.*, 1998; Kokai-Kun *et al.*, 2000; Friedrich et al., 2002).

At least 14 stx_2 variants have been described (refer to Table 1.4). The most prevalent Stx2 variants are stx_{2c}, stx_{2d} and stx_{2e} (Weinstein *et al.*, 1988; Schmitt *et al.*, 1991; Pierard *et al.*, 1998). stx_{2c} was isolated from *E. coli* O157:H- strain E32511 and is closely related to stx_2 and $stx_{2vh\text{-}a}$ (Schmitt *et al.*, 1991). The stx_{2d} cluster as defined by Pierard *et al.* (1998) comprises $stx_{2d\text{-}O111}$ (Paton *et al.*, 1993), $stx_{2d\text{-}OX3a}$ (Paton *et al.*, 1992) and $stx_{2d\text{-}Ount}$ variants and these subtypes were identified in non-O157 STEC isolated from humans and meat (Pierard *et al.*, 1998). However, stx_{2d} positive STEC are not observed in the most virulent serogroups for humans including O157, O26, O103, O111 and O145 and have been reported to be less frequently associated with diarrhoea and HUS (Pierard *et al.*, 1998;

Friedrich *et al.*, 2002). stx_{2e} is predominantly associated with oedema disease in swine (Weinstein *et al.*, 1988) and is rarely recovered from humans.

The importance of characterising Stx2 types has been recently highlighted by the observation that mouse or human colonic mucin (Melton-Celsa *et al.*, 1996) can activate some Stx2 toxins. The Vero cell cytotoxicity of intestinal mucus-treated Stx2vha/b was reported to increase 35-350-fold compared to non mucin-treated Stx2vha/b. Mucin activation provides an explanation for the observation that STEC expressing Stx2vh are highly virulent (LD_{50} < 10 colony forming units) when fed to streptomycin-treated CD-1 mice compared the STEC expressing Stx2c (LD_{50} of 10^{10} colony forming units) (Lindgren *et al.*, 1993; Melton-Celsa *et al.*, 1998).

Recent studies of sheep in eastern Australia have demonstrated that the predominant STEC serotypes containing accessory virulence factors (either enterohaemolysin and/or intimin) are O5:H-, O75:H8, O91:H-, O123:H- and O128:H2 (Djordjevic *et al.*, 2001) and several of these serotypes have been occasionally isolated from clinically-affected patients. More than 60 different serotypes of STEC have been isolated from humans with clinical infections (Acheson *et al.*, 2000). Many STEC of ovine origin contain stx_2 and express toxin (Djordjevic *et al.*, 2001). However, only a few reports have examined stx_2 subtypes among STEC recovered from ruminant sources, particularly sheep. The aims of this study were (i) to determine the stx_2 subtype(s) among STEC derived from ovine sources and (ii) to determine the stx_2 subtypes among STEC isolates from humans that possess a serotype commonly associated with sheep with the purpose to determine if sheep represent a source of STEC for human infections.

3.2 MATERIALS AND METHODS

3.2.1 STEC isolates

One hundred and sixty seven STEC isolates were used in this study (Table 2.1). Of these 77 STEC isolates (68 sheep and 9 human) were investigated in Chapter 2 (refer Appendix A). The Elizabeth Macarthur Agricultural Institute (New South Wales, Australia) provided the 124 isolates which were isolated using methods described by Djordjevic *et al.* (2001) (refer section 2.2.1). Of these, 121 were isolated from healthy sheep and 3 were isolated from diagnostic submissions where STEC were not necessarily implicated as the cause of the disease. Thirty-four isolates were obtained from the Microbiological Diagnostic Unit (MDU) (Melbourne, Australia). These consisted of 12 isolates of human origin, 9 isolates from lamb meat, 2 isolates from sheep faeces, 1 isolate from a meat sausage and 10 isolates were derived from lamb carcasses. Dr. Andre Burnens from the National Reference Laboratory for Foodborne Diseases (Berne, Switzerland) provided 9 ioslates from humans from patients with diarrhoea or HUS (Burnens *et al.*, 1992; Essers *et al.*, 2000). The Swiss isolates possessed serotypes not commonly found in STEC recovered from ovine sources and were included in this study for comparative purposes only. The Swiss isolates were serotyped by Kim Ziebel and Roger Johnson from the Guelph Laboratory, Health Canada, Guelph, Ontario, Canada. Dr. Karl Bettelheim from MDU, Victoria, Australia, serotyped all other isolates.

3.2.2 DNA extraction and multiplex PCR analysis of STEC

Multiplex PCR for the detection of STEC virulence factors was applied to isolates not investigated in Chapter 2 (refer to Appendix A). Isolates were prepared and subjected to

Table 3.1 Virulence factor profiles and stx_2 subtypes of ovine and human STEC.

Serotype	Source[a]	Total no. of isolates	Virulence Profile				Number of isolates containing indicated stx_2 variant genes				
			stx_1	stx_2	*ehxA*	*eae*	stx_2	stx_{2d}-Ount	stx_{2d}-OX3a/O111	stx_{2vha}	stx_{2vhb}
O5:H-	Sheep feces, NSW (E)	17	+	+	+	-			17		
O5:H-	Sheep feces, NSW (E)	1	-	+	+	-	1				
O5:H-	Human, Australia, HUS (V)	1	+	+	+	-			1		
O7:H-	Human, HUS (S)	1	-	+	+	+	1				
O8:H14	Human, HUS (S)	1	-	+	-	-					1
O26:H-	Human, HUS (S)	2	-	+	-	+	2				
O75:H-	Sheep feces, NSW (E)	1	+	+	+	-		1			
O75:H8	Sheep feces, NSW (E)	16	+	+	+	-		16			
O75:H40	Sheep feces, NSW (E)	1	+	+	+	-		1			
O91:H-	Sheep feces, NSW (E)	36	+	+	+	-		34	2		
O91:H-	Human, Australia, Symptomless (V)	1	+	+	-	-		1			
O91:H-	Human, Australia, Diarrhoea (V)	1	+	+	-	-		1			
O91:H-	Lamb carcasses, Queensland (V)	9	+	+	+	-		8	1		
O91:H-	Lamb carcasses, Queensland (V)	1	-	+	-	-		1			
O91:H-	Lamb meat, New Zealand (V)	6	+	+	+	-		6			
O91:H-	Lamb meat, New Zealand (V)	3	+	+	-	-		3			
O91:H-	Sheep feces, New Zealand (V)	1	+	+	+	-		1			
O91:H-	Sheep feces, USA (V)	1	+	+	+	-		1			
O91:H-	Meat sausage, Australia (V)	1	+	+	-	-		1			
O91:H2	Sheep feces, NSW (E)	1	+	+	+	-			1		
O91:H10	Human, Australia, Diarrhoea (V)	1	-	+	-	-	1				
O91:H21[b]	Human, New Zealand, Diarrhoea (V)	1	-	+	+	-	1				1
O103:H38	Sheep feces, NSW (E)	1	+	+	+	-		1			
O121:H19	Human, HUS (S)	1	-	+	+	+	1				
O121:H19	Human, Diarrhoea (S)	1	-	+	-	+	1				

TABLE 3.1 Virulence factor profiles and stx_2 subtypes of ovine and human STEC.

Serotype	Source[a]	Total no. of isolates	Virulence Profile				Number of isolates containing indicated stx_2 variant genes				
			stx_1	stx_2	*ehxA*	*eae*	stx_2	stx_{2d}-Ount	stx_{2d}-OX3a/O111	stx_{2vha}	stx_{2vhb}
O123:H-	Sheep feces, NSW (E)	22	+	+	+	-		21	1		
O123:H-	Sheep feces, Diagnostic, NSW (E)	3	+	+	+	-		3			
O123:H-	Human, Australia, Diarrhoea (V)	1	+	+	+	-		1			
O128:H2	Sheep feces, NSW (E)	12	+	+	+	-		12			
O128:H2	Human, Australia, Diarrhoea (V)	3	+	+	+	-		3			
O128:H2	Human, New Zealand, Diarrhoea(V)	1	+	+	+	-		1			
O128:H2	Human, Australia, Diarrhoea (V)	1	+	+	-	-		1			
O128:H2	Human, New Zealand, Diarrhoea (V)	1	+	+	-	-		1			
O128:H-	Sheep feces, NSW (E)	2	+	+	+	-		2			
O145:H-	Human, HUS/Diarrhoea (S)	2	-	+	-	+	1				1
O153:H-	Sheep feces, NSW (E)	2	+	+	+	-		2			
O153:H25	Sheep feces, NSW (E)	1	+	+	+	-		1			
O157:H-	Sheep feces, NSW (E)	2	+	+	+	+				2	
O157:H-	Sheep feces, NSW (E)	1	-	+	+	+				1	
O157:H21	Sheep feces, NSW (E)	1	+	+	+	+				1	
OR:H2	Sheep feces, NSW (E)	4	+	+	+	-		4			
OX3:H8	Human, HUS (S)	1	+	+	-	-		1			

[a] E, Isolates obtained from Elizabeth Macarthur Agricultural Institute, NSW, Australia;
V, Isolates obtained from Victorian Infectious Diseases Laboratory, Victoria, Australia;
S, Isolates obtained from National Reference Laboratory for Foodborne Diseases, Berne, Switzerland.
[b] Strain had 2 different stx_2 subtype.

multiplex PCR for the detection of STEC virulence factors *stx*$_1$, *stx*$_2$, *ehxA* and *eae* as described by Paton and Paton (1998) with the modifications outlined in section 2.2.3. Amplified DNA fragments were resolved by agarose gel electrophoresis (Sambrook *et al.*, 1989) using 2% (w/v) agarose. Gels were stained with 0.5 µg/ml ethidium bromide and visualized with UV illumination and imaged using a GelDoc 1000 image analysis station (Bio-Rad, Richmond, CA).

3.2.3 *stx*$_2$ subtyping

Ovine and human STEC (Table 3.1) containing *stx*$_2$ were subjected to *stx*$_2$ subtyping as described by Pierard *et al.* (1998) and Bastian *et al.* (1998). The primers sequences are listed in (Table 3.2). In this chapter *stx*$_{2d}$ (*stx*$_{2d\text{-}Ount}$/*stx*$_{2d\text{-}O111}$/*stx*$_{2d\text{-}OX3a}$) is defined as a nucleotide sequence variant of *stx*$_2$ as described by Pierard *et al.* (1998) and does not refer to the mucin-inducible Stx2d toxin subtype (encoded by *stx*$_{2\text{-}vha}$/*stx*$_{2\text{-}vhb}$) as defined by Melton-Celsa *et al.* (1998).

stx$_2$ amplified with VT2-e and VT2-f primers (Table 3.2) was subjected to restriction endonuclease digestion with *Hae*III and *Pvu*II as described by Pierard *et al.* (1998). PCR product obtained with the LinF and LinR primers (Table 3.2) was digested with *Hinc*II and *Acc*I as described by Bastian *et al.* (1998). PCR products (10 µl) were incubated with 5 U of appropriate enzyme in the buffer provided by the manufacturer. Restriction fragments were separated by agarose gel electrophoresis. *stx*$_2$ subtypes were identified based on their restriction profiles (Table 3.3).

Table 3.2 Primers used to amplify *stx*$_2$.

Primer	Sequence (5' to 3')	Product size (bp)	Reference
Primers for typing *stx*$_2$			
VT2-e	AATACATTATGGGAAAGTAATA	348	Pierard *et al.*, 1998
VT2-f	TAAACTGCACTTCAGCAAAT		
Lin F	GAACGAAATAATTTATATGT	900[a]	Lin *et al.*, 1993
Lin R	TTTGATTGTTACAGTCAT		
Primers for sequencing *stx*$_2$			
Stx2F	TATCTGCGCCGGGTCT	1280	This study
Stx2R	CAAAKCCKGARCCTGA[b]		
Paton F	GGCACTGTCTGAAACTGCTCC	255	Pierard *et al.*, 1998
Paton R	TCGCCAGTTATCTGACATTCTG		
Gannon F	CCATGACAACGGACAGCAGTT	779	Gannon *et al.*, 1992
Gannon R	CCTGTCAACTGAGCACTTTG		

[a] The size of the Lin amplicon varies by a few nucleotides depending on the variant.
[b] Redundant nucleotides. K= G+T, R= A+G.

3.2.4 *stx*$_2$ DNA sequence analysis

An O91:H- isolate (isolate CS164) was chosen as a source of *stx*$_2$ for sequencing studies for the following reasons. Firstly, O91:H- is the most common ovine STEC serotype recovered from Australian sheep. Secondly, the *stx*$_{2d}$ RFLP profile indicated that it possessed a *stx*$_{2d\text{-}Ount}$ subtype, which was the most common *stx*$_2$ subtype observed among STEC from the faeces of Australian sheep. The A and B subunits of *stx*$_2$ of isolate CS164 were amplified using oligonucleotide primers Stx2F2 and Stx2R (Table 3.2). PCR assays were carried out in a 50 µl total volume containing 5 µl nucleic acid (extracted with InstaGene matrix) from the isolate, 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 2 mM $MgCl_2$, 10 pmol of each primer, 200 µM of each dNTP and 1 U of Taq DNA polymerase. After an initial denaturing step of

TABLE 3.3 Sizes of restriction fragments used for RFLP analysis of stx_2.

Primers used to amplify fragment	Restriction enzyme	Expected fragment size(s) (bp) for:					Reference
		stx_2	stx_{2vha}	stx_{2vhb}	$stx_{2d\text{-}Ount}$	$stx_{2d\text{-}OX3a/O111}$	
VT2-e, VT2-f	*HaeIII*	348	216, 132	216, 132	216, 132	167, 132, 49[a]	Pierard *et al.*, (1998)
	PvuII	323, 25[a]	323, 25[a]	250, 73, 25[a]	200, 120, 28[a]	200, 120, 28[a]	
Lin F, Lin R	*HincII*	556, 263, 62[a], 25[a]	556, 322, 25[a]	556, 347	881, 25[a]	881, 25[a]	Bastian *et al.*, (1998)
	AccI	554, 352	551, 352	551, 352	906	554, 352	

[a] This fragment was too small to visualize under the electrophoresis conditions used

5 min at 95°C, the samples were subjected to 35 cycles of denaturation (95°C, 30 sec), annealing (60°C, 45 sec) and extension (72°C, 90 sec), followed by a single final extension step of 5 min at 72°C. PCR products were analysed by agarose gel electrophoresis. PCR product was purified using QIA quick DNA purification kit (Qiagen, Germany). Primers used for sequencing are listed in Table 3.2. DNA sequence reactions were performed using the BigDye terminator cycle sequencing ready reaction DNA sequencing kit and electrophoresed on an ABI prism 377 DNA sequencer (Perkin-Elmer, CA). Compilation and analysis of DNA sequence data was performed as described in section 2.2.4.

3.2.5 Nucleotide sequence accession number

The sequence of *stx*$_2$ from the ovine O91:H- isolate (CS164) has been submitted to the GenBank database under the accession no. AF298816.

3.3 RESULTS

3.3.1 Detection of STEC virulence factors using multiplex PCR

All 146 ovine STEC contained *stx*$_2$. Of these 143 (97.9%) contained *stx*$_1$ and *stx*$_2$, 139 of 146 (95.2%) contained *stx*$_1$, *stx*$_2$, and *ehxA* and 3 of 146 (2%) contained all 4 virulence factors. All 21 human STEC isolates contained *stx*$_2$. Eleven (52.4%) of these contained *stx*$_1$ and *stx*$_2$, 2 of 21 (9.5%) contained *stx*$_2$, *ehxA* and *eae* and none contained all 4 virulence factors. The virulence factor profiles for all isolates are presented in Table 3.1.

3.3.2 Subtyping of stx_2

The most common stx_2 subtype observed among STEC isolated from sheep was $stx_{2d\text{-}Ount}$ (Figures 3.1 and 3.2; Table 3.1). Specifically, 55 of 58 (94.8%) O91:H-, 16 of 16 (100%) O75:H8, 24 of 25 (96%) O123:H-, 12 of 12 (100%) O128:H2 and 4 of 4 (100%) OR:H2 STEC isolates from sheep contained $stx_{2d\text{-}Ount}$. Seventeen of 18 (94.4%) O5:H-, 3 of 58 (5.1%) O91:H- and 1/25 (4%) O123:H- STEC from sheep were found to contain either $stx_{2d\text{-}O111}$ or $stx_{2d\text{-}OX3a}$. These latter two stx_2 variants were not differentiated due to their high nucleotide sequence homology (99%). Of the 11 ioslates from humans with serotypes commonly isolated from sheep, 10 (90%) also contained $stx_{2d\text{-}Ount}$. The human O5:H- isolate contained $stx_{2d\text{-}O111}/stx_{2d\text{-}OX3a}$. The four ovine O157:H-/H21 isolates possessed stx_{2vha}. Other ioslates from humans with serotypes not commonly found in sheep contained either stx_2 or stx_{2vhb} variants (Figures 3.3 and 3.4; Table 3.1). One strain of serotype O91:H21 from a human source contained stx_2 in combination with stx_{2vhb}.

3.3.3 stx_2 sequence analysis

DNA sequence analysis of stx_2 from the O91:H- (CS164) isolate showed 99% homology with $stx_{2d\text{-}Ount}$ (accession no. AFO43627) and showed a single amino acid change (C^{41} to S) compared to $stx_{2d\text{-}Ount}$ in the A subunit (Figure 3.5). The stx_2 DNA sequence was also highly homologous (97%) to $stx_{2d\text{-}OX3a}$ (accession no. X65949) and $stx_{2d\text{-}O111}$ (accession no. L11078). These stx_2 variants are grouped together as stx_{2d} as described by Pierard *et al.* (1998).

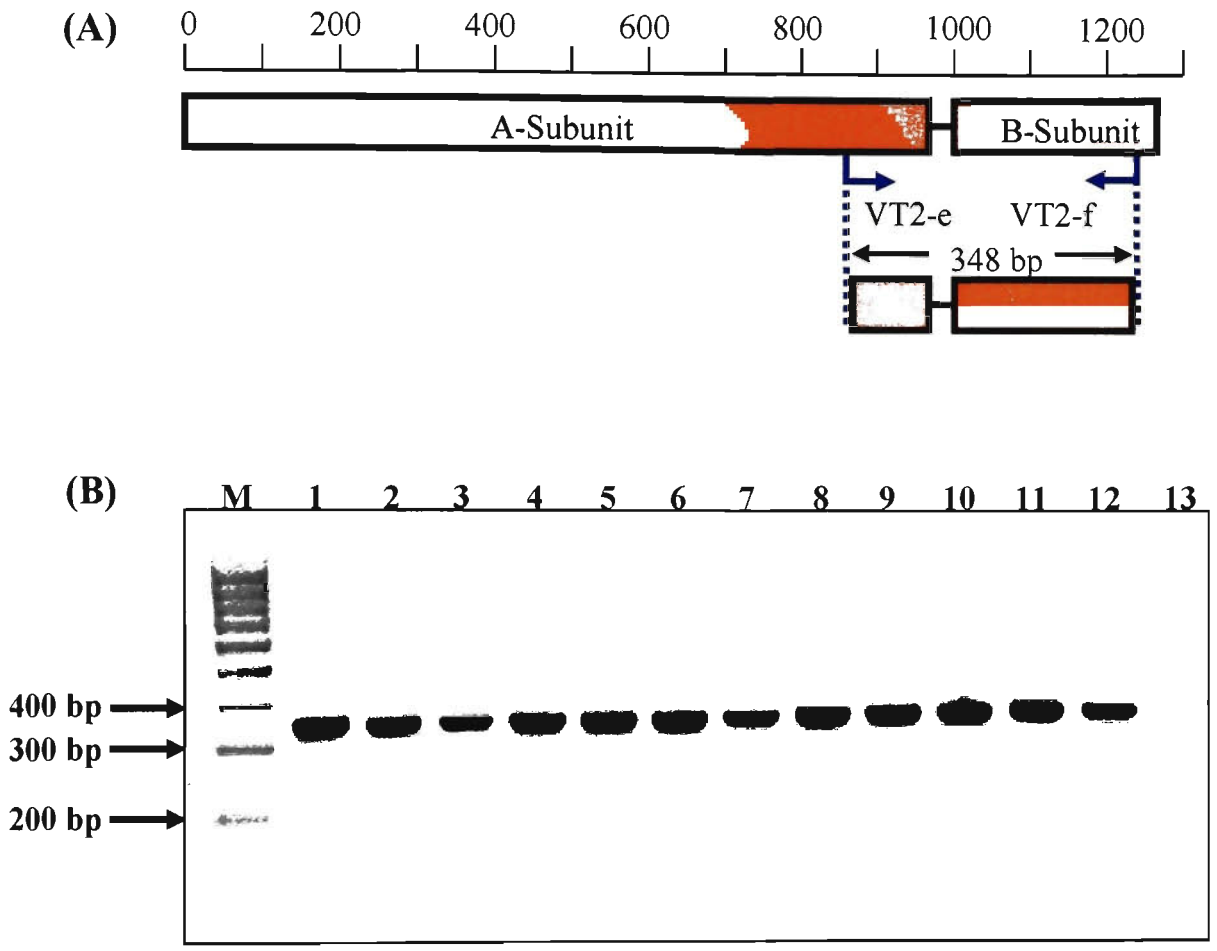


Figure 3.1 Amplification of *stx*$_2$ gene family by primers VT2-e and VT2-f described by Pierard *et al.* (1998). (A) Schematic representation of amplification .The blue arrows denotes the primer annealing positions and extension directions. (B) PCR products electrophoresed through 2% agarose gel. Lanes: M, 100 bp plus marker; 1, O91:H- (ovine); 2, O123:H- (ovine); 3, O128:H2 (ovine); 4, O75:H8 (ovine); 5, O5:H- (ovine); 6, O91:H- (human); 7, O123:H- (human); 8, O128:H2 (human); 9, O5:H- (human); 10, OX3:H8 (human); 11, O91:H10 (human); 12, O91:H21 (human); 13, negative control (no nucleic acid present in PCR mixture).

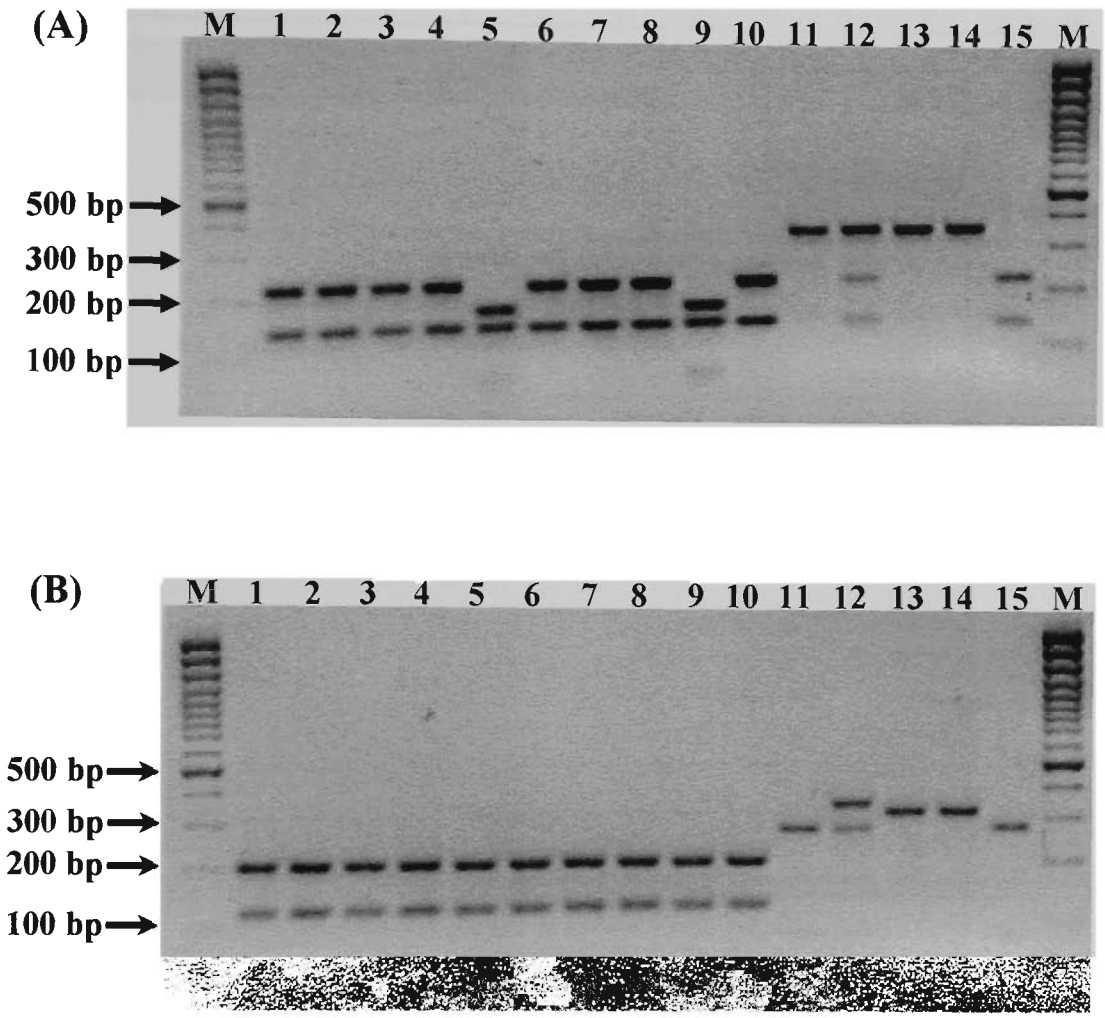


Figure 3.2 *Hae*III (A) and *Pvu*II (B) digests of PCR product obtained with VT2-e and VT2-f primers. Lanes: M, 100 bp plus marker; 1, O91:H- (ovine); 2, O123:H- (ovine); 3, O128:H2 (ovine); 4, O75:H8 (ovine); 5, O5:H- (ovine); 6, O91:H- (human); 7, O123:H- (human); 8, O128:H2 (human); 9, O5:H- (human); 10, OX3:H8 (human); 11, O91:H10 (human); 12, O91:H21 (human); 13, O121:H19 (human); 14, O145:H- (human); 15, O8:H14 (human).

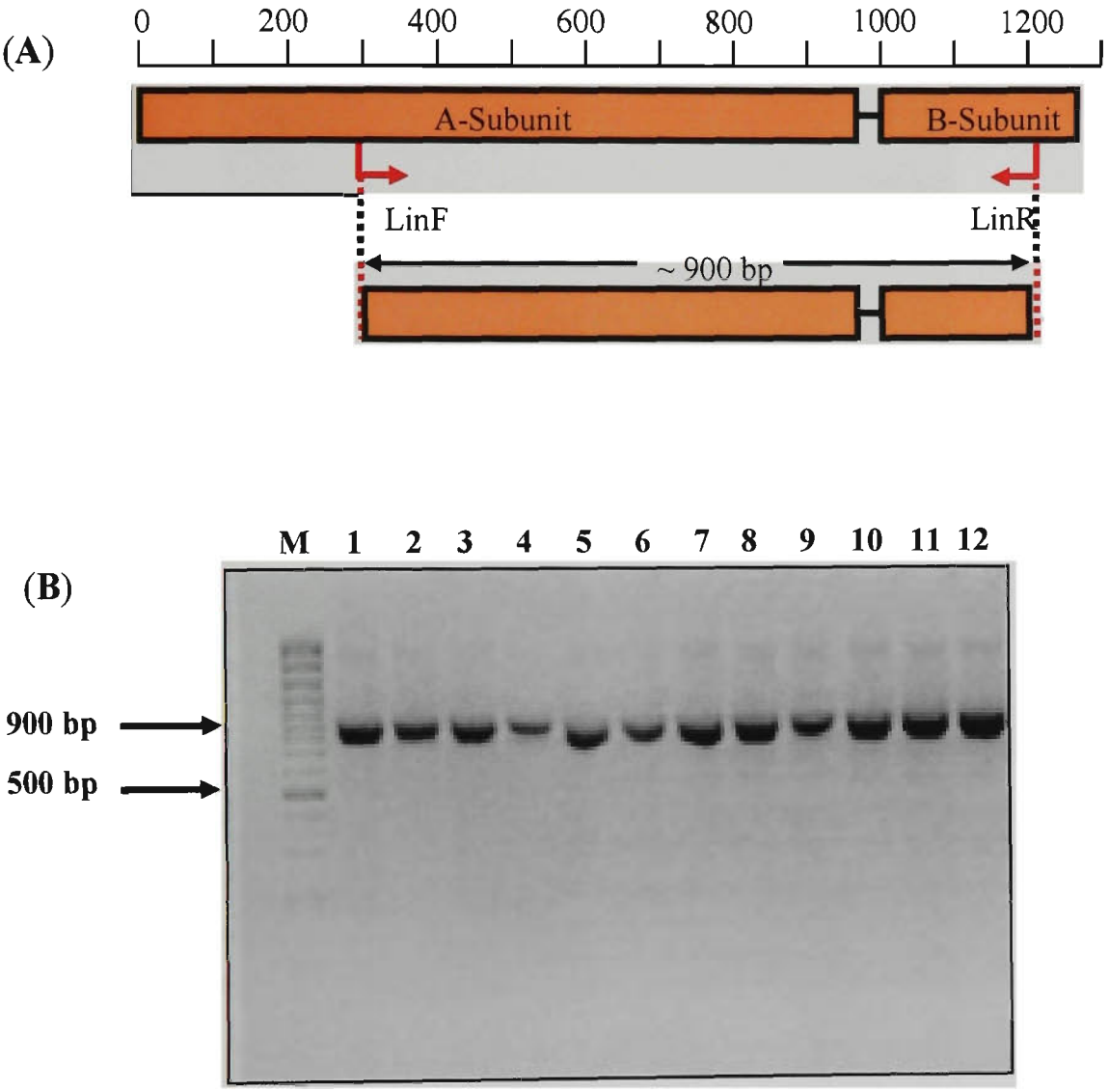


Figure 3.3 Amplification of *stx*$_2$ gene family by primers LinF and LinR described by Lin *et al.* (1993). (A) Schematic representation of amplification. The red arrows denote the primer annealing positions and extension directions. (B) PCR products electrophoresed through 2% agarose gel. Lanes: M, 100 bp plus marker; 1, O121:H19 (human); 2, O145:H- (human); 3, O7:H- (human); 4, O145:H- (human); 5, O26:H- (human); 6, O121:H19 (human); 7, O26:H- (human); 8, O8:H14 (human); 9, O26:H11 (negative control, human isolate with *stx*$_1$ only, since the LinF and LinR primers also amplify *stx*$_1$); 10, O111:H8 (positive *stx*$_2$ control, human isolate with both *stx*$_1$ and *stx*$_2$); 11, O91:H- (ovine); 12, O5:H- (ovine).

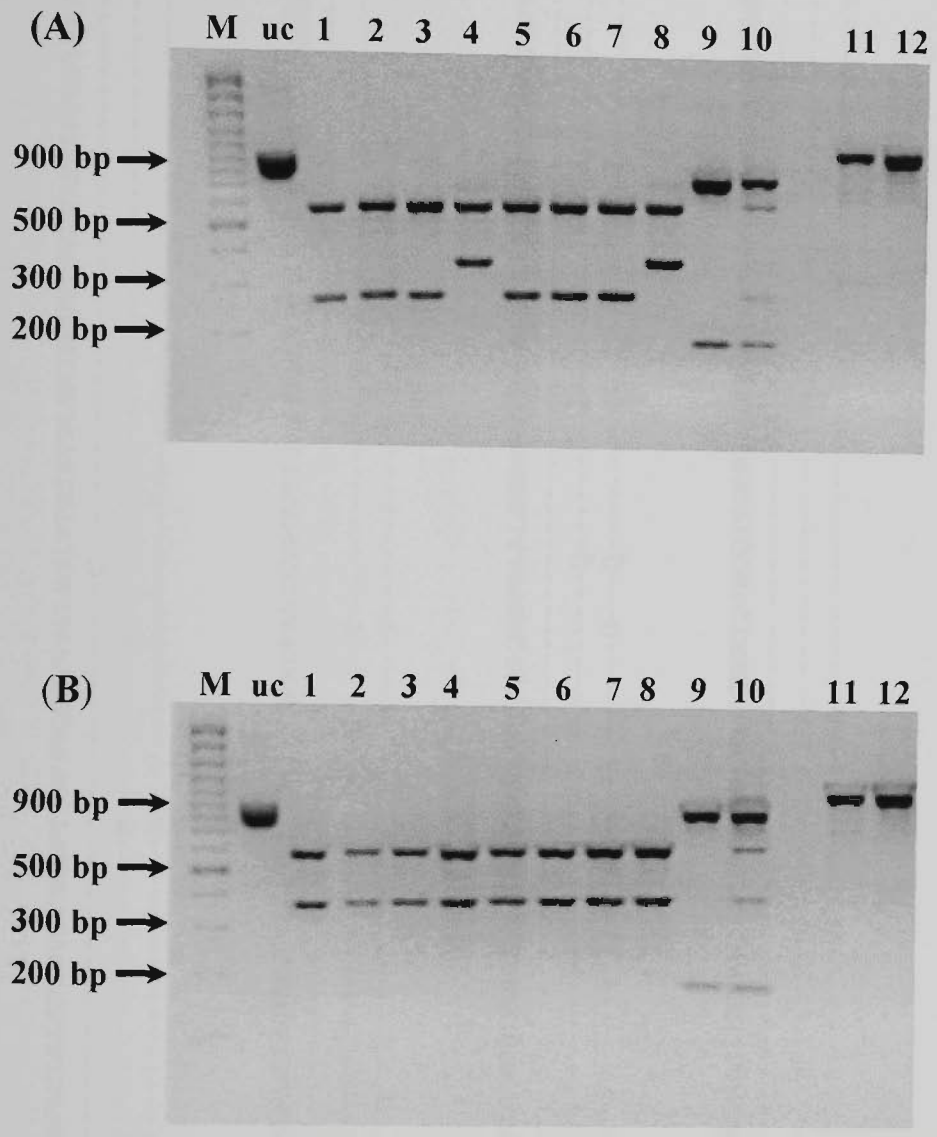


Figure 3.4 *Hinc*II (A) and *Acc*I (B) digests of PCR product obtained with Lin F and Lin R primers. Lanes: M, 100 bp plus marker; uc, undigested PCR product; 1, O121:H19 (human); 2, O145:H- (human); 3, O7:H- (human); 4, O145:H- (human); 5, O26:H- (human); 6, O121:H19 (human); 7, O26:H- (human); 8, O8:H14 (human); 9, O26:H11 (negative control, human isolate with stx_1 only, and the restriction pattern distinguishes stx_1 from stx_2 profiles); 10, O111:H8 (positive stx_2 control, human isolate with both stx_1 and stx_2); 11, O91:H- (ovine); 12, O5:H- (ovine).

A SUBUNIT

```
Stx2d-Ount (This study)  MKCILLKWVLCLLLGFSSVSYSREFTIDFSTQQSYVSSLNSIRTEISTPLEHISQGTTSVSVINHTPPGSYFAVDIRGLD
Stx2d-Ount (AF043627)    ----------------------------------------C-----------------------------------
Stx2d-Ox3a (X65949)      -----F------------------M-----------------------------------------------------
Stx2d-O111 (L11078)      -----F------------------M-----------------------------------------------------

Stx2d-Ount (This study)  VYQARFDHLRLIIEQNNLYVAGFVNTATNTFYRFSDFAHISVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSS
Stx2d-Ount (AF043627)    ------------------------------------------------------------------------------
Stx2d-Ox3a (X65949)      ----------------------------------T-------------------------------------------
Stx2d-O111 (L11078)      ----------------------------------T-------------------------------------------

Stx2d-Ount (This study)  YLALMEFSGNAMTRDASRAVLRFVTVTAEALRFRQIQREFRLALSETAPVYTMTPEEVDLTLNWGRISNVLPEFRGEGGV
Stx2d-Ount (AF043627)    ------------------------------------------------------------------------------
Stx2d-Ox3a (X65949)      ----------T-------------------------------Q-------------------------------------
Stx2d-O111 (L11078)      ----------T-------------------------G---Q-------------------------------------

Stx2d-Ount (This study)  RVGRISFNNISAILGTVAVILNCHHQGARSVRSVNEEIQPECQITGDRPVIRLNNTLWESNTAAAFLNRRAHSLNTSGE
Stx2d-Ount (AF043627)    ------------------------------------------------------------------------------
Stx2d-Ox3a (X65949)      -------------------------------A-------------------I-------------------------
Stx2d-O111 (L11078)      -------------------------------A-------------------I-------------------------
```

B SUBUNIT

```
Stx2d-Ount (This study)  MKKIFVAALFAFVSVNAMAADCAKGKIEFSKYNENDTFTVKVAGKEYWTNRWNLQPLLQSAQLTGMTVTIKSNTCASGSG
Stx2d-Ount (AF043627)    ----------------------------------------------------------------------------
Stx2d-Ox3a (X65949)      ----------------------P-----------------------------------------------------
Stx2d-O111 (L11078)      ----------------------------------------------------------------------------


Stx2d-Ount (This study)  FAEVQFN
Stx2d-Ount (AF043627)    -------
Stx2d-Ox3a (X65949)      -------
Stx2d-O111 (L11078)      -------
```

Figure 3.5 Comparison of the deduced amino acid sequences of the A and B subunits of Stx2d-Ount from an ovine source (this study) and published sequences for Stx2d-Ount (Pierard *et al.*, 1998), Stx2d-OX3a (Paton *et al.*, 1992) and Stx2d-O111 (Paton *et al.*, 1993). The accession numbers for the published sequences are given in brackets. Amino acids are represented by single letter code. The dashes (-) denote amino acids identical to that of ovine Stx2d-Ount amino acid sequence from this study.

3.4 DISCUSSION

Although STEC may contain at least four well-characterised virulence factors (Shiga toxins 1 and 2, intimin and enterohaemolysin), Stx2 is considered the most important factor affecting human health (Ostroff *et al.*, 1989; Boerlin *et al.*, 1999; Yoshimura *et al.*, 2000). In this study the stx_2 subtypes of 146 STEC from sheep and 21 isolates from humans were determined. stx_{2d} variants were most predominant among ovine isolates (141 of 146; 96.6%). Of these, 119 were $stx_{2d\text{-}Ount}$ positive, which was found in association with serotypes; O75:H-/H8/H40, O91:H-, O123:H-, O128:H2/H-, OR:H2 and O153:H25/H-. $stx_{2d\text{-}O111/OX3a}$ subtypes were found in the remaining 22 ovine isolates of serotypes O5:H-, O91:H-, O91:H2 and O123:H-. The four ovine isolates with O157:H-/H21 possessed a stx_{2vha} subtype and the single ovine O5:H- isolate possessed a stx_2 subtype.

Of the 21 human STEC, 11 possessed serotypes commonly associated with STEC derived from ovine faeces (Djordjevic *et al.*, 2001). Nine STEC isolates (six with serotype O128:H2, two O91:H-, and one O123:H-) were recovered from seven patients with diarrhoea and two asymptomatic carriers and possessed an $stx_{2d\text{-}Ount}$ subtype. Furthermore, isolates OX3:H8 (Switzerland) and O5:H- (Australia) were each recovered from HUS patients and possessed $stx_{2d\text{-}Ount}$ and $stx_{2d\text{-}OX3a}$/$stx_{2d\text{-}O111}$ subtypes respectively. The O5:H- isolate from the HUS patient is genetically indistinguishable from several epidemiologically unrelated O5:H- isolates recovered from sheep by pulse field gel electrophoresis (Starr *et al.*, 1998). Collectively these observations suggest this isolate had an ovine origin.

Ten isolates from humans were of serotypes not commonly associated with sheep (O7:H-, O8:H14, O26:H-, O91:H10, O91:H21, O121:H19 and O145:H-). These were recovered from patients with symptoms ranging from diarrhoea to HUS and also included an isolate from a symptomless carrier. All these isolates possessed stx_2 and stx_{2vhb} subtypes and one isolate (O91:H21) contained two subtypes stx_2 and stx_{2vhb}. However, it is important to emphasise that none of the isolates from humans from Switzerland possessed a serotype representative of the vast majority of isolates recovered from ovine sources. These data are consistent with studies by Pierard *et al.* (1998) showing that STEC normally associated with human disease (serogroups O157, O111, O26, O103 and O145) do not possess a stx_{2d} subtype and that stx_{2d}-positive isolates are less frequently associated with HUS. These and previous studies reinforce the hypothesis that certain serotypes of STEC seem to be associated with their animal host species (Montenegro *et al.*, 1990; Beutin *et al.*, 1993; Djordjevic *et al.*, 2001). Furthermore, stx_{2d} subtypes are rarely observed among STEC recovered from bovine sources in Australia (Brett *et al.*, 2003). Collectively, these results are consistent with the observation that different stx_2 subtypes associate with certain serotypes and these data have significant ramifications in epidemiological studies of STEC infections. These observations also suggest that lambdoid phages carrying different stx_2 subtypes lysogenise distinct *E. coli* populations, which may be determined by their serotype.

Vero cell assays of ovine isolates possessing stx_{2d} subtypes are generally toxigenic with titres down to 10^{-7} (Djordjevic *et al.*, 2001). This study did not determine the contribution of Stx1 toxin (which is present in almost all sheep isolates used in this study) to Vero cell

toxicity. However, Paton *et al.* (1993; 1992) reported a low cytoxicity to Vero cells for the two stx_{2d} variants ($stx_{2d\text{-}O111}$ and $stx_{2d\text{-}OX3a}$) as did Pierard *et al.* (1998) for the single isolate tested in that study. Pierard *et al.* (1998) suggested that Stx2d producing strains may be a marker for less pathogenic STEC since they often failed to possess associated virulence factors. *eae* gene was not observed among any of the ovine STEC isolates that possessed stx_{2d} in this study. This result is consistent with observations of Pierard *et al.* (1998) who failed to observe *eae* among the 65 isolates displaying the stx_{2d} variant genes. However, in contrast to Pierard *et al.* (1998), 141 of 146 isolates recovered from ovine sources possessed the *ehxA* gene. These data suggest that further studies need to be carried out to determine the pathogenicity of STEC of ovine origin to humans.

Chapter 4

Development of a universal intimin typing scheme for *Escherichia coli* and the characterisation of intimin from *E. coli* isolated from ovine and human sources

4 – Development of a universal intimin typing scheme for *Escherichia coli* and the characterisation of intimin from *E. coli* isolated from ovine and human sources

4.1 INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) represent two pathovars of the diarrhoeagenic *E. coli* (Nataro and Kaper, 1998) that are commonly recovered from the faeces of food-producing animals and that pose potential threats to the health of humans and livestock (Pearson *et al.*, 1989; Dean-Nystrom *et al.*, 1997; Nataro and Kaper, 1998; O'Loughlin and Robbins-Browne, 2001). EPEC are a common cause of diarrhoea especially among infants in the developing world and EHEC represent a subset of STEC recovered from patients with serious gastro-intestinal and systemic diseases such as HC and HUS (Paton and Paton 1998; O'Loughlin and Robbins-Browne, 2001).

Unlike other diarrhoeagenic *E. coli*, EPEC and STEC share the ability to induce the formation of a characteristic histological feature known as an attaching and effacing (A/E) lesion. A/E lesions are characterised by localised destruction of brush border microvilli and the formation of polymerised actin pedestals beneath the intimately adhering bacteria (Knutton *et al*, 1995; Frankel *et al*, 1998a). *eae* was the first gene to be associated with A/E activity and encodes the bacterial adhesin known as intimin (Jerse *et al.*, 1990). Pathogenicity islands known as the loci of enterocyte effacement (LEE) govern the formation of A/E lesions (McDaniel *et al.*, 1995) and the number of genes within these islands varies considerably among strains of EPEC and STEC (Perna *et al.*, 1998; Elliot *et*

al., 1998), The island comprises five polycistronic operons known as *LEE*1, *LEE*2, *LEE*3, *tir* and *LEE*4 (Mellies *et al.*, 1999). Components of a type III secretion system are encoded by *LEE*1, *LEE*2 and *LEE*3, the *tir* operon encodes translocated intimin receptor (Tir) (Deibel *et al.*, 1998; Kenny *et al.*, 1997), intimin (Jerse and Kaper 1991; Jerse *et al.*, 1990) and the Tir chaperone CesT (Abe *et al.*, 1999; Elliot *et al.*, 1999), and *LEE*4 encodes several secreted proteins (EspA, EspB and EspD) involved in epithelial cell signal transduction (Kenny *et al*, 1996; Lai *et al*, 1997; Elliot *et al.*, 2001, Frankel *et al.*, 2001). LEE islands are not restricted to EPEC and STEC and have been reported in other coliforms including *Citrobacter rodentium* (formerly *C. freundii*) and *Escherichia alvei* (formerly *Hafnia alvei*) (Kaper, 1998a, Janda *et al.*, 1999).

Intimin consists of a conserved N-terminal region and a variable C-terminal region (McGraw *et al.*, 1999; Frankel *et al.*, 2001). The receptor-binding domain of the intimin molecule is localised to the C-terminal 280 amino acids (Int_{280}) (Frankel *et al.*, 1994; Frankel *et al.*, 1995). Furthermore, based on sequence variation within Int_{280}, six distinct intimin subtypes designated α, β, γ, δ, ε and θ have been identified (Adu-Bobie *et al.*, 1998; Oswald *et al.*, 2000; Tarr and Whittam, 2002). Int-α has been divided into two subtypes α1 and α2 due to different restriction fragment length polymorphism (RFLP) profiles exhibited by the strains harbouring Int-α (Oswald *et al.*, 2000). Further, five intimin alleles designated Int-ζ (accession no. AJ298279) (J. Jores, K. Zehmke, L. Roumer and L. Wieler, unpublished data), Int-ι (accession no. AJ308551) (W. L. Zhang, H. Karch and H. Schmidt, unpublished data), Int-η (accession no AJ271407) (H. Schmidt, H. Karch, E. Oswald), Int-λ (accession no. AF439538) (J. Pan, unpublished data) and Int-κ (accession no. AJ308552)

(W. L. Zhang, H. Karch and H. Schmidt, unpublished data) have been submitted to the GenBank database. However, comparison of the Int_{280} regions of Int-κ with Int-δ reveal 99.6% sequence similarity and therefore are referred to as Int-δ in this study. Further, comparison of the Int_{280} region of Int-η with Int-ζ reveal 100% amino acid sequence homology and therefore is cited as Int-ζ in this study. Interestingly, the different intimin subtypes were found to be preferentially associated with specific EPEC and STEC serotypes and the phylogeny of the strains (Adu-Bobie *et al.*, 1998; McGraw *et al.*, 1999; Oswald *et al.*, 2000; Reid *et al.*, 2000; Tarr and Whittam, 2002).

The presence of intimin is essential for colonisation in newborn piglets, calves and adult cattle (Donnenberg *et al.*, 1993; Dean-Nystrom *et al.*, 1997; Dean-Nystrom *et al.*, 1998; Phillips *et al.*, 2000). Furthermore, *eae*-ve EPEC and STEC strains are unable to colonise any region of the mucosa when inoculated onto human intestinal *in vitro* organ cultures (IVOC)(Hicks *et al*, 1998; Phillips and Frankel, 2000; Fitzhenry *et al*, 2002). In addition to a role in A/E lesion formation, intimin may play an important role in tissue tropism. O157:H7 typically expresses Int-γ and preferentially generates large intestinal lesions in a gnotobiotic piglet model (Tzipori *et al.*, 1995). However, O157:H7 engineered to express Int-α produces A/E lesions on both the small and large intestines of gnotobiotic piglets (Tzipori *et al.*, 1995). Using IVOC, O157:H7 preferentially binds to and induces A/E lesions on human follicle-associated epithelium of Peyer's patches whereas EPEC strains expressing Int-α typically colonise explants derived from the small but not the large intestine (Phillips *et al.*, 2000; Phillips and Frankel, 2000). Intimin exchange studies using IVOC cultured with *eae*-ve EPEC strain CVD206 carrying a recombinant plasmid

expressing Int-γ bound preferentially to the Peyer's patch mucosa (Phillips and Frankel, 2000). Similarly, EHEC O157:H7 engineered to express Int-α produced A/E lesions on both Peyer's patch mucosa and small intestinal explants (Fitzhenry *et al.*, 2002). Intimin has also been reported to bind to β-integrins and perhaps another host cell receptor(s) in the absence of Tir and these interactions may play an important role in tissue tropism (Hartland *et al.*, 1999; Phillips and Frankel, 2000; Diebel *et al.*, 2001).

There is a paucity of information describing intimin types from *E. coli* recovered from cattle and sheep, two meat producing species that represent major reservoirs of *E. coli* that enter the human food chain. Several intimin subtyping schemes have been described (Adu-Bobie *et al.*, 1998; Reid *et al.*, 1999; Oswald *et al.*, 2000), however a universal scheme capable of subtyping these ten intimin subtypes (α1, α2, β, γ, θ, δ, ε, ζ, ι, λ) has not been described. This study describes a PCR and restriction fragment length polymorphism (RFLP) assay that is capable of reliably identifying these ten intimin subtypes. Further, this assay was applied to characterise intimin from 153 STEC and non-STEC isolates representing 58 different serotypes derived predominantly from sheep and humans. This study also describe the nucleotide sequence of two previously undescribed intimin subtypes identified here as ε2 and ι2 in *E. coli* isolates with serotypes O2-related:H19 (CNC155, ovine) and OR:H- (NC38, ovine) respectively. Phylip program was utilised to compare these genes with intimin sequences deposited in GenBank to examine the phylogenic relationships of the intimin gene family.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial Strains

One hundred and fifty three *E. coli* isolates were used in this study (Tables 4.1 and 4.2). The Elizabeth Macarthur Agricultural Institute (New South Wales, Australia) provided 105 *E. coli* isolates recovered from healthy sheep, which were isolated using methods described by Djordjevic *et al.* (2001) (refer to section 2.2.1). Thirty five isolates from humans were obtained from the Microbiological Diagnostic Unit (MDU) (Melbourne, Australia). These consisted of 28 isolates from patients with HUS, bloody diarrhoea, infantile diarrhoea, infantile gastroenteritis or diarrhoea, 6 isolates from healthy babies and 1 isolate from a human with an unknown history. Andre Burnens from the National Reference Laboratory for Food Borne Diseases (Bern, Switzerland) kindly provided 13 isolates from patients with diarrhoea or HUS (Burnens *et al*, 1992; Essers *et al.*, 2000). The Swiss isolates were serotyped by Kim Ziebel and Roger Johnson from the Guelph Laboratory, Health Canada, Guelph, Ontario, Canada. Dr. Karl Bettelheim from MDU, Victoria, Australia, serotyped all other isolates.

4.2.2 Multiplex PCR analysis of *E. coli* isolates

DNA from isolates not investigated in Chapters 2 and 3 (refer to Appendix A) was prepared and subjected to multiplex PCR for the detection of STEC virulence factors stx_1, stx_2, *ehxA* and *eae* as described by Paton and Paton (1998) (refer to section 2.2.3), except that InstaGene matrix (Bio-Rad, Richmond, Calif.) was used for the preparation of template DNA as described previously by Fagan *et al.* (1999) (refer to section 2.2.2). Amplified DNA fragments were resolved by gel electrophoresis (Sambrook *et al.*, 1989) using 2%

Table 4.1 Distribution of Shiga toxin and *ehxA* genes and intimin subtypes among 153 *eae*-containing *E. coli* strains.

Serotype	Source [a]	Total no. of isolates	PCR Results				*eaeA* subtype
			stx$_1$	*stx*$_2$	*eae*	*ehxA*	
O2-related:H19	Ovine (E)	1	-	-	+	-	Int-ε2
O3:H8	Ovine (E)	1	-	-	+	-	Int-θ
O5:H-	Human, New Zealand, HUS (V)	1	+	-	+	+	Int-β
O5:H11	Ovine (E)	3	-	-	+	+	Int-β
O5:H11	Ovine (E)	1	-	-	+	+	Int-ζ
O5:Hnt	Ovine (E)	1	-	-	+	-	NAP[b]
O5:HR	Ovine (E)	1	+	+	+	+	NAP
O7:H-	Human, HUS (S)	1	-	+	+	+	Int-β
O7:H8	Ovine (E)	1	-	-	+	-	NAP
O15:H-	Human, HUS (S)	1	-	-	+	-	Int-β
O15:H2	Human, Australia, Healthy Baby (V)	1	-	-	+	-	Int-β
O15:H2	Ovine (E)	1	-	-	+	+	Int-ε1
O26:H-	Human, Australia, Bloody Diarrhea (V)	1	+	-	+	+	Int-β
O26:H-	Human, Australia, Diarrhea (V)	2	+	-	+	+	Int-β
O26:H-	Human, England, Diarrhea (V)	2	-	-	+	-	Int-β
O26:H-	Human, Denmark, Diarrhea (V)	1	-	-	+	-	Int-β
O26:H-	Human, HUS (S)	2	-	+	+	-	Int-β
O26:H-	Human, UK, Infantile Diarrhea (V)	2	-	-	+	-	Int-β
O26:H-	Ovine (E)	2	+	-	+	+	Int-β
O26:H11	Human, Diarrhea (S)	1	+	-	+	+	Int-β
O26:H11	Human, Diarrhea (S)	1	+	-	+	-	Int-β

Table 4.1 Distribution of Shiga toxin and *ehxA* genes and intimin subtypes among 153 *eae*-containing *E. coli* strains.

Serotype	Source [a]	Total no. of isolates	PCR Results				*eaeA* subtype
			stx$_1$	*stx*$_2$	*eae*	*ehxA*	
O26:H11	Human, Australia, Bloody Diarrhea (V)	2	+	-	+	-	Int-β
O26:H11	Human, Australia, Bloody Diarrhea (V)	3	+	-	+	+	Int-β
O26:H11	Human, Australia, Diarrhea (V)	2	+	-	+	+	Int-β
O26:H11	Human, Canada, Diarrhea (V)	1	+	-	+	-	Int-β
O26:H11	Ovine (E)	5	+	-	+	+	Int-β
O26:H11	Ovine (E)	4	-	-	+	+	Int-β
O28:H31	Ovine (E)	1	-	-	+	-	Int-ζ
O37:H-	Ovine (E)	3	-	-	+	-	Int-δ
O37:H10	Ovine (E)	1	+	+	+	-	NAP
O55:H6	Human, UK, Infantile Diarrhea (V)	1	-	-	+	-	Int-α1
O55:H12	Ovine (E)	1	-	-	+	-	NAP
O55:H20	Ovine (E)	1	+	+	+	+	NAP
O69:H8	Ovine (E)	1	-	-	+	-	NAP
O77:H12	Ovine (E)	2	-	-	+	-	NAP
O84:H-	Ovine (E)	2	+	-	+	+	Int-ζ
O84:H2	Ovine (E)	2	-	-	+	+	Int-ζ
O84:H25	Ovine (E)	2	-	-	+	+	Int-θ
O85:H49	Ovine (E)	4	-	-	+	-	Int-α1
O85:H49	Ovine (E)	1	-	+	+	-	Int-α1
O86:H-	Human, UK, Infantile Diarrhea (V)	1	-	-	+	-	Int-δ
O88:H8	Ovine (E)	1	+	+	+	-	NAP
O98:H-	Ovine (E)	1	-	-	+	+	Int-γ

Table 4.1 Distribution of Shiga toxin and *ehxA* genes and intimin subtypes among 153 *eae*-containing *E. coli* strains.

Serotype	Source [a]	Total no. of isolates	PCR Results				*eaeA* subtype
			stx$_1$	*stx*$_2$	*eae*	*ehxA*	
O103:H2	Human, Diarrhea (S)	1	+	-	+	+	Int-ε1
O103:H2	Human, Diarrhea (S)	1	+	-	+	-	Int-ε1
O103:H2	Ovine (E)	2	+	-	+	+	Int-ε1
O103:H2	Ovine (E)	7	-	-	+	+	Int-ε1
O103:H2	Ovine (E)	1	-	-	+	+	Int-θ
O106:HR	Ovine (E)	1	+	-	+	-	NAP
O106:HR	Ovine (E)	1	-	-	+	-	NAP
O109:H-	Ovine (E)	4	-	-	+	+	Int-β
O111:H-	Human, UK, Infantile Diarrhea (V)	2	-	-	+	-	Int-β
O112ab:H2	Ovine (E)	1	+	+	+	+	NAP
O118:H16	Human, Diarrhea (S)	1	+	-	+	+	Int-β
O121:H19	Human, Diarrhea (S)	1	-	+	+	-	Int-ε1
O121:H19	Human, HUS (S)	1	-	+	+	+	Int-ε1
O121:H19	Ovine (E)	2	-	-	+	+	Int-ε1
O125:H6	Human, Australia, Healthy Baby (V)	1	-	-	+	-	Int-α2
O126:H2	Human, UK, Infantile Diarrhea (V)	1	-	-	+	-	Int-β
O127:H-	Human, UK, Infantile Diarrhea (V)	1	-	-	+	-	Int-α1
O127:H-	Human, USA, Infantile Diarrhea (V)	1	-	-	+	-	Int-α1
O128:H2	Human, Australia, Healthy Baby (V)	1	-	-	+	-	Int-β
O128:H2	Human, England, Infantile Gastroenteritis (V)	1	-	-	+	-	Int-β
O128:H2	Human, UK, Infantile Diarrhea (V)	2	-	-	+	-	Int-β

Table 4.1 Distribution of Shiga toxin and *ehxA* genes and intimin subtypes among 153 *eae*-containing *E. coli* strains.

Serotype	Source [a]	Total no. of isolates	PCR Results				*eaeA* subtype
			stx$_1$	*stx*$_2$	*eae*	*ehxA*	
O145:H-	Human, HUS (S)	1	-	+	+	-	Int-γ
O145:H-	Human, Diarrhea (S)	1	-	+	+	-	Int-γ
O145:H-	Ovine (E)	2	-	-	+	+	Int-β
O153:H11/21	Human, Australia, Healthy Baby (V)	1	-	-	+	-	Int-θ
O156:H1	Human, Australia, Healthy Baby (V)	1	-	-	+	-	Int-ζ
O157:H-	Human, Australia, HUS (V)	1	-	+	+	+	Int-γ
O157:H-	Ovine (E)	3	+	+	+	+	Int-γ
O157:H-	Ovine (E)	1	-	+	+	-	Int-γ
O157:H-	Ovine (E)	1	+	+	+	-	Int-γ
O157:H-	Ovine (E)	1	-	+	+	+	Int-γ
O157:H11	Ovine (E)	4	-	-	+	+	Int-β
O157:H21	Ovine (E)	1	+	+	+	+	Int-γ
O158:HR	Ovine (E)	1	+	-	+	+	NAP
O158:HR	Ovine (E)	1	-	-	+	-	NAP
O162:H10	Ovine (E)	1	-	-	+	-	NAP
O166:H49	Ovine (E)	1	-	-	+	+	Int-ε1
O172:H1	Ovine (E)	1	-	-	+	+	Int-γ
Ont:H-	Human, New Zealand (V)	1	+	-	+	+	Int-ζ
Ont:H-	Ovine (E)	1	-	-	+	+	Int-β
Ont:H6	Ovine (E)	1	-	-	+	-	NAP
Ont:H8	Ovine (E)	1	+	+	+	-	NAP
Ont:H8	Ovine (E)	2	-	-	+	-	Int-ι1

Table 4.1 Distribution of Shiga toxin and *ehxA* genes and intimin subtypes among 153 *eae*-containing *E. coli* strains.

Serotype	Source [a]	Total no. of isolates	PCR Results				*eaeA* sutype
			stx$_1$	*stx*$_2$	*eae*	*ehxA*	
Ont:H25	Ovine (E)	10	-	-	+	+	Int-ζ
Ont:H25	Ovine (E)	2	-	-	+	-	Int-ζ
Ont:H25	Ovine (E)	1	-	-	+	+	Int-θ
Ont:H49	Ovine (E)	1	+	+	+	-	NAP
Ont:HR	Ovine (E)	1	-	-	+	-	Int-δ
Ont:Hnt	Human, Australia, Healthy Baby (V)	1	+	-	+	+	Int-β
OR:H-	Ovine (E)	1	-	-	+	-	Int-ι2
OR:H-	Ovine (E)	1	+	+	+	+	Int-ε1
OR:H2	Ovine (E)	1	-	-	+	+	Int-ζ
OR:Hnt	Ovine (E)	1	-	-	+	+	Int-γ

[a] E, isolates obtained from Elizabeth Macarthur Agricultural Institute, New South Wales, Australia; V, isolates obtained from Melbourne Diagnostic Unit, Australia; S, isolates obtained from National Reference Laboratory for Food borne Diseases, Bern, Switzerland.

[b] NAP, *eae* not amplified by subtyping primers EaeVF/EaeVR/EaeZetaVR/EaeJotaVR

(wt/vol) agarose. Gels were stained with ethidium bromide (5 µl/ml), visualized with UV illumination, and imaged using a GelDoc 1000 image analysis station (Bio-Rad).

Table 4.2 Distribution of *stx*$_1$, *stx*$_2$ and *ehxA* among 153 *eae*-containing *E. coli* isolates.

Source	No. of isolates	No. (%) of animals/samples containing *E. coli* isolates positive for:							
		stx*$_1$, *eae	***stx*$_2$, *eae***	***stx*$_1$, *stx*$_2$, *eae***	***stx*$_1$, *eae*, *ehxA***	***stx*$_2$, *eae*, *ehxA***	***stx*$_1$, *stx*$_2$, *eae*, *ehxA***	***eae*, *ehxA***	***eae***
Human	48	5 (10.4)	5 (10.4)	-	14 (29.1)	3 (6.2)	-	-	21 (43.7)
Ovine	105	1 (0.9)	2 (1.9)	5 (4.7)	12 (11.4)	1 (0.9)	8 (7.6)	50 (47.6)	26 (24.7)

4.2.3 Amplification and subtyping of the *eae* gene by PCR-RFLP analysis

The forward primer EaeVF and three reverse primers EaeVR, EaeZetaVR and EaeJotaVR (Sigma Genosys, St Louise, Mo) (Table 4.3) were designed in this study to specifically amplify an approximately 834-876 bp fragment (fragment size varies depending on the variant amplified) representing the 3' variable region (encoding the C-terminal Int_{280} amino acids) of all reported intimin variants. InstaGene DNA preparations (5 µl) (refer section 2.2.2) were each amplified in a reaction mixture that contained 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 2 U of Taq DNA polymerase and 50 pmol of each primer. The reaction volume was made up to 50 µl with distilled H_2O. Thermal cycling conditions used to amplify this region of the intimin variants is shown in Table 4.3. A final extension cycle was performed at 72°C for 5 min. Amplified DNA fragment was resolved by agarose gel electrophoresis as described above.

Table 4.3 Primers used for amplifying and sequencing intimin subtypes

Primer	Sequence (5' to 3')	Orientation	Product size (bp)	PCR cycle number and cycling conditions	Reference
EaeVF	AGYATTACTGAGATTAAG	Forward			This study
EaeVR	AAATTATTYTACACARAY	Reverse	840 – 880[a]	35 (94°C, 60 s; 41°C, 60 s; 72°C, 60 s)	This study
EaeZetaVR	AGTTTATTTTACGCAAGT	Reverse	840 – 880[a]		This study
EaeJotaVR	TTAAATTATTTTATGCAAAC	Reverse	840 – 880[a]		This study
EaeUniVF	ACTCATTGTGGTGGAGC	Forward	434[b]	35 (94°C, 50 s; 52°C, 60 s; 72°C, 45 s)	This study
PatonR	CCACCTGCAGCAACAAGAGG	Reverse			Paton *et al.*, 1998
Reid F	CTGAACGGCGATTACGCGAA	Forward	917[c]	30 (94°C, 60 s; 53°C, 120 s; 72°C, 180s)	Reid *et al.*,
Reid R	CCAGACGATACGATCCAG	Reverse			1999
Gannon F	GTGGCGAATACTGGCGAGACT	Forward	890[d]	35 (94°C, 60 s; 58°C, 60 s; 72°C, 120 s)	Gannon *et*
Gannon R	CCCCATTCTTTTTCACCGTCG	Reverse			*al.*, 1997
EaeVRF1	CACCTGGTCAGCAGA	Forward	331[e]	30 (94°C, 20 s; 62°C, 30 s; 72°C, 20 s)	This study
EaeVRR1	ACCTCTGCCGTTCCAT	Reverse			This study
EaeVRF2	AACAATGTACAGCTCACTAT	Forward	543[f]	30 (94°C, 30 s; 56°C, 40 s; 72°C, 30 s)	This study
EaeVRR2	TACCGAGGCAAGACCATC	Reverse			This study
Eae64/4VRF1	CGCAGTACGCAGAAGATT	Forward	793[g]	30 (94°C, 30 s; 60°C, 40 s; 72°C, 30 s)	This study
Eae64/4VRR1	CCGAGCCAGATGTCAGTT	Reverse			This study

Table 4.3 Primers used for amplifying and sequencing intimin subtypes

Primer	Sequence (5' to 3')	Orientation	Product size (bp)	PCR cycle number and cycling conditions	Reference
H41VRF2	ATTACCGTTCTGTCGAAT	Forward	439[h]	30 (94°C, 45 s; 55°C, 40 s; 72°C, 45 s)	This study
H41VRR1	ATACCGGCTGACCATT	Reverse			This study

[a] Primers used to amplify and sequence the 3' end of intimin variants; the size of the amplicon varied depending on the *eae* gene variant.
[b-e] Primers used to sequence the conserved 5' region of all intimin gene variants.
[f] Primers used to sequence intimin ι2; location within gene, 1618-2160 bp
[g] Primers used to sequence intimin ε2; location within gene, 1399-2191 bp
[h] Primers used to sequence intimin α2; location within gene, 1635-2074 bp

Alignment of Int_{280} nucleotide sequences using the PileUp program (www.angis.org.au) representing all known subtypes enabled to select restriction endonucleases which were predicted by computational analyses (Mapplot program; www.angis.org.au) to be capable of differentiating the known intimin types. PCR products (10 µl) generated using the primer cocktail EaeVF, EaeVR, EaeZetaVR and EaeJotaVR were incubated with 3 U of restriction enzymes (RE) *Alu*I and *Rsa*I (Progen) in the buffer provided by the manufacturer for a minimum of 4 hours at 37°C. Restriction fragments were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Intimin subtypes were identified according to their RE profiles (Table 4.4).

Table 4.4 Predicted sizes of restriction fragments used for RFLP analysis of *eaeA*

Intimin type	Restriction enzyme used and expected fragment size(s) (bp)	
	***Alu*I**	***Rsa*I**
Int-α1	736, 113	725, 84[a], 40[a]
Int-α2	375, 334, 133, 7[a]	590, 135, 84, 40[a]
Int-β	475, 374	528, 246, 75[a]
Int-γ	834 (Uncut)	432, 402
Int-θ	527, 165, 110, 21[a], 14[a]	405, 354, 78[a]
Int-δ	342, 214, 162, 131	231, 201, 159, 120, 84[a], 54[a]
Int-ε1	384, 270, 190, 32[a]	774, 102
Int-ε2	844, 32[a]	774, 102
Int-ζ	605, 203, 38[a]	345, 279, 135, 87[a]
Int-ι1	602, 241	525, 318
Int-ι2	824, 19[a]	525, 318
Int-λ	232, 214, 204, 151, 45[a]	441, 318, 87[a]

[a] Fragment was too small to visualize under the electrophoresis conditions used.

4.2.4 Southern hybridisation analysis

Intimin probe was generated by PCR of genomic DNA extracted by InstaGene matrix (Fagan *et al.*, 1999) from a bovine O157:H7 STEC isolate. Primers eaeF (5' GACCCGGCACAAGCATAAGC 3') and eaeR (5' CCACCTGCAGCAACAAGAGG 3') (Sigma) previously described by Paton and Paton. (1998) were utilised in the PCR to yield a 384 bp fragment of the *eae* gene. PCR was carried out in a 50 µl volume with 2 µl of InstaGene matrix DNA preparation, 200 µM concentration of DIG-labelled dNTP (Roche, Mannheim, Germany), 250 nM concentration of each primer, 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 2 mM $MgCl_2$, 1 U of Taq DNA polymerase and distilled H_2O to volume. Samples were subjected to 35 PCR cycles, each consisting of 1 min of denaturation at 95°C; 2 min of annealing at 60°C; and 1 min of elongation at 72°C. The PCR product was purified using the QIAquick DNA purification kit (Qiagen, Hilden, Germany).

Intimin from the isolates that did not amplify a visible product using the primer cocktail EaeVF, EaeVR, EaeZetaVR and EaeJotaVR were amplified using the primers eaeF and eaeR using the PCR conditions described above. PCR products were electrophoresed on a 0.8% agarose gel. The gel was acid treated, denatured, neutralized and blotted to Hybond-N+ membrane (Amersham). After UV cross-linking, the membrane was prehybridised for 2 h at 58°C in EasyHyb solution (Roche) prior to the addition of probe. Hybridisation with the intimin probe was for 18 h at 42°C and was followed by three washes in 2x standard saline citrate with 0.1% SDS (15 min per wash). Following the washes the membrane was blocked in blocking buffer (Roche) for 1 h and incubated with anti-DIG antibody solution for 30 min. Prior to the addition of detection buffer (Roche) the membrane was washed

twice with wash buffer (Roche) (15 min per wash). The chemiluminescent substrate CSPD (Roche) was added to the membrane along with the detection buffer. The membrane was then analysed by exposing photographic film (Hyperfilm-ECL, Amersham) to the membrane for varying periods of time. The photographic film was then developed by placing it in Kodak GBX developer for 2 min prior to washing with water. The film was then transferred to Kodak GBX fixer until it appeared transparent. Finally the film was washed in water before being allowed to air dry.

4.2.5 DNA sequencing of *eae* genes

Complete nucleotide sequences of *eae* from two *E. coli* isolates with serotypes O2-related:H19 and OR:H- was determined since their respective RFLP profiles did not match with any of the reported intimin variants. DNA sequencing was also undertaken for the *eae* gene from a human *E. coli* isolate with serotype O125:H6 possessing Int-α2 since no prototype sequence was available in the public databases for this subtype. The strategy used to sequence these intimin genes involved the amplification of the 3' region of the genes spanning nucleotides 1975 to 2805-2847 (fragment sizes vary depending on the variant gene amplified) which encoded the C-terminal variable region known as Int_{280}. These same primers (Table 4.3) were also used to sequence this region of the *eae* genes and the remaining sequence was obtained by primer walking. To generate sequencing templates spanning the 5' conserved regions of intimin subtypes, previously published primers and primers generated by primer walking were used. PCR reaction mixtures contained 100-500 ng of template DNA, 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 2 U of Taq DNA polymerase, 50 pmol of each primer and reaction volume was

made up to 50 µl with distilled H_2O. Primer sequences and the cycling conditions used to generate sequencing templates are described in Table 4.3. PCR products were analysed by agarose gel electrophoresis and purified using a QIAquick DNA purification kit (Qiagen). DNA sequence reactions were performed as described in section 2.2.4. Compilation and analysis of DNA sequence data were performed as described in section 2.2.4.

4.2.6 Sequence analysis

Clustal W (Thompson *et al.*, 1994) was used to produce multiple sequence alignment of 45 inferred Int_{280} amino acid sequences, which included the 3 sequences determined in this study and 42 sequences retrieved from GenBank (refer to Appendix B). Evolutionary gene trees were then estimated using the Phylip package (http://bioweb.pasteur.fr/seqanal/phylogeny/phylip-uk.html). Pairwise distances were calculated using the "protdist" program, specifying the Dayhoff PAM matrix as the distance model. Int_{280} gene trees were then constructed using BIONJ, due to its superior performance compared with neighbour-joining, particularly when substitution rates vary among lineages (Gascuel, 1997). Bootstrap analyses were subsequently performed (1,000 replicates) to assess the relative support for the nodes in the gene tree.

4.2.7 Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been submitted to GenBank under accession numbers AF530553 to AF530555.

4.3 RESULTS

4.3.1 Prevalence of genes encoding Shiga toxins and enterohaemolysin among *eae*-containing *E. coli* by multiplex PCR

All 153 isolates contained *eae* and comprised of 58 serotypes (Tables 4.1 and 4.2). These consisted of 18 (11.7%) isolates that also contained stx_1 and/or stx_2, 38 (24.8%) isolates that contained *ehxA*, stx_1 and/or stx_2, 50 (32.6%) isolates that contained *ehxA* in combination with *eae* and 47 (30.7%) isolates contained *eae* alone. Of 48 isolates from humans 21 (43.7%) contained only *eae*, 14 (29.1%) contained stx_1, *eae* and *ehxA*, five (10.4%) contained all four genes stx_2, *eae*, stx_1 and *eae* and three (6.2%) possessed stx_2, *eae* and *ehxA*. The relationship between serotypes and virulence factor profile for all 153 isolates is shown in Table 4.1.

4.3.2 Development of a PCR-RFLP assay for subtyping *eae* genes

Primers were designed (Table 4.3) to amplify an approximately 840-880 bp of the 3' end of all known intimin subtypes (Figure 4.1). This region of the intimin gene encodes the C-terminal Int_{280} amino acids and was selected for RFLP analysis because it possesses the greatest degree of sequence variation between intimin types. An amplification product was generated for all twelve known subtypes (α1, α2, β, γ, θ, δ, ε, ζ, ι, λ) including two new subtypes (see below) designated, ι2 and ε2 (Figure 4.1; Table 4.1). Computational analyses of aligned intimin gene sequences using the mapplot program predicted that the restriction endonucleases *Alu*I and *Rsa*I could potentially differentiate all known intimin subtypes (Table 4.4). *Alu*I differentiated all 12 subtypes. However, since intimin subtypes γ, ι2 and ε2 (Figure 4.2 A, lanes 4, 10 and 11) produced bands around 824-844 bp, which were close

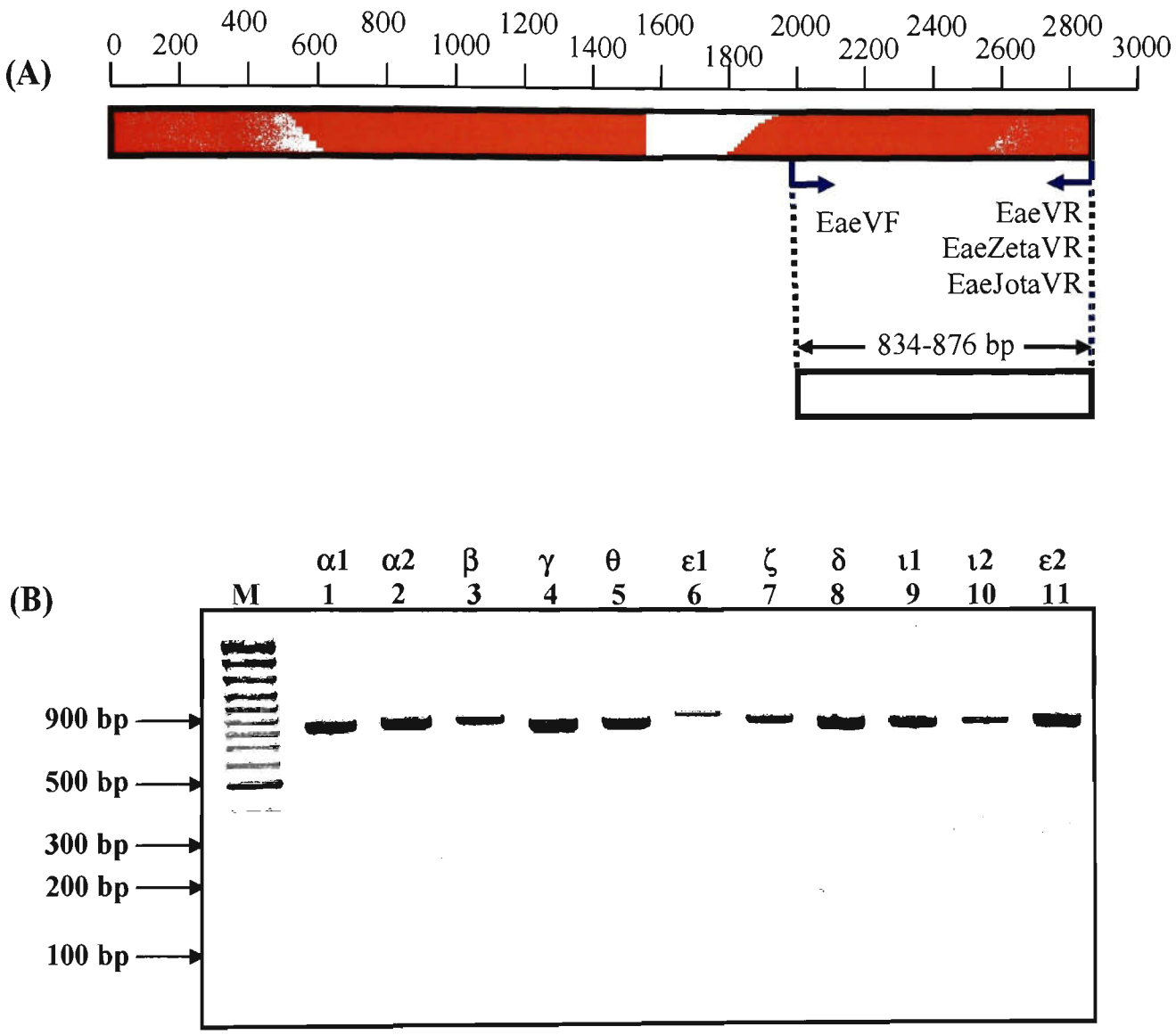


Figure 4.1 *eae* amplified with subtyping primers EaeVF, EaeVR, EaeVRZeta and EaeVRJota from *E. coli* strains representative of the different intimin types. (A) Schematic representation of amplification. The blue arrows denote the primer annealing positions and extension directions. (B) PCR products electrophoresed through 2% agarose gel. Lanes: M, 100 bp + molecular weight marker; 1, O127:H- (α1) (human); 2, O125:H6 (α2) (human); 3, O26:H11 (β1) (ovine); 4, O157:H- (γ) (ovine); 5, O3:H8 (θ) (ovine); 6, O103:H3 (ε1) (ovine); 7, O84:H2 (ζ) (ovine); 8, O37:H- (δ) (ovine); 9, Ont:H8 (ι1) (ovine); 10, OR:H- (ι2) (ovine); 11, O2-related:H19 (ε2) (ovine).

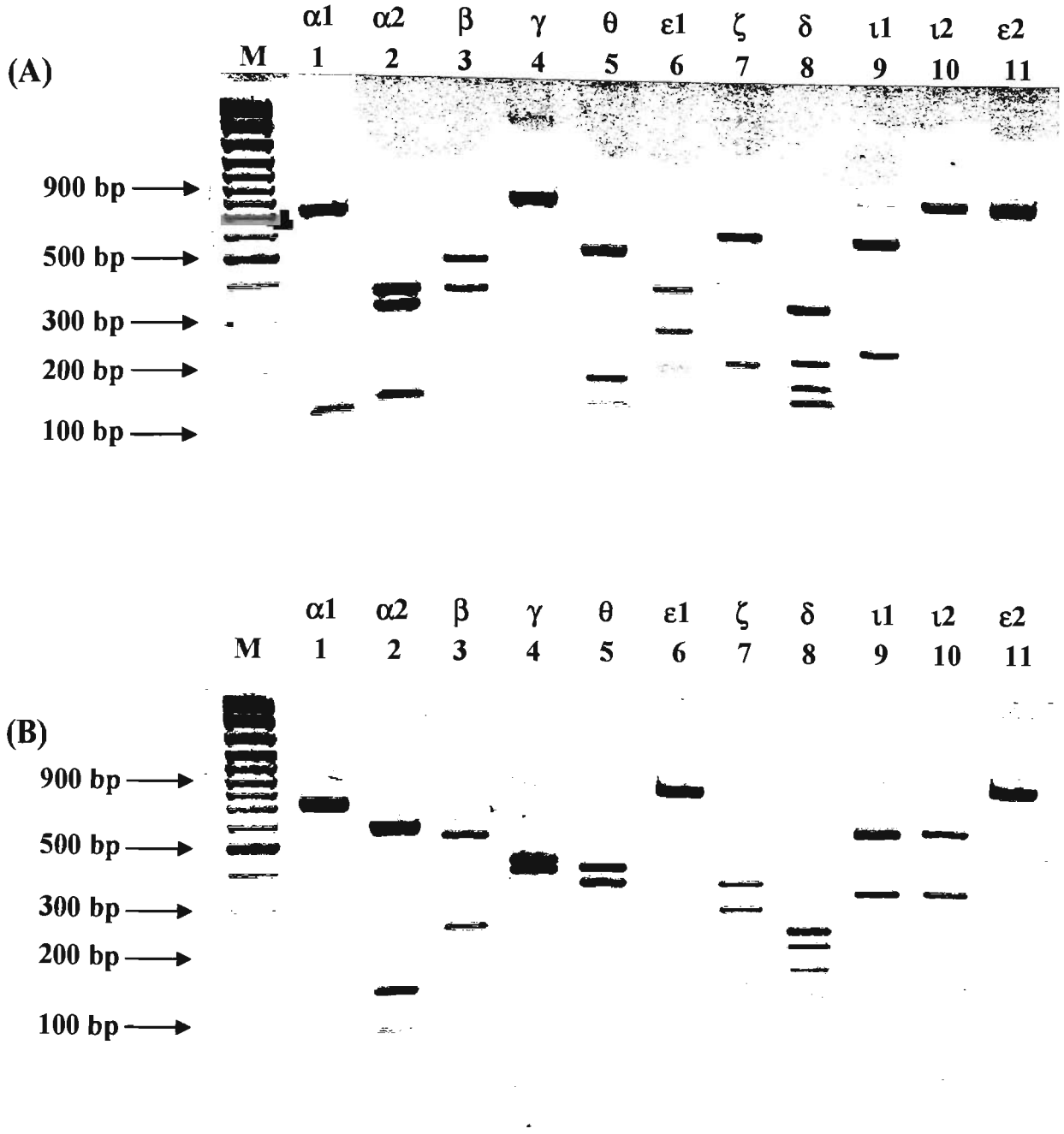


Figure 4.2 RFLP analysis of the 3' Int_{280} 840-880 bp of all known intimin subtypes using *Alu*I (A) and *Rsa*I (B). Lanes: M, 100 bp + molecular weight marker; 1, O127:H- (α1) (human); 2, O125:H6 (α2) (human); 3, O26:H11 (β1) (ovine); 4, O157:H- (γ) (ovine); 5, O3:H8 (θ) (ovine); 6, O103:H3 (ε1) (ovine); 7, O84:H2 (ζ) (ovine); 8, O37:H- (δ) (ovine); 9, Ont:H8 (ι1) (ovine); 10, OR:H- (ι2) (ovine); 11, O2 related:H19 (ε2) (ovine).

to each other, a second RE enzyme, *Rsa*I was used to facilitate better discrimination. *Rsa*I distinguished 9 of 12 intimin subtypes. *Rsa*I failed to differentiate ι1 and ι2 (Figure 4.2 B, lanes 9 and 10) and ε1 and ε2 (Figure 4.2 B, lanes 6 and 11). Therefore both enzymes were required to differentiate all 12 intimin subtypes. The intimin subtypes determined from 134 of 153 *eae* containing *E. coli* isolates are shown in Table 4.1. Representative RFLP profiles generated using these enzymes are shown in Figures 4.2 A and B.

Int-β was the most commonly identified subtype among *E. coli* isolates represented in this study (58 of 153; 37.9%) and was found associated with the greatest diversity of serotypes (n = 16). Int-ζ (21 of 153; 13.7%) was the second most common subtype and was represented among 7 serotypes. Int-ε1 was identified as the third most common subtype (18 of 153; 11.7%), followed by Int-γ (13 of 153; 8.5%) and each subtype was found in association with five and six serotypes respectively. Int-θ was identified among 6 isolates representing five different serotypes. Int-δ (five isolates), Int-α1 (three isolates), Int-α2 (one isolate) and Int-ι1 (two isolates) were infrequently identified. None of the 134 *E. coli* isolates simultaneously harboured more than one type of intimin. The relationship between intimin subtypes and *E. coli* serotype is shown in Table 4.5.

Intimin from nineteen (12.4%) *E. coli* isolates representing 16 different serotypes (Tables 4.1 and 4.5) did not amplify a visible product using the primer cocktail EaeVF, EaeVR, EaeZetaVR and EaeJotaVR. The presence of intimin was confirmed in these strains by southern hybridisation using *eae* gene probe (Figure 4.3).

Table 4.5 Association between intimin subtypes and *E. coli* serotypes.

Intimin type/subtype	Serotype
Int-α1	O55:H6, O85:H49, O127:H-
Int-α2	O125:H6
Int-β	O5:H-, O5:H11, O7:H-, O15:H-, O15:H2, O26:H-, O26:H11, O109:H-, O111:H-, O118:H16, O126:H2, O128:H2, O145:H-, O157:H11, Ont:H-, Ont:Hnt
Int-γ	O98:H-, O145:H-, O157:H-, O157:H21, O172:H1, OR:Hnt
Int-θ	O3:H8, O103:H2, O84:H25, O153:H11/21, Ont:H25
Int-δ	O37:H-, O86:H-, Ont:HR
Int-ε1	O15:H2, O103:H2, O121:H19, O166:H49, OR:H-
Int-ε2	O2 related:H19
Int-ζ	O28:H31, O84:H-, O84:H2, O156:H1, Ont:H-, Ont:H25, OR:H2
Int-ι1	Ont:H8
Int-ι2	OR:H-
Non typeable	O5:Hnt, O5:HR, O7:H8, O37:H10, O55:H12, O55:H20, O69:H8, O77:H12, O88:H8, O106:HR, O112ab:H2, O158:HR, O162:H10, Ont:H6, Ont:H8, Ont:H49

4.3.3 Sequence and phylogenetic analysis of novel *eae* genes

RFLP profiles of *eae* genes from two *E. coli* isolates with serotypes O2-related:H19, and OR:H- could not be identified suggesting that these isolates possess novel intimin types. DNA sequencing of *eae* from these strains was performed using a panel of primers published previously and by primer walking (Table 4.3). Alignment of the predicted amino acid sequences of the C-terminal Int_{280} regions of these two genes showed considerable sequence divergence from known intimin types. The predicted amino acid sequence of the intimin gene characterised from ovine isolate (CNC155) with serotype O2-related:H19 shared the highest overall amino acid sequence similarity (92.5%) with Int-ε. These two

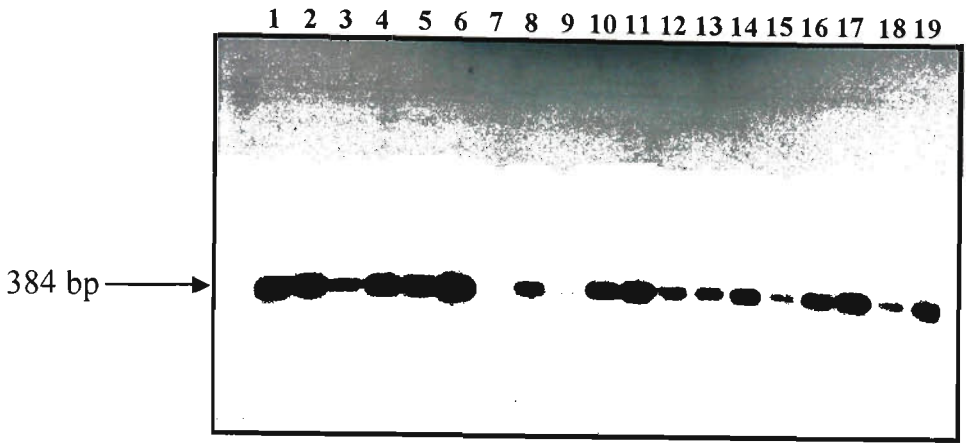


Figure 4.3 Southern blot of PCR product obtained from ovine *E. coli* strains belonging to various serotypes hybridised with the *eae* gene probe. Lanes: 1, NC142 (O5:Hnt); 2, CS123 (O5:HR); 3, NC695 (O7:H8); 4, CS139 (O37:H10); 5, CS180 (O55:H20); 6, NC133 (O69:H8); 7, NC70 (O77:H12; 8, CS129 (O88:H8); 9, NC134 (O106:HR); 10, NC135 (O106:HR); 11, CS89 (O112ab:H2); 12, CS74 (O158:HR); 13, NC69 (O158:HR); 14, NC132 (O162:H10); 15, NC1234 (Ont:H-); 16, NC75 (Ont:H6); 17, CS126 (Ont:H8); 18, CS136 (Ont:H49); 19, NC77 (O77:H12).

sequences shared 85.2% similarity in the C-terminal Int_{280} amino acids. Phylogenetic analysis using the Phylip package (http://bioweb.pasteur.fr/seqanal/phylogeny/phylip-uk.html) confirmed the close relationship of these two Int-ε subtypes (Figure 4.4).

The predicted amino acid sequence of the intimin gene from ovine isolate (NC38) with serotype OR:H- showed 91.7% and 85% similarity to the full *eae* and Int_{280} sequences of Int-ι respectively. Phylogenetic analysis resolved these two intimin subtypes as closest relatives (Figure 4.6).

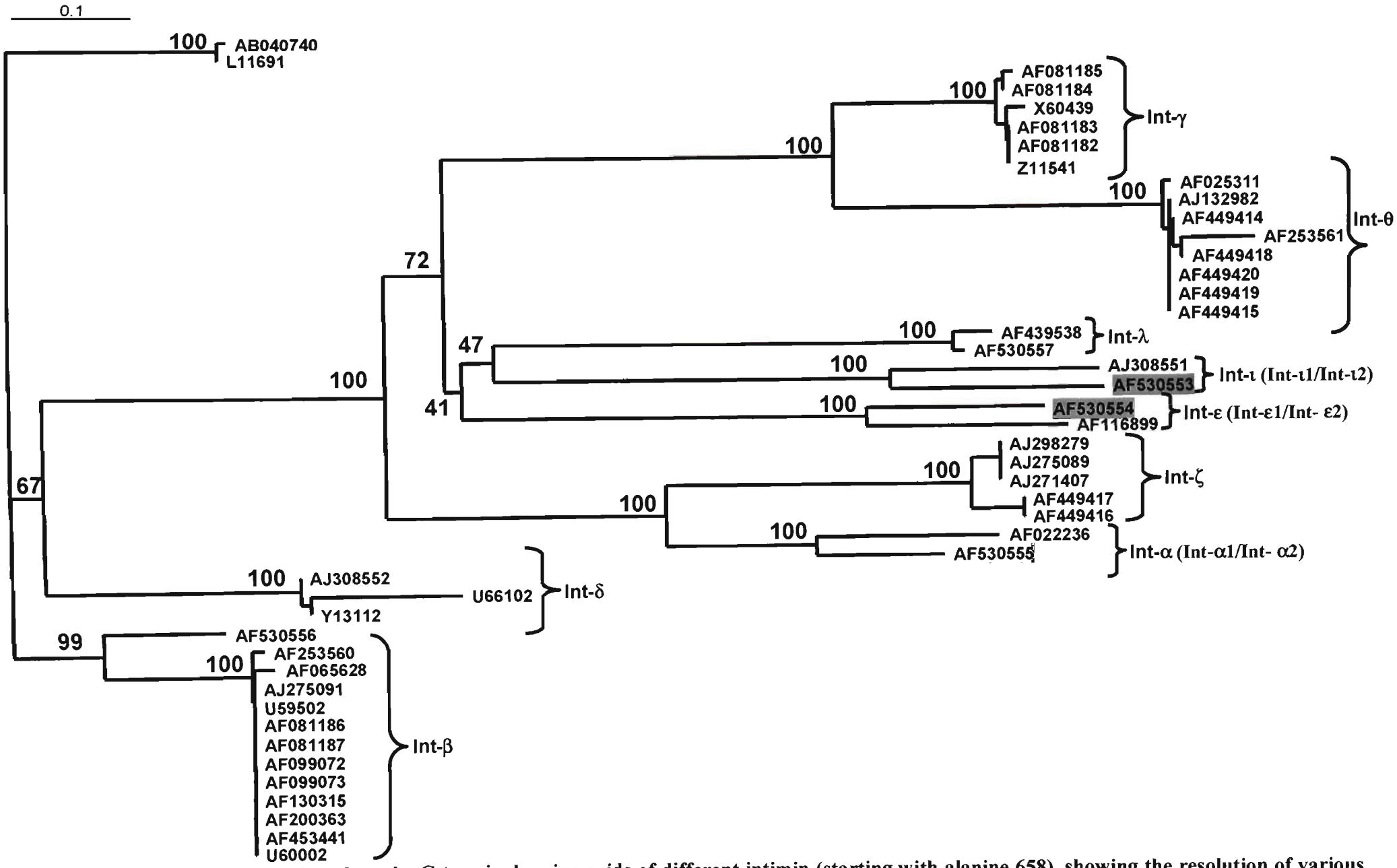


Figure 4.4 Neighbour-joining gene tree based on the C-terminal amino acids of different intimin (starting with alanine 658), showing the resolution of various intimin families. Numbers following the branches are the accession numbers in GenBank for the intimin sequences. The highlighted accession numbers represent the sequences determined in this study Numbers at nodes correspond to bootstrap proportions. The scale bar indicates the number of amino acid replacements per site.

Intimin sequences from ovine isolates O2- related:H19 and OR:H- showed enough amino acids sequence divergence from intimin subtypes existing in public databases to suggest that these sequences receive new subtype designations and propose they be identified as Int-ε2 and Int-ι2 respectively.

4.4 DISCUSSION

The intimin sequence family has been extensively studied (Adu-Bobie *et al.*, 1998; McGraw *et al.*, 1999; Oswald *et al.*, 2000; Philips and Frankel, 2000; Reece *et al.*, 2001; Tarr and Whittam, 2002) because of their key roles in colonisation, pathogenesis, tissue tropism and for their importance as phylogenetic markers of different clonal lineages of EPEC and EHEC/STEC. The intimin typing scheme developed in this study is the first system capable of successfully identifying all ten intimin variants (α1, α2, β, γ, θ, δ, ε, ζ, ι, λ). The application of this typing scheme to 134 of 153 *E. coli* strains representing 43 different serotypes is the most comprehensive subtyping study so far reported and enabled the identification of two previously unreported intimin subtypes identified as Int-ε2 and Int-ι2 in this study.

Int-β was identified among 16 different serotypes of which only O15:H-, O26:H-/H11, O111:H-, O118:H16 and O128:H2 have previously been reported to possess this subtype (Oswald *et al.*, 2000). Currently, serotypes O5:H-/H11, O7:H-, O15:H2, O109:H-, O126:H2, O145:H- and O157:H11 have not been reported to possess Int-β subtype. It is interesting that most serotypes possessing Intβ also possess either H2 or H11 flagella antigens although Oswald *et al.* (2000) reported several *E. coli* serotypes with Int-

ε (referred to as ε1 in this study) that also possessed a H2 flagella antigen. Int-ζ was the second most common intimin subtype identified in this study and was shown to be associated with 7 serotypes. Int-ζ was originally identified in a bovine STEC strain with serotype O84:NM and this study is the first to link it with serotypes O28:H31, O84:H2, O156:H1 and Ont:H-/H25. Tarr and Whittam (2002) identified intimin ζ in strains of serotypes O111:H9 and O111:H- isolated from patients with diarrhoeal disease, particularly the O111:H9 *E. coli* strain which was isolated from an outbreak of diarrhoea in Finland that affected more than 700 people (Viljanen *et al.*, 1990). Two isolates from humans of serotypes Ont:H- and O156:H- isolated from a human with an unknown history and a healthy baby respectively also possessed the Int-ζ variant.

Intimin ε1 was first identified in a STEC of serotype O103:H2 isolated from a patient with HUS and was shown to be found only in Stx producing human and bovine *E. coli* strains of serotypes O8:H2, O11:H2, O45:H2, O103:H-/H2/H18/Hnd, O121:H19 and O165:H- (Oswald *et al.*, 2000). In this study, intimin ε1 was identified in ovine *E. coli* strains possessing serotypes O15:H2, O103:H2, O121:H19, O166:H49 and OR:H- along with STEC strains of serotypes O103:H2 and O121:H19 isolated from patients with diarrhoea. Importantly a non-STEC isolate with serotype O2-related:H19 was shown to possess Int-ε2, a new intimin variant.

Int-γ is a variant associated with STEC serogroup O157 capable of causing serious illness in humans (Adu-Bobie *et al.*, 1998; Oswald *et al.*, 2000) and was identified in 7 of 11 ovine isolates of serogroup O157 with an exception of four isolates of serotype O157:H21 which

possessed Int-β. Serotypes O55:H7, O86:H40, O111:H-/H8, O127:H40, O128:H-/H8, O145:H- and O157:H-/H7 have previously been shown to possess an Int-γ subtype (Oswald *et al.*, 2000). This study confirmed that isolates with serotypes O145:H- and O157:H- possessed Int-γ and showed that serotypes O98:H-, O157:H21, O172:H1 and OR:Hnt also possessed Int-γ.

This study identified five *E. coli* serotypes (O3:H8, O103:H2, O84:H25, O153:H11/H21 and Ont:H25) to possess an Int-θ subtype, all of which have not been previously reported to possess Int-θ. Only O111:H- has been reported to possess an Int-θ subtype (Tarr and Whittam, 2002). However, this variant was previously characterised as Int-γ2 due to difference in RFLP pattern (Oswald *et al.*, 2000) and is similar to the *eae* sequence from a STEC of serotype O111:H- published by Voss *et al.* (1998). Int-θ shares closely homology to Int-β in the N-terminal 550 amino acids but is more related to Int-γ in the C-terminal 400 amino acids encompassing Int_{280}.

Only a limited number of *E. coli* serotypes (O55:H6, O127:H-/H6, and O157:H-/H45) have been reported to possess Int-α1 and most display a H6 flagella type (Oswald *et al.*, 2000). With the exception of O157:H-/H45, this study identified these same serotypes and confirmed them to possess Int-α1. An ovine isolate with serotype O85:H49 was the only strain possessing Int-α1 that has not previously been reported. More importantly, this isolate possessed stx_2 gene and as such this isolate represents the first description of an STEC strain possessing Int-α1 (McGraw *et al.*, 1999). Int-α2 was found in a human EPEC

strain of serotype O125:H6 (Oswald *et al.*, 2000) and this study identified and confirmed the same serotype to possess Int-α2.

Int-δ is usually expressed by EPEC strain belonging to serotype O86:H34 (Adu-Bobie *et al.*, 1998). Further, Oswald *et al.* (2000) identified this variant in a human EPEC strain of serotype O86:H- and a EPEC strain of serogroup O49 isolated from a dog. In this study, Int-δ was identified in only five isolates and these comprised of three different serotypes (O37:H-, O86:H- and Ont:HR).

Int-ι1 was only identified in two ovine *E. coli* strains of serotype Ont:H8. However, this variant was initially identified in a *E. coli* isolate of serotype O154:H4 (W. L. Zhang, H. Karch and H. Schmidt, unpublished data). A new variant designated Int-ι2 was identified in this study, in a *E. coli* isolate of serotype OR:H- recovered from sheep. Intimin λ was not identified in any of the *E. coli* isolates investigated in this study. However, a bovine isolate of serotype O2/74:H- was identified to possess this variant (Kim Brett, personal communication) demonstrating that the typing scheme developed in this study is capable of detecting all of the reported intimin subtypes.

Intimin from 19 of 105 (18%) ovine *E. coli* strains representing 16 different serotypes could not be typed by the PCR-RFLP system indicating an even larger variety of intimins. Further studies need to be carried out on these isolates to determine if these intimins belong to diverse subsets of the intimin subtypes already reported or whether they are entirely novel group/groups of intimin subtypes.

Phylogenetic analysis of all available intimin sequences confirmed the previous division of intimins into the 6 types α, β, γ, δ, ε and θ (Adu-Bobie *et al.*, 1998; Oswald *et al.*, 2000; Tarr and Whittam, 2002) and each subtype was resolved as a well-supported group (100% bootstrap support; Figure 4.5). In addition, this analysis confirmed the validity of the newly designated ξ subtype (Jores *et al.*, unpublished), resolving it as distinct, and most closely related to the α intimins. Similarly, the newly designated ι and λ groups were resolved as distinct groups (Figure 4.5), but their affinities were less clear. The analysis indicates that the λ, ι and ε intimins are most closely related to each other than to any other intimin subtype (72% bootstrap support), but that the relationships among these three intimins are unclear. The sequence data suggest that the λ subtype is most closely related to the ι subtype, with ε more remotely related to the other two, but bootstrap support for this is unconvincing (47%). The branches with low support (41% and 47% bootstrap proportions) are very short, suggesting that these intimin subtypes diverged over a short period of time. Further, the branches leading to the λ, ι and ε groups are very long, indicating that each of these groups are quite divergent from each other.

The presence of several distinct *eae* genes may be due to the strong selective pressure for amino acid diversity to enable A/E pathogens to colonise various tissue and/or evade the host immune system. This phenomenon is characteristic of bacterial genes encoding proteins that are secreted or exposed on the cell surface (Whittam, 1995). The majority of the sequence diversity is observed on the 3' end of the *eae* gene, which is expected since the C-terminal forms the extracellular (EC) domain of intimin (Luo *et al.*, 2000) that is exposed to the host immune system and also involved in binding to receptors. It has been

suggested that divergence of *eae* genes is accelerated by recombination (McGraw *et al.*, 1999). Several immunodominant regions within the EC domains of intimin α and β have been identified (Adu-Bobie *et al.*, 1998) confirming that diversity is essential to elude the host immune response. It has also been shown that intimin types influences the tissue and host tropism of A/E pathogens (Tzipori *et al.*, 1995; Philips *et al.*, 2000; Fitzhenry *et al.*, 2002). However, the tissue tropism activities have only been investigated extensively with Int-α and Int-γ (Philips *et al.*, 2000; Fitzhenry *et al.*, 2002). Further studies need to be carried out to explore the tissue tropism properties of the other reported intimin variants.

Although Int-ε2 and ι2 subtypes showed considerable sequence diversity in the C-terminal Int_{280} region, they each contained key amino acid residues considered important for intimin-Tir interactions and that are conserved across all known intimin types (Frankel *et al.*, 1995; Luo *et al.*, 2000; Reece *et al.*, 2002) (refer to Appendix B). These residues include two cysteine residues which form the disulfide bond required for epithelial cell binding activity (Frankel *et al.*, 1995) and four tryptophan residues (W117/776, W136/795, W222/881, and W240/899) (positions numbered according to $Int_{280α}$/whole intimin α), which reside within the receptor-binding superdomain of intimin (Batchelor *et al.*, 2000). W240/899, which is located on a conserved loop on the D3 domain, is important in A/E lesion formation and intimin-Tir interactions (Batchelor *et al.*, 2000) and its substitution with alanine (W240/899A) in site-directed mutagenesis studies generated a phenotype where intimin could no longer bind Tir nor induce A/E lesions on Hep-2 cells or colonic hyperplasia *in vivo* (Reece *et al.*, 2002). Similarly, the phenotype associated with W136/795A showed intimin-Tir interaction but no A/E lesion formation and this

tryptophan residue is believed to play a central role in maintaining the integrity of the D2/D3 super domain within intimin (Reece *et al.*, 2002). The remaining two tryptophan residues are postulated to play roles in Tir-independent host-receptor interactions (Reece *et al.*, 2002). The preservation of these tryptophan residues among 12 different intimin types supports the hypothesis that these residues are essential for the biological functions of intimin. Given the central role of intimin in EPEC/STEC interactions with gastro-intestinal epithelial cells, it will be interesting to see how different intimin variants affect biological function(s) and host range. Other studies have shown that different STEC serotypes preferentially colonise cattle, and sheep and many of these do not possess intimin (Djordjevic *et al.*, 2001; Hornitzky *et al.*, 2001). However, the ability of a subset of STEC to cause severe gastro-intestinal disease in humans may be more directly linked to intimin subtype since the common serotypes associated with HUS and HC (O157:H-/H7, O111:H-/H2, O26:H11, and others) typically possess Int-γ, β or θ subtypes, although there are well described cases of EHEC strains causing these diseases that do not possess intimin (e.g. O113:H21) (Paton *et al.*, 2001). Further studies are required to expand the knowledge of the associations between serotype and intimin subtype before such comparisons can be rigorously examined.

Chapter 5

Molecular typing of non-O157 STEC strains isolated from ovine and human sources

5 – Molecular typing of non-O157 STEC strains isolated from ovine and human sources

5.1 INTRODUCTION

Identification of an epidemic strain is often critical to the success of epidemiological investigations aimed at preventing the spread of infection and eradicating its source. The introduction of genotyping methods based on DNA analysis has significantly increased the resolution of epidemiological typing. Methods that have been used to genotype STEC O157:H7 include Shiga toxin genotyping (Samadpour, 1995), multilocus enzyme electrophoresis (Whittam *et al.*, 1988), bacteriophage λ restriction fragment length polymorphism profiling (Samadpour *et al.*, 1993; Paros *et al.*, 1993), ribotyping (Martin *et al.*, 1996) and octamer based genome scanning (Kim *et al.*, 1999).

PFGE is another genotyping method used for genetic subtyping of STEC O157:H7 due to its high discriminatory power and reproducibility (Bohm and Karch, 1992; Barrett *et al.*, 1994; Meng *et al.*, 1995; Izumiya *et al.*, 1997; Allison *et al.*, 2000). This method involves the use of rare cutting restriction enzymes to generate a limited number (10-20) of high molecular weight restriction fragments. The resulting electrophoretic profiles have been shown to be highly specific for a wide variety of organisms (Versalovic *et al.*, 1993) and also allow identification of variations within strains of an organism caused by random genetic events such as point mutations, insertions and deletions of DNA. This technique can aid in the epidemiological identification of specific strains implicated in disease outbreaks.

Outbreaks of STEC O157:H7 and non-O157 have been epidemiologically linked to the consumption of contaminated foods (Griffin and Tauxe, 1991; Johnson *et al.*, 1996; Armstrong *et al.*, 1996). Ruminants, particularly cattle and sheep, have been shown to be natural reservoirs for STEC of different serotypes (Gyles *et al.*, 1998; Kudva *et al.*, 1997; Djordjevic *et al.*, 2001) and foods that become contaminated with faeces from these animals may serve as the primary source of human infection. The most common STEC serotype isolated from sheep are O5:H-, O91:H- and O128:H2 (Beutin *et al.*, 1997; Kudva *et al.*, 1997; Bettelheim *et al.*, 2000; Djordjevic *et al.*, 2001); these serotypes have been recovered from patients with HC and HUS (www.microbionet.com.au).

An alternate approach to PFGE is PCR based fingerprinting. Lately, repetitive sequence based PCR (rep-PCR) fingerprinting, such as repetitive extragenic palindromic (REP) PCR and enterobacterial repetitive intragenic consensus (ERIC) PCR have been extensively used to characterise strains of various bacterial species (Georghiou *et al.*, 1994; Pooler *et al.*, 1996; Sadowsky *et al.*, 1996; Sander *et al.*, 1998). rep-PCR fingerprinting has been reported as being relatively simple, rapid and sensitive for discriminating between closely related strains (Georghiou *et al.*, 1994; Pooler *et al.*, 1996; Sadowsky *et al.*, 1996). REP and ERIC PCR utilises primers homologous to defined sequences, which are present in multiple copies in the bacterial genome (Versalovic *et al.*, 1991; Lupski and Weinstock, 1992; Versalovic *et al.*, 1993). In a recent study, ERIC PCR was used to fingerprint 80 STEC isolates of serogroup O157 recovered from symptomatic patients in northern Palestine and the analysis revealed three major clonal populations (Adwan *et al.*, 2002). Further, rep-PCR have been used in molecular typing of uropathogenic *E. coli* strains (Johnson *et al.*, 1997; Johnson *et al.*, 1998) and *E. coli* isolated from chickens (Carvalho de

Moura *et al.*, 2001). However, this method has not been applied to date on non-O157 STEC strains recovered from sheep and humans.

PFGE has been successfully applied to fingerprint STEC O157:H7 isolates from humans (Izumiya *et al.*, 1997), cattle (Akiba *et al.*, 1999) and sheep (Kudva *et al.*, 1997). Izumiya *et al.* (1997) identified six PFGE groups among the 825 O157:H7 isolates recovered from 19 outbreaks and 608 sporadic cases in Japan. Akiba *et al.* (1999) reported 50 PFGE profiles among the seventy seven O157:H-/H7 isolates recovered from cattle, while Kudva *et al.* (1997) reported 11 PFGE patterns among the 140 *E. coli* O157:H7 isolates recovered from sheep. Several studies have also investigated clonal relationship among non-O157 STEC isolates from humans (Eklund *et al.*, 2001; Welinder-Olsson *et al.*, 2002; Khan *et al.*, 2002) and cattle (Pradel *et al.*, 2001; Khan *et al.*, 2002) using PFGE methodology. In a recent study investigating the clonal relatedness among 63 non-O157 STEC strains isolated from cattle and humans in India using PFGE revealed high level of genetic heterogeneity between the strains indicating diverse clonality (Khan *et al.*, 2002). PFGE has not been applied to date on non-O157 STEC isolates recovered from sheep to determine the clonal relatedness among strains of the same serotype and linking these strains with STEC isolates of the same serotype isolated from humans to evaluate the potential of sheep as a source of human STEC infections.

The aim of this study was to (i) determine the clonal relatedness and diversity among non-O157 STEC isolates (O5:H-, O91:H- and O128:H2) of the same serotypes recovered from human and ovine sources using PFGE and (ii) compare PFGE fingerprinting and PCR

based finger printing using REP PCR and ERIC PCR to determine suitable methods for genotyping non-O157 STEC strains.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains

Sixteen STEC isolates of serotype O5:H-, 17 *E. coli* (16 STEC and 1 non-STEC) isolates of serotype O91:H- and 18 STEC isolates of serotype O128:H2 were used in this study (Table 5.1). Of the sixteen O5:H- STEC isolates, 11 were from healthy sheep, 4 isolates were from patients with HUS and 1 isolate was from a healthy cow. Of the seventeen O91:H- *E. coli* isolates, 11 were from healthy sheep, 1 isolate was from a meat sausage, 1 isolate was from a lamb carcass, 1 isolate was from a healthy cow, 1 isolate was from a patient with diarrhoea and 2 isolates were from healthy humans. The eighteen O128:H2 STEC isolates comprised of 12 isolates from healthy sheep and 6 isolates from patients with diarrhoea. Elizabeth Macarthur Agricultural Institute (New South Wales, Australia) provided the sheep and cattle isolates which were isolated using methods described by Djordjevic *et al.* (2001) (refer to section 2.2.1). Thirteen isolates from humans were obtained from the Microbiological Diagnostic Unit (MDU) (Melbourne, Australia) along with 1 isolate from a meat sausage, 1 isolate from a lamb carcass, 1 isolate from sheep faeces from New Zealand and 1 isolate from sheep faeces from the USA. Dr. Karl Bettelheim from MDU, Victoria, Australia, serotyped all isolates used in this study.

Table 5.1 Virulence profile and fingerprint categories obtained by different genotyping methods for non-O157 *E. coli* isolated from ovine, bovine and human sources.

Isolate No.	Serotype	Source[a]	Virulence Type				Banding patterns identified by visual inspection		
			stx$_1$	*stx*$_2$	*eae*	*ehxA*	PFGE	REP-PCR	ERIC-PCR
531	O5:H-	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dO111/OX3a}$	-	+	O5-A	O5-R1	O5-E1
497	O5:H-	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dO111/OX3a}$	-	+	O5-B	O5-R2	O5-E2
1496	O5:H-	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dO111/OX3a}$	-	+	O5-C	O5-R2	O5-E3
1264	O5:H-	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dO111/OX3a}$	-	+	O5-D1	O5-R2	O5-E3
VN23	O5:H-	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dO111/OX3a}$	-	+	O5-E	O5-R2	O5-E2
VN6	O5:H-	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dO111/OX3a}$	-	+	O5-D2	O5-R3	O5-E2
773	O5:H-	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dO111/OX3a}$	-	+	O5-F	O5-R4	O5-E4
CS106	O5:H-	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dO111/OX3a}$	-	+	O5-G1	O5-R5	O5-E5
CS468	O5:H-	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dO111/OX3a}$	-	+	O5-G2	O5-R5	O5-E5
CS147	O5:H-	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dO111/OX3a}$	-	+	O5-G3	O5-R5	O5-E5
CS310	O5:H-	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dO111/OX3a}$	-	+	O5-H	O5-R6	O5-E6
H1	O5:H-	Human, New Zealand, HUS (V)	*stx*$_{1c}$	-	Int-β	+	O5-I1	O5-R7	O5-E7
H3	O5:H-	Human, Australia, HUS (V)	*stx*$_{1c}$	*stx*$_{2dO111/OX3a}$	-	+	O5-J	O5-R8	O5-E8
H2	O5:H-	Human, Australia, HUS (V)	*stx*$_{1c}$	-	Int-β	-	O5-K	O5-R9	O5-E9
H4	O5:H-	Human, Australia, HUS (V)	*stx*$_{1c}$	-	-	+	O5-K	O5-R9	O5-E9
310	O5:H-	Cattle faeces, NSW (E)	*stx*$_{1c}$	-	Int-β	+	O5-I2	O5-R7	O5-E7
639	O91:H-	Cattle faeces, NSW (E)	*stx*$_1$	*stx*$_{2dOunt}$	-	+	O91-A	O91-R1	O91-E1
726	O91:H-	Sheep faeces, NSW (E)	*stx*$_1$	*stx*$_{2dOunt}$	-	+	O91-B1	O91-R1	O91-E2
849	O91:H-	Sheep faeces, NSW (E)	*stx*$_1$	*stx*$_{2dOunt}$	-	+	O91-B2	O91-R1	O91-E2
VN56	O91:H-	Sheep faeces, NSW (E)	*stx*$_1$	*stx*$_{2dOunt}$	-	+	O91-C	O91-R2	O91-E3

Table 5.1 Virulence profile and fingerprint categories obtained by different genotyping methods for non-O157 *E. coli* isolated from ovine, bovine and human sources.

Isolate No.	Serotype	Source[a]	Virulence Type				Pattern or group by indicated method		
			stx$_1$	*stx*$_2$	*eae*	*ehxA*	PFGE	REP-PCR	ERIC-PCR
755	O91:H-	Sheep faeces, NSW (E)	*stx*$_1$	*stx*$_{2dOunt}$	-	+	O91-D	O91-R2	O91-E3
1034	O91:H-	Sheep faeces, NSW (E)	*stx*$_1$	*stx*$_{2dOunt}$	-	+	O91-B3	O91-R1	O91-E4
K16	O91:H-	Sheep faeces, New Zealand (V)	*stx*$_1$	*stx*$_{2dOunt}$	-	+	O91-E	O91-R3	O91-E5
K13	O91:H-	Mettwurst sausage, Australia (V)	*stx*$_1$	*stx*$_{2dOunt}$	-	-	O91-F	O91-R5	O91-E6
917	O91:H-	Sheep faeces, NSW (E)	*stx*$_1$	*stx*$_{2dOunt}$	-	+	O91-G	O91-R4	O91-E4
K14	O91:H-	Sheep faeces, USA (V)	*stx*$_1$	*stx*$_{2dOunt}$	-	+	O91-H	O91-R4	O91-E6
K6	O91:H-	Lamb carcasses, Queensland (V)	*stx*$_1$	*stx*$_{2dOunt}$	-	+	O91-I	O91-R3	O91-E5
CS21	O91:H-	Sheep faeces, NSW (E)	*stx*$_1$	*stx*$_{2dOunt}$	-	+	O91-J	O91-R4	O91-E7
CS36	O91:H-	Sheep faeces, NSW (E)	*stx*$_1$	*stx*$_{2dOunt}$	-	+	O91-J	O91-R4	O91-E7
M501	O91:H-	Sheep faeces, NSW (E)	*stx*$_1$	*stx*$_{2dOunt}$	-	+	O91-K	O91-R4	O91-E7
H5	O91:H-	Human, Australia, Diarrhoea (V)	*stx*$_1$	*stx*$_{2dOunt}$	-	-	O91-F	O91-R5	O91-E7
H17	O91:H-	Human, Denmark, Healthy (V)	-	-	-	-	O91-L	O91-R6	O91-E8
H6	O91:H-	Human, Australia, Symptomless (V)	*stx*$_1$	*stx*$_{2dOunt}$	-	-	O91-M	O91-R1	O91-E1
H8	O128:H2	Human, Australia, Diarrhoea (V)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	+	O128-A	O128-R1	O128-E1
H9	O128:H2	Human, New Zealand, Diarrhoea (V)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	+	O128-B	O128-R1	O128-E1
H10	O128:H2	Human, Australia, Diarrhoea (V)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	+	O128-C1	O128-R1	O128-E2
H11	O128:H2	Human, Australia, Diarrhoea (V)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	-	O128-D	O128-R1	O128-E2
H12	O128:H2	Human, Australia, Diarrhoea (V)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	+	O128-E	O128-R1	O128-E2
H13	O128:H2	Human, Australia, Diarrhoea (V)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	-	O128-C2	O128-R1	O128-E3
CS193	O128:H2	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	+	O128-F1	O128-R1	O128-E3

Table 5.1 Virulence profile and fingerprint categories obtained by different genotyping methods for non-O157 *E. coli* isolated from ovine, bovine and human sources.

Isolate No.	Serotype	Source[a]	Virulence Type				Pattern or group by indicated method		
			stx$_1$	*stx*$_2$	*eae*	*ehxA*	PFGE	REP-PCR	ERIC-PCR
CS241	O128:H2	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	+	O128-F2	O128-R1	O128-E3
CS462	O128:H2	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	+	O128-F2	O128-R1	O128-E3
M570	O128:H2	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	+	O128-G1	O128-R1	O128-E4
M527	O128:H2	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	+	O128-H	O128-R1	O128-E4
M537	O128:H2	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	+	O128-G2	O128-R2	O128-E4
VN8	O128:H2	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	+	O128-I	O128-R1	O128-E4
VN47	O128:H2	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	+	O128-J	O128-R1	O128-E4
796	O128:H2	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	+	O128-K	O128-R1	O128-E4
442	O128:H2	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	+	O128-L	O128-R1	O128-E4
700	O128:H2	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	+	O128-M1	O128-R1	O128-E4
1138	O128:H2	Sheep faeces, NSW (E)	*stx*$_{1c}$	*stx*$_{2dOunt}$	-	+	O128-M2	O128-R1	O128-E5

[a] E, isolates obtained from Elizabeth Macarthur Agricultural Institute, New South Wales, Australia; V, isolates obtained from Microbiological Diagnostic Unit, Victoria, Australia.

5.2.2 Virulence factor detection and subtyping

Isolates that were not subjected to virulence factor genotyping in Chapters 2, 3 and 4 (refer to Appendix A) were subjected to multiplex PCR for the detection of STEC virulence factors *stx*$_1$, *stx*$_2$, *ehxA* and *eae* as described by Paton and Paton (1998) (refer to section 2.2.3). *stx*$_1$ and *stx*$_2$ subtyping was undertaken for 26 and 18 STEC isolates respectively. These isolates were not included in the *stx*$_1$ and *stx*$_2$ subtyping described in Chapters 2 and 3 respectively. The procedures undertaken for the subtyping are described in Chapters 2 and 3. *eae* from a single bovine STEC isolate of serotype O5:H- (310) was subtyped by Kim Brett from the Elizabeth Macarthur Agricultural Institute using the intimin typing system described in Chapter 4.

5.2.3 PFGE

E. coli colonies grown overnight on MacConkey agar plates were suspended in 100 µl of TE buffer (10 mM Tris-HCl and 0.1 mM EDTA [pH 7.5]) to obtain a turbid solution. Agarose plugs were made by mixing an equal volume of pre-warmed 1.5% low melt preparative grade agarose (Bio-Rad, Richmond, CA.) with the cell suspension. The mixture was immediately poured into a PFGE mould and allowed to solidify for 10 min at 4°C. After solidification, the plugs were transferred into 1.5 ml Eppendorf tubes containing 380 µl lysis buffer (6 mM Tris-HCl [pH 7.6], 1 mM NaCl, 100 mM EDTA [pH 8.5], 0.5% Brij 58, 0.5% sodium lauryl sarcosine, 0.2% deoxycholic acid and 0.5 mg of lysozyme/ml) and the tubes were incubated for 3 hours at 37°C. Following lysis, the plugs were washed once with 1 ml TE buffer and incubated overnight at 50°C with 280 µl of deproteination solution (500 mM EDTA (pH 8.5), 1% sodium lauryl sarcosine and 1 mg of proteinase K/ml). The

plugs were then washed once for 30 min in TE buffer containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and three times for 30 min in TE buffer without PMSF. Unless they were used immediately, the plugs were stored at 4°C in 500 µl of 1M EDTA (pH 8.5).

Prior to digestion with restriction enzymes, 3 x 3.5 mm slices were cut from the plugs and equilibrated for 1 hour in 200 µl of 1 X restriction buffer supplied by the manufacturer. The individual slices were then incubated overnight at 37°C in fresh 1 X restriction buffer (100 µl) containing 20 U of *Not*I (Roche, Mannheim, Germany). Restriction fragments were separated in 1% PFGE certified agarose (Bio-Rad) in 0.5 X Tris-borate-EDTA (0.1 M Tris [pH 8.0], 0.1 M boric acid and 0.2 M EDTA) buffer at 10°C using the Gene Navigator™ system (Pharmacia LKB). The buffer in the electrophoretic chamber was kept at a constant temperature of 10°C throughout the electrophoresis run using a water cooler. The run time was 25 hours, with a voltage of 200V and a linearly ramped pulse time of 5 to 35 s. Low-range PFG markers (New England Biolabs) were included as a DNA size standard.

Following electrophoresis, gels were stained for 30 min in 0.5 X Tris-borate-EDTA buffer containing 5 µg/ml of ethidium bromide with gentle agitation. Gels were destained for 30 min in 0.5 X Tris-borate-EDTA buffer and visualised under UV illumination using the GelDoc 1000 image analysis station (Bio-Rad).

5.2.4 REP-PCR

Isolation of DNA for REP-PCR was carried out as previously described by Fagan *et al.* (1999) (refer section 2.2.2). REP-PCR was performed with the primers REP1R-I and REP2-I (Table 5.2) at a concentration of 50 pmol as described previously by Versalovic *et al.* (1991), with the following modification. For the DNA preparation, InstaGene matrix (Bio-Rad) was used as described by Fagan *et al.* (1999). Amplification was performed in a 50 µl reaction mixture, containing 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 1.25 mM of each dNTP, 6 mM $MgCl_2$, 2 U of Taq DNA polymerase, 5 µl of nucleic acid and distilled H_2O to volume. PCR amplification was performed using an automated thermal cycler with an initial denaturing step at 95°C for 6 min, followed by 30 cycles of 94°C for 1 min, 40°C for 1 min, and 65°C for 8 min. Final extension was performed at 65°C for 16 min. Amplified PCR products (15 µl) were then resolved by agarose gel electrophoresis (Sambrook *et al.*, 1989) using 1% w/v agarose and stained with ethidium bromide (5 µg/ml). Visualisation was achieved by UV illumination and the images captured using a GelDoc 1000 image analysis station (Bio-Rad).

5.2.5 ERIC-PCR

Isolation of DNA for ERIC-PCR was carried out as previously described by Fagan *et al.* (1999) (refer section 2.2.2). ERIC-PCR was performed with the primers ERIC1R and ERIC2 (Table 5.2) at a concentration of 50 pmol as described previously by Versalovic *et al.* (1991) with the modification mentioned in section 5.2.4. PCR amplification was performed with an initial denaturation (95°C, 7 min), followed by 30 cycles of denaturation (94°C, 1 min), annealing (52°C, 1 min) and extension (65°C, 8 min), with a single final

extension (65°C, 16 min). PCR amplicons were resolved and visualised as described in section 5.2.4.

Table 5.2 Repetitive sequence based oligonucleotide primers used in REP and ERIC PCR.

Primer Name	Primer Sequence (5' to 3')	Reference
REP1R-I	IIICGICGICATCIGGC[a]	Versalovic *et al.*, 1991
REP2-I	ICGICTTATCIGGCCTAC	Versalovic *et al.*, 1991
ERIC1R	ATGTAAGCTCCTGGGGATTCAC	Versalovic *et al.*, 1991
ERIC2	AAGTAAGTGACTGGGGTGAGCG	Versalovic *et al.*, 1991

[a] I, deoxyinosine

5.2.6 Analysis of the DNA fingerprints generated by PFGE

The fingerprints obtained with PFGE were compared following the criteria of Tenover and colleagues for bacterial strain typing (Tenover *et al.*, 1995) and analysed with the Diversity One software package (Bio-Rad). Genetic similarity was compared by clustering methods (unweighted pair-group method with arithmetic means) using the Dice coefficient. A tolerance in the band positions of 2% was applied for comparison of fingerprint profiles. DNA fragments smaller than 97.0 kb were disregarded in the genetic comparison for O5:H- and O91:H- isolates and DNA fragments smaller than 48.5 kb were disregarded for O128:H2 isolates.

5.2.7 Analysis of fingerprints generated by REP and ERIC PCR

The fingerprint profiles obtained by REP and ERIC were compared for similarity by visual inspection of band patterns. Sizes of DNA fragments amplified by PCR were determined by direct comparison with the DNA marker. Fingerprints were considered highly similar when all visible bands obtained had the same migration distance for each isolate. Disparity in the

intensity and shape of bands among isolates were not considered differences. The presence or absence of one or more distinct bands was considered a difference.

5.3 RESULTS

5.3.1 Analysis of STEC virulence factors and subtypes

Of the 51 isolates used in this chapter, 46 contained *stx*$_1$, *stx*$_2$ and *ehxA*, 3 isolates contained *stx*$_1$, *eae* and *ehxA*, a single isolate contained *stx*$_1$ and *ehxA* and a single isolate did not possess any of the investigated virulence factors. All the O5:H- and O128:H2 isolates containing *stx*$_1$ possessed the variant *stx*$_{1c}$, while the O91:H- isolates possessed the *stx*$_1$ subtype. The O5:H- isolates containing *stx*$_2$ harboured the *stx*$_{2d\text{-}O111/OX3a}$ variant, while the O91:H- and O128:H2 isolates possessed the *stx*$_{2d\text{-}Ount}$ variant. The three O5:H- isolates containing *eae* possessed the Int-β variant. The virulence factor combination and variants are shown in Table 5.1.

5.3.2 PFGE analysis

PFGE typing was carried out to determine clonal relatedness among ovine and isolates from humans of same serotypes (O5:H-, O91:H- and O128:H2). Sixteen O5:H- STEC, 17 O91:H- and 18 O128:H2 STEC isolates were subjected to PFGE to analyse their *Not*I restriction patterns (Figures 5.1, 5.2 and 5.3).

5.3.2.1 PFGE analysis of O5:H- STEC isolates

PFGE of 16 O5:H- STEC isolates produced 9 to 15 fragments ranging in size from approximately 97.0 to 582.0 kb (DNA fragments smaller than 97.0 kb were disregarded in the genetic comparison) (Figure 5.1). Fifteen different band patterns were observed by visual inspection (Figure 5.1 and Table 5.1). Only two human STEC isolates (H2 and H4) produced identical fingerprint patterns designated O5-L (Figure 5.1). This was expected since these two isolates were recovered from the same patient with HUS and only differed in their ability to ferment sorbitol (Karl Bettelheim, personal communication). Three ovine STEC isolates CS106, CS468 and CS147 produced closely related restriction patterns designated O5-H1, O5-H2 and O5-H3 respectively (Figure 5.1; Lanes 8, 9 and 10). Isolates CS106 and CS468 differed from one another by just 2 bands, whilst isolates CS468 and CS147 differed by 3 bands. These findings are consistent with one independent genetic event (Tenover *et al.*, 1995) and are therefore are considered to be closely related. These three isolates were obtained from different sheep from a single farm. The restriction digestion patterns of ovine STEC isolates VN6 and 1264 differed with each other by 3 bands (Figure 5.1; lanes 4 and 6) and the bovine STEC isolate 310 and a STEC isolate H1 from a patient with HUS in New Zealand also differed by 3 bands (Figure 5.1; lanes 16 and 12) suggesting a close genetic relatedness (Tenover *et al.*, 1995). The remainder of the O5:H- isolates were not genetically related according to the criteria of Tenover *et al.* (1995), since their PFGE profiles had more than six band differences by visual inspection.

5.3.2.2 PFGE analysis of O91:H- *E. coli* isolates

Among the 17 O91:H- isolates, 15 different patterns were observed by visual inspection (Figure 5.2 and Table 5.1). PFGE produced 9 to 18 fragments ranging in size from

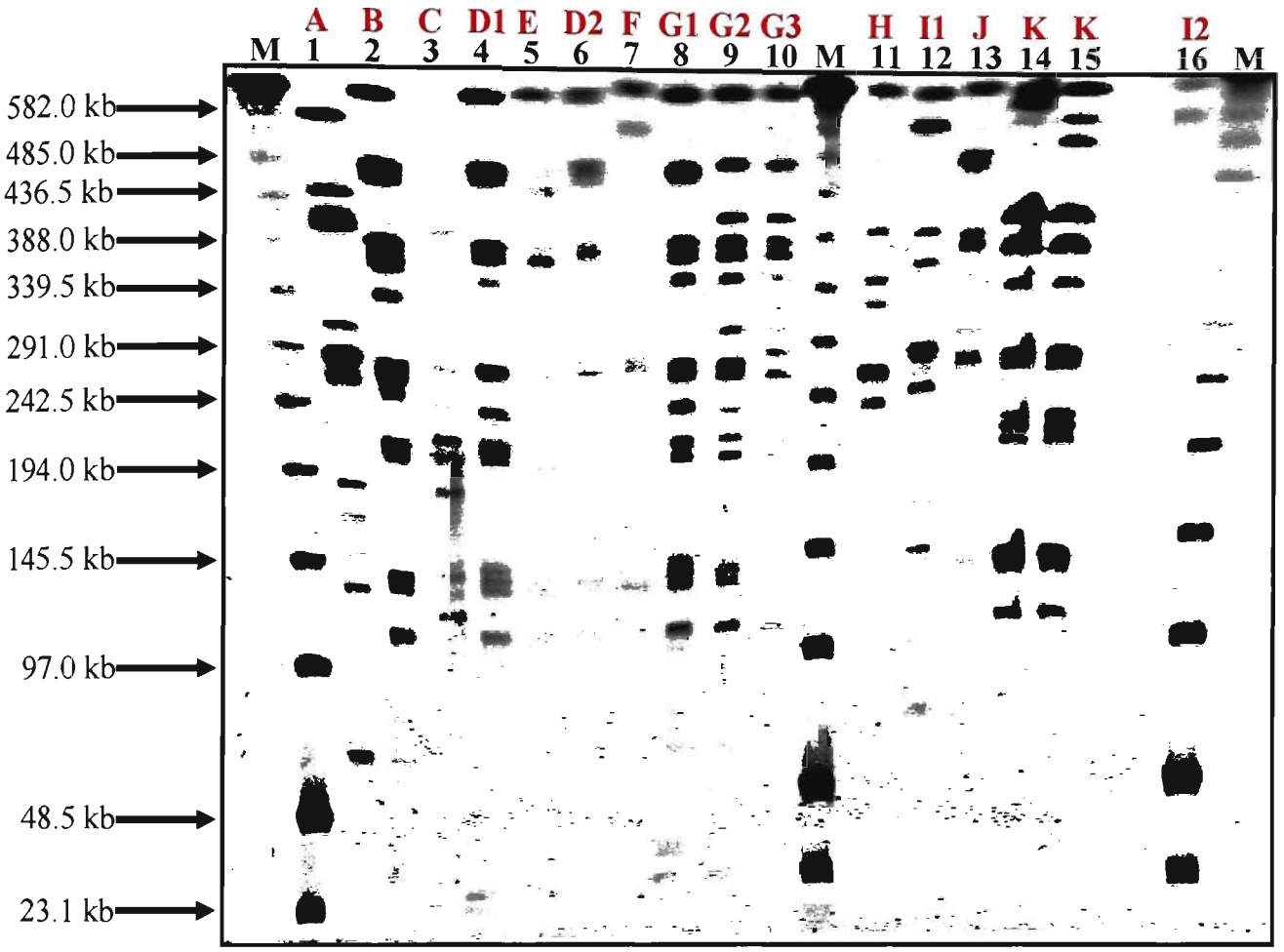


Figure 5.1 PFGE fingerprint patterns of *Not*I-cleaved genomic DNA of *E. coli* O5:H- isolates. Lanes: M, Low range lambda ladder PFG marker; 1, 531 (ovine); 2, 497 (ovine); 3, 1496 (ovine); 4, 1264 (ovine); 5, VN23 (ovine); 6, VN6 (ovine); 7, 773 (ovine); 8, CS106 (ovine); 9, CS468 (ovine); 10, CS147 (ovine); 11, CS310(ovine); 12, H1 (human); 13, H3 (human); 14, H2 (human); 15, H4 (human); 16, 310 (bovine). The designated banding profiles observed by visual inspection are shown in red.

approximately 97.0 to 630.5 kb (fragments smaller than 97.0 kb were disregarded in the genetic comparison) (Figure 5.2). Two ovine isolates (CS21 and CS36; Figure 5.2, Lanes 12 and 13) produced identical fingerprint profiles. Both these isolates are from different sheep from the same farm. The O91:H- STEC isolate (K13) recovered from mettwurst

sausage associated with the Garibaldi outbreak in South Australia and the O91:H- STEC isolate (H5) recovered from a patient with diarrhoea linked to the Garibaldi outbreak shared an identical pattern designated O91-G (Figure 5.2; lanes 8 and 15). Ovine STEC isolates 726 and 849 (Figure 5.2; lanes 2 and 3) produced closely related patterns and differed by

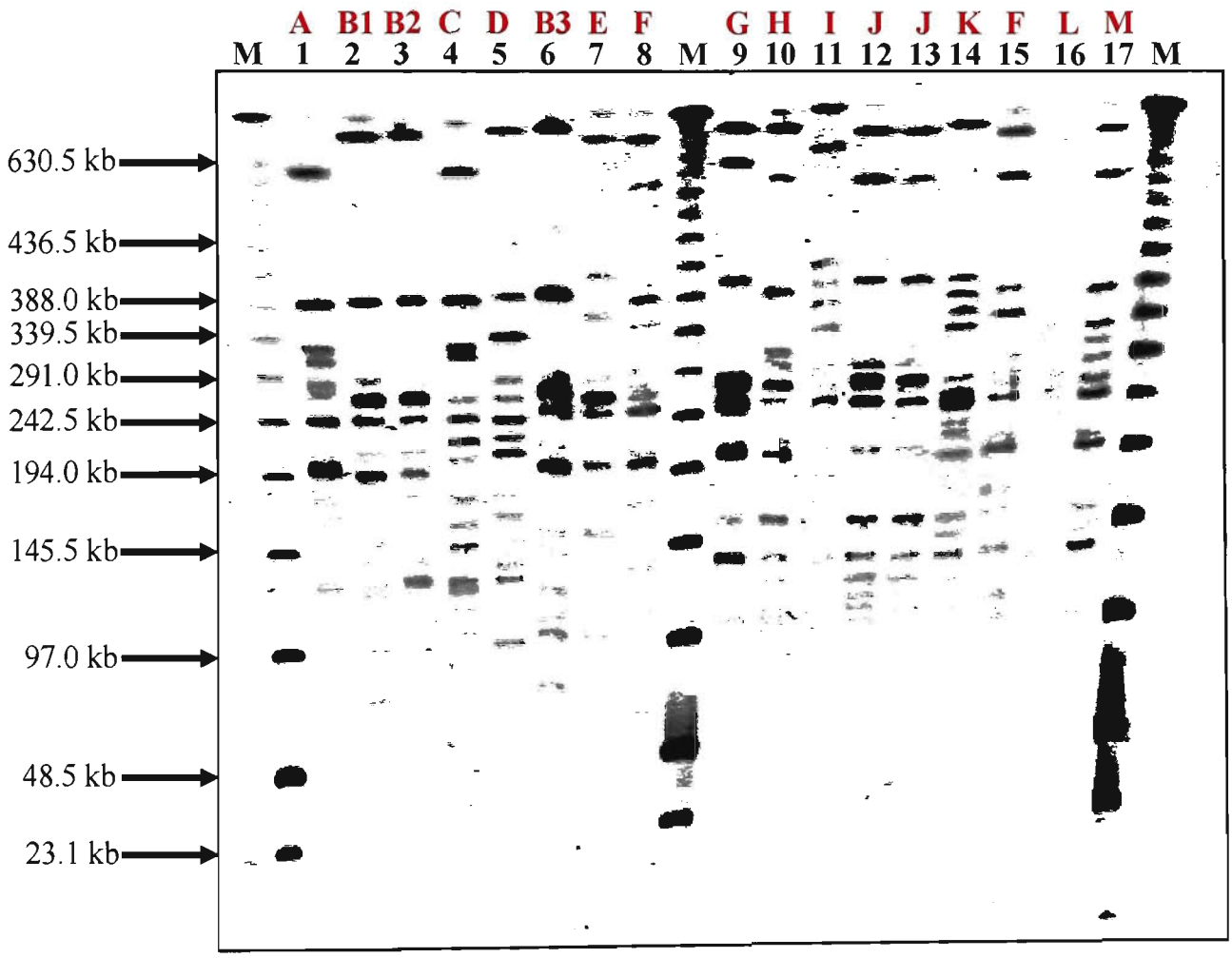


Figure 5.2 PFGE fingerprint patterns of *Not*I-cleaved genomic DNA of *E. coli* O91:H- isolates. Lanes: M, Low range lambda ladder PFG marker; 1, 639 (bovine); 2, 726 (ovine); 3, 849 (ovine); 4, VN56 (ovine); 5, 755 (ovine); 6, 1034 (ovine); 7, K16 (ovine); 8, K13 (ovine); 9, 917 (ovine); 10, K14 (ovine); 11, K6 (ovine); 12, CS21 (ovine); 13, CS36 (ovine); 14, M501 (ovine); 15, H5 (human); 16, H17 (human); 17, H6 (human). The designated banding profiles observed by visual inspection are shown in red

only 3 bands, referred to as band patterns O91-B1 and O91-B2 respectively. Ovine STEC isolate 1034 (Figure 5.2; lane 6) produced banding patterns that differed from isolates 726 and 849 by 4 and 6 bands respectively. The banding pattern is therefore referred to as band pattern O91-B3 (Figure 5.2; lane 6). These three isolates were from different sheep located in the same farm. The remainder of the O91:H- isolates did not produce closely related banding patterns and exhibited band differences of more than 6 bands.

5.3.2.3 PFGE analysis of O128:H2 STEC isolates

Digestion of DNA from the 18 O128:H2 STEC isolates with *Not*I produced between 11 to 14 chromosomal fragments (Figure 5.3). The molecular sizes of the fragments ranged from approximately 48.5 to 582.0 kb (fragments smaller than 48.5 kb were disregarded in the genetic comparison) (Figure 5.3). Among the 18 strains, 17 different patterns were observed by visual inspection (Table 5.1). The only identical band pattern (O128-F2) observed in this group was between two ovine isolates (CS241 and CS462; Figure 5.3, lanes 8 and 9). A third ovine STEC isolate (CS193; Figure 5.3, lane 7) produced a closely related restriction pattern (O128-F1) with O128-F2 and there were just 5 band differences indicating two independent genetic events (Tenover *et al.*, 1995). These three isolates were obtained from different animals from the same farm. Ovine isolates pairs M537 and M570 produced closely related patterns (O128-G1 and O128-G2) (Figure 5.3, lanes 10 and 12; Table 5.1). Ovine isolates 700 and 1138 also produced closely related pattern pairs (O128-M1 and O128-M2) (Figure 5.3, lanes 17 and 18; Table 5.1). Isolates M537 and M570 differed by only 3 bands and isolates 700 and 1138 differed by 2 bands with each other, which is consistent with a single independent genetic event (Tenover *et al.*, 1995). M537

and M570 were isolated from 2 merino sheep that were the descendants of the first flock of merino sheep bought into Australia from Spain. However, STEC isolate M527 (Figure 5.3,

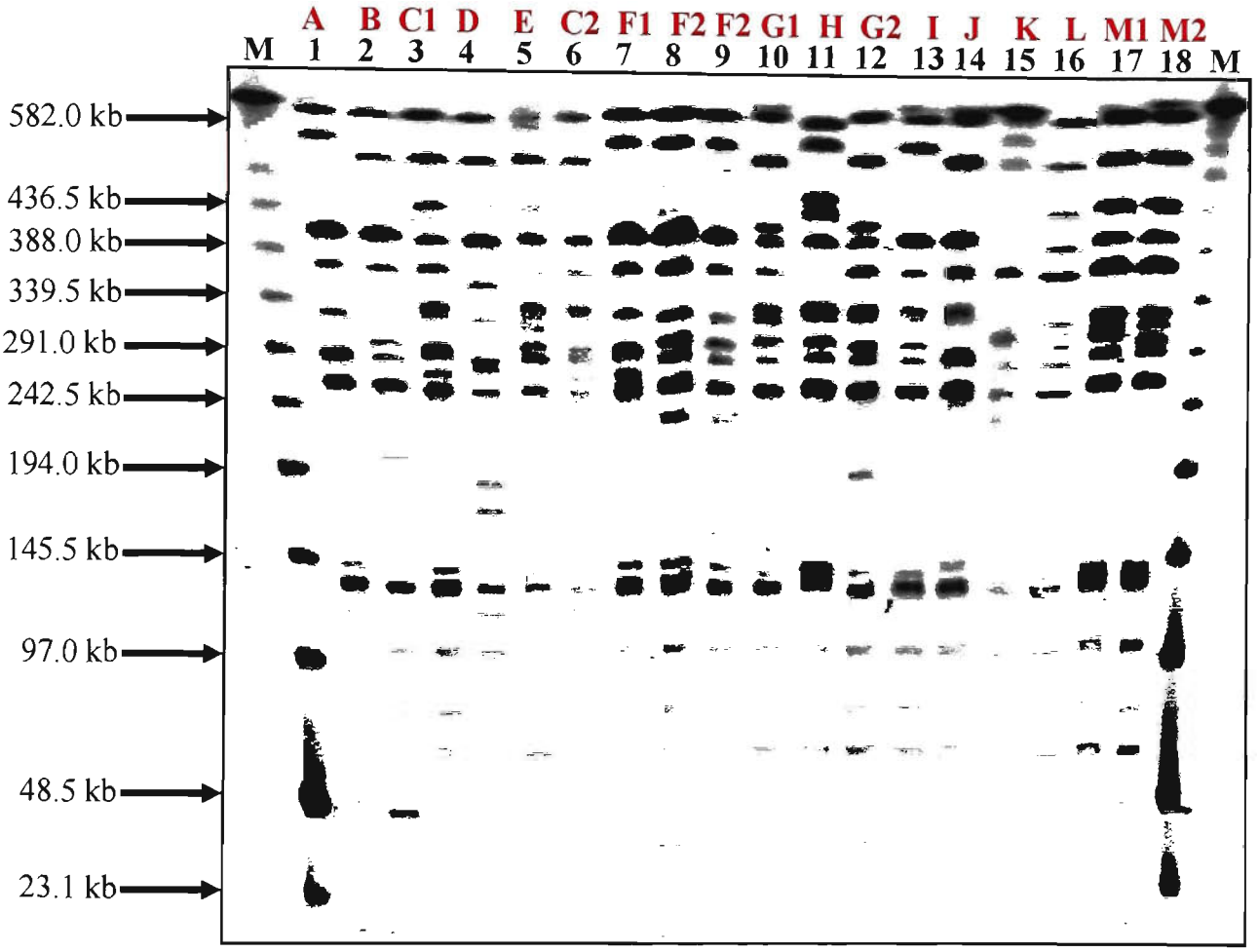


Figure 5.3 PFGE fingerprint patterns of *Not*I-cleaved genomic DNA of STEC O128:H2 isolates. Lanes: M, Low range lambda ladder PFG marker; 1, H8 (human); 2, H9 (human); 3, H10 (human); 4, H11 (human); 5, H12 (human); 6, H13 (human); 7, CS193 (ovine); 8, CS241 (ovine); 9, CS462 (ovine); 10, M570 (ovine); 11, M527 (ovine); 12, M537 (ovine); 13, VN8 (ovine); 14, VN47 (ovine); 15, 796 (ovine); 16, 442 (ovine); 17, 700 (ovine); 18, 1138 (ovine). The designated banding profiles observed by visual inspection are shown in red.

lane 11) another O128:H2 STEC isolate from the merino flock, produced unrelated banding patterns with 7 band differences (band pattern O128-H). Isolates 700 and 1138 are from geographically unrelated animals. Two human STEC isolates, H13 and H10, from patients with diarrhoea may possibly be related since they differed from one another by 3 bands indicating one independent genetic event (Tenover *et al.*, 1995). Ovine isolates 796, 442 and 1138 were from animals from the same flock, but showed different banding patterns O128-K, O128-L and O128-M2, respectively (Figure 5.3; lanes 15, 16 and 18) with more than 6 band differences. The rest of the human and ovine O128:H2 STEC isolates produced unique patterns (Table 5.1) with more than 6 band differences and were therefore considered genetically unrelated (Tenover *et al.*, 1995).

5.3.3 Analysis of PFGE patterns by Dice coefficient method

Analysis of the genetic relatedness of *E. coli* isolates of the same serotype by the Dice coefficient method demonstrated that isolates that produced restriction digestion patterns showing > 70% coefficient of similarity were possibly related (fewer than 6 bands difference) as defined by Tenover *et al.* (1995). PFGE patterns showing < 70% coefficient of similarity (more than 6 bands difference) appeared to be unrelated isolates. The patterns of PFGE fragments showing a 100% coefficient of similarity were likely to be clones of the same strain (Tenover *et al.*, 1995). With the dice coefficient the different banding patterns observed by visual inspection for each PFGE gel could be discriminated into different clusters, with possibly related clusters exhibiting a cut off level of 70% coefficient similarity (Figures 5.4, 5.5 and 5.6). The O5:H- STEC isolates fell into eleven different clusters (Figure 5.4). There was only one cluster (IV), which contained human STEC isolates H4 and H2 with 100% coefficient similarity. The most closely related isolates fell

into Cluster I (bovine STEC isolate 310 and human STEC isolate H1) exhibiting 83% coefficient similarity, while the potentially related clusters were VIII (Ovine STEC isolates VN6 and 1264) with 70% coefficient similarity and XI (Ovine STEC isolates CS147, CS468 and CS106) with 72 to 83% coefficient similarity (Figure 5.4). The rest of the clusters were more heterogeneous (< 70% coefficient similarity) (Figure 5.4). In the cluster analysis of O91:H- isolates, twelve fingerprint profile clusters were identified (Figure 5.5). Clusters V (Ovine STEC isolates CS21 and CS36) and XII (STEC isolate K13 from a meat sausage and human STEC isolate H5) showing 100% coefficient similarity indicating a clonal relationship. The potentially related isolates were the three ovine STEC isolates 1034, 849 and 726 (72 to 83% similarity) within cluster VI while the rest of the clusters were more heterogenous (< 70% similarity). Thirteen clusters were identified in the coefficient similarity analysis of O128:H2 STEC isolates (Figure 5.6). Clusters I (ovine STEC isolates 700 and 1138), V (human STEC isolates H13 and H10) and X (ovine STEC isolates M537 and M570) contained closely related isolates with 73 to 88% coefficient similarity. Cluster XIII contained ovine STEC isolates CS193, CS241 and CS462. The latter two showed 100% coefficient similarity while isolate CS193 exhibited 70% similarity indicating possible genetic relationship with isolates CS241 and CS462. The rest of the clusters exhibited <70% similarity indicating unrelated isolates.

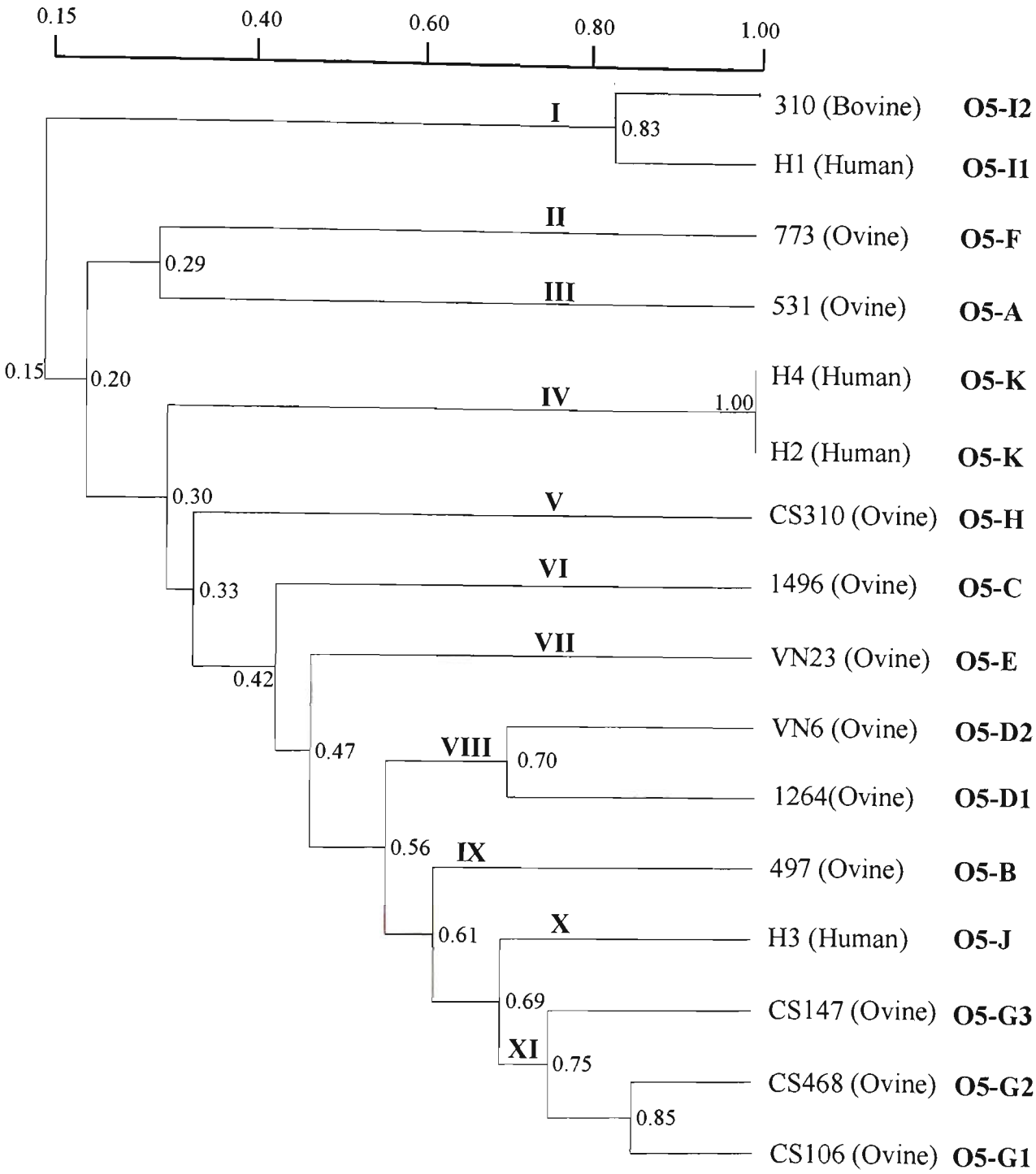


Figure 5.4 Dendrogram showing the estimated genetic relationship of 16 STEC isolates of serotype O5:H- isolated from sheep, humans and cattle. The dendogram was constructed with the use of unweighted pair group method with arithmetic means using the Dice coefficient. The banding patterns observed by visual inspection are shown in bold on the right hand corner. The genetically related/unrelated clusters as defined by Tenover *et al.* (1995) are shown in roman numerical numbers.

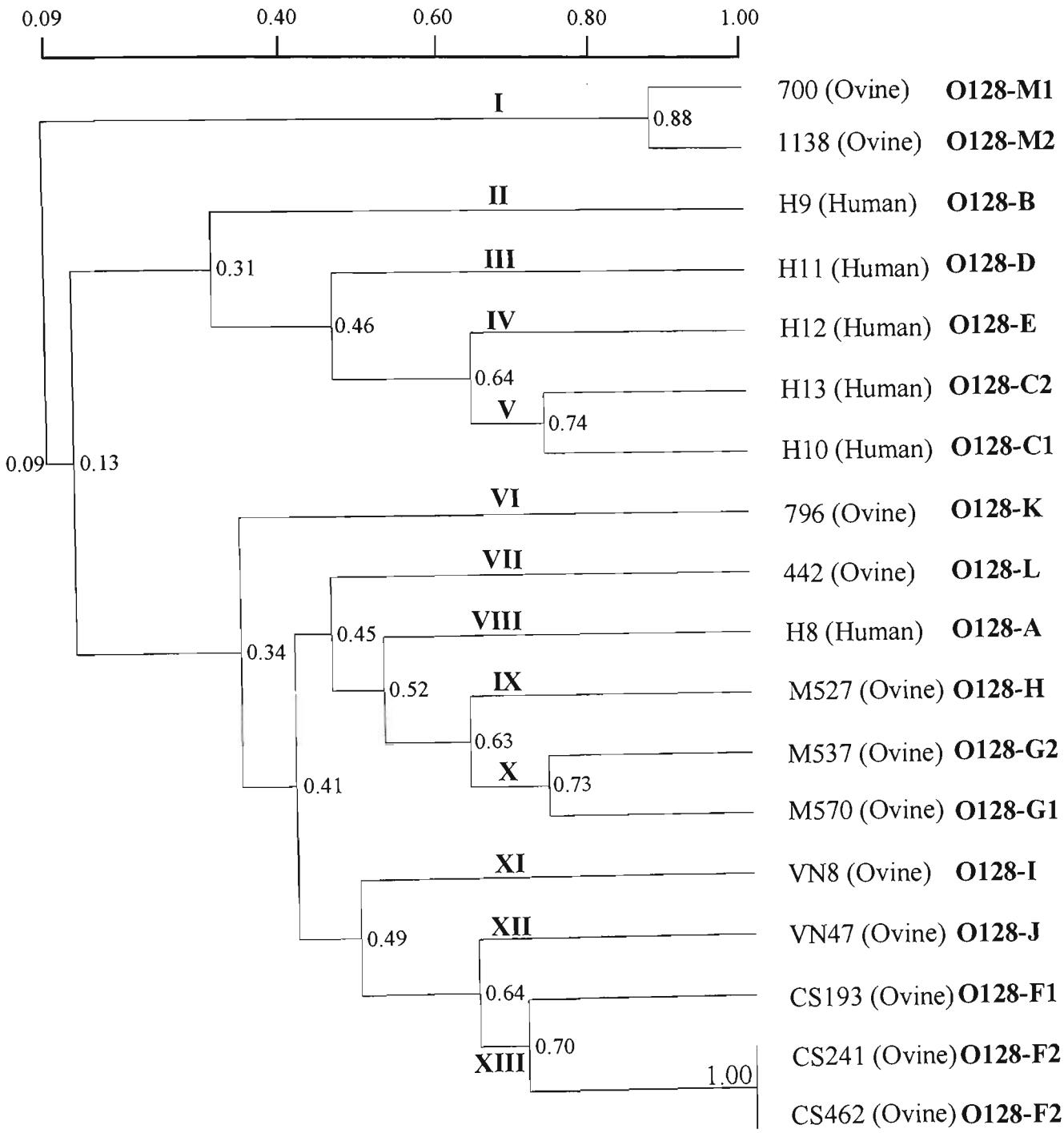


Figure 5.6 Dendrogram showing the estimated genetic relationship of 18 STEC isolates of serotype O128:H2 isolated from sheep and humans. The dendogram was constructed with the use of unweighted pair group method with arithmetic means using the Dice coefficient. The banding patterns observed by visual inspection are shown in bold on the right hand corner. The genetically related/unrelated clusters as defined by Tenover *et al.* (1995) are shown in roman numerical numbers.

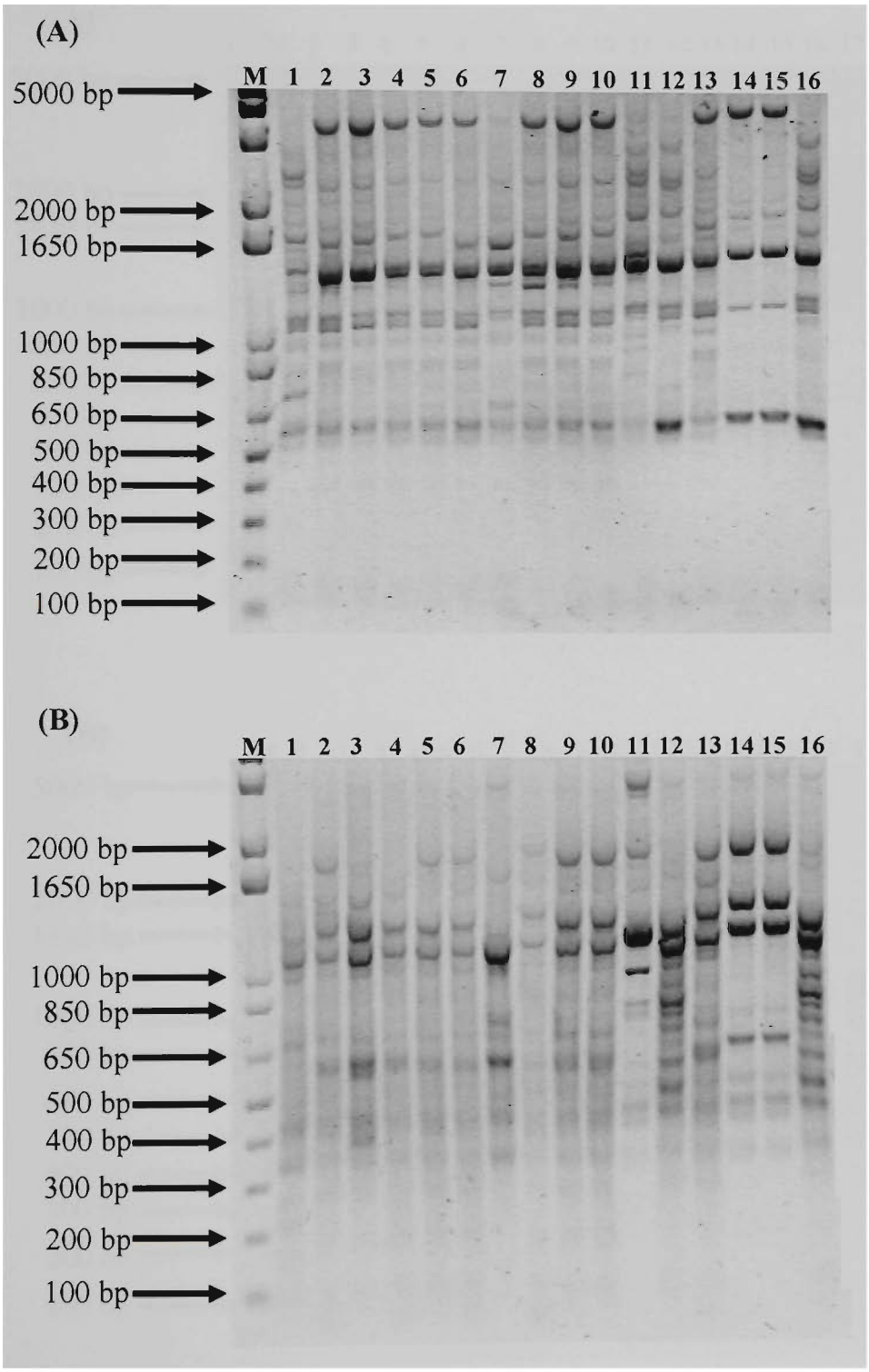


Figure 5.7 DNA fingerprint analysis of *E. coli* O5:H- isolates by REP-PCR (A) and ERIC-PCR (B). Lanes: M, I Kb plus DNA ladder; 1, 531 (ovine); 2, 497 (ovine); 3, 1496 (ovine); 4, 1264 (ovine); 5, VN23 (ovine); 6, VN6 (ovine); 7, 773 (ovine); 8, CS106 (ovine); 9, CS468 (ovine); 10, CS147 (ovine); 11, CS310 (ovine); 12, H1 (human); 13, H3 (human); 14, H2 (human); 15, H4 (human); 16, 310 (bovine).

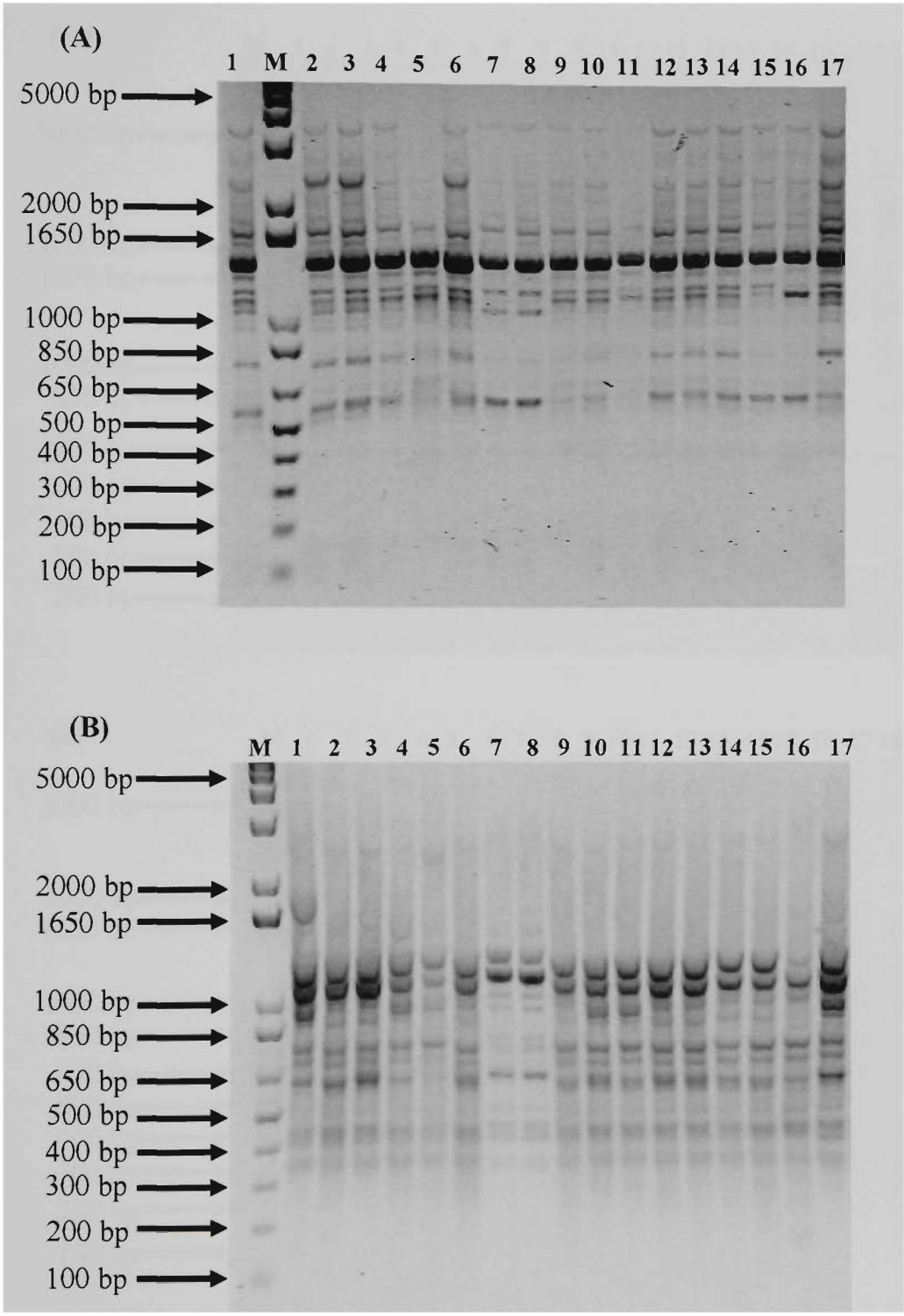


Figure 5.8 DNA fingerprint analysis of *E. coli* O91:H- isolates by REP-PCR (A) and ERIC-PCR (B). Lanes: M, I Kb plus DNA ladder; 1, 639 (bovine); 2, 726 (ovine); 3, 849 (ovine); 4, VN56 (ovine); 5, 755 (ovine); 6, 1034 (ovine); 7, K16 (ovine); 8, K6 (lamb carcass); 9, 917 (ovine); 10, K14 (ovine); 11, K13 (mettwurst sausage); 12, CS21 (ovine); 13, CS36 (ovine); 14, M501 (ovine); 15, H5 (human); 16, H17 (human); 17, H6 (human).

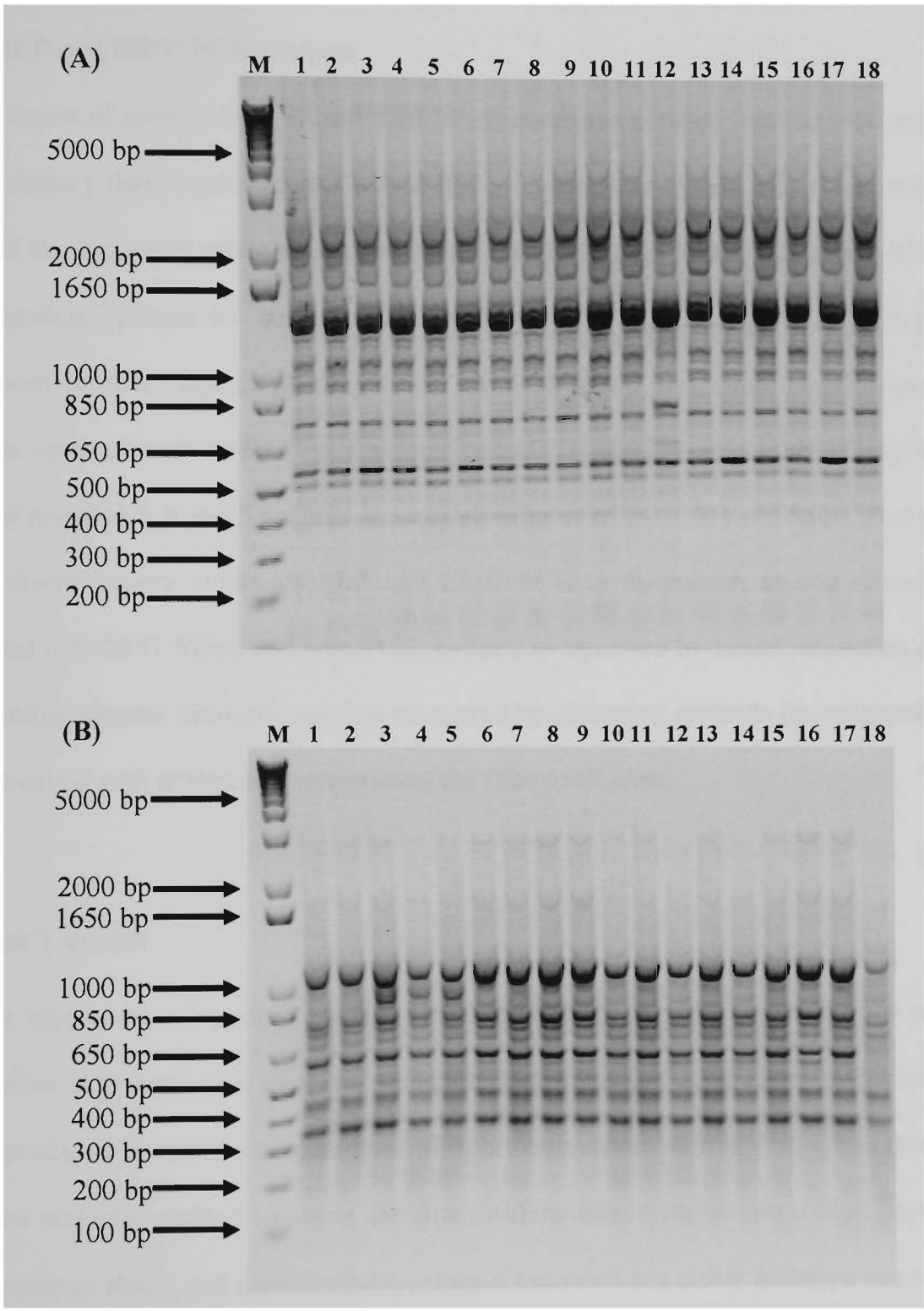


Figure 5.9 DNA fingerprint analysis of STEC O128:H2 isolates by REP-PCR (A) and ERIC-PCR (B). Lanes: M, I Kb plus DNA ladder; 1, H8 (human); 2, H9 (human); 3, H10 (human); 4, H11 (human); 5, H12 (human); 6, H13 (human); 7, CS193 (ovine); 8, CS241 (ovine); 9, CS462 (ovine); 10, M570 (ovine); 11, M527 (ovine); 12, M537 (ovine); 13, VN8 (ovine); 14, VN47 (ovine); 15, 796 (ovine); 16, 442 (ovine); 17, 700 (ovine); 18, 1138 (ovine).

5.3.4 REP and ERIC PCR analysis

Investigation of isolates by REP and ERIC PCR generated patterns less complex and less discriminatory than those obtained with PFGE (Figures 5.7, 5.8 and 5.9). The molecular sizes of the fragments produced ranged from 300 to 5000 bp. Visual inspection of REP-PCR banding patterns for serotypes O5:H-, O91:H- and O128:H2 revealed 9, 6 and 2 fingerprint profiles respectively (Figures 5.7A, 5.8A and 5.9A). Analysis of fingerprint patterns obtained with ERIC-PCR for serotypes O5:H-, O91:H- and O128:H2 by visual scrutiny revealed 9, 8 and 5 profiles respectively (Figures 5.7B, 5.8B and 5.9B). Due to the lower discriminatory ability of REP and ERIC PCR to distinguish among related and unrelated non-O157 STEC and non-STEC isolates as observed by visual inspection of the gel pictures, genetic similarity was not compared by clustering methods (unweighted pair-group method with arithmetic means) using the Dice coefficient.

5.4 DISCUSSION

Isolates need to be subtyped to provide ample strain discrimination to identify the source and modes of transmission of STEC infection. Determination of serotypes and virulence factor profiles add only a moderate level of information about clonality. PFGE is probably the most powerful method available for strain differentiation and shows a high degree of discriminatory ability and reproducibility, since it examines at a stable genotype rather than variably expressed phenotypic attributes. PFGE is a derivative of conventional agarose gel electrophoresis in which the direction of the electric field is changed repeatedly, enabling the resolution of large DNA fragments generated by restriction enzyme digestion of the bacterial chromosomal DNA (Versalovic *et al.*, 1993).

This technique has been successfully applied to genetically characterise epidemiologically related and unrelated STEC O157:H7 strains associated with human infections (Meng *et al.*, 1995; Banatvala *et al.*, 1996; Izumiya *et al.*, 1997; Welinder-Olsson *et al.*, 2002). Although molecular typing of non-O157 STEC isolates using PFGE has been reported (Eklund *et al.*, 2001; Pradel *et al.*, 2001; Khan *et al.*, 2002; Welinder-Olsson *et al.*, 2002), no studies have been undertaken to evaluate the genetic relatedness of non-O157 STEC isolates in particular O5:H-, O91:H- and O128:H2 which are the most common serotypes found in sheep (Bettelheim *et al.*, 2000; Djordjevic *et al.*, 2001). These serotypes have been recovered from (or strongly implicated in) patients with severe human infections such as HUS and HC (www.microbionet.com.au). In the present study, sixteen O5:H-, seventeen O91:H- and eighteen O128:H2 isolates recovered from sheep, cattle and humans were molecular typed by PFGE. Considerable variations in the PFGE profiles were observed for isolates of the same serotype from animals from different farms and even among isolates within the same farm, although isolates with identical PFGE patterns were also identified.

Interpretation of chromosomal patterns can be complicated when isolates differ by only a few DNA fragments. Such differences could occur within a single strain due to inversions, deletions, re-arrangements of the chromosome or from the acquisition or loss of a prophage, transposon or insertion sequence. In this study, PFGE fingerprints were interpreted according to the methods of Tenover *et al.* (1995). Fingerprint patterns exhibiting fewer than 6 bands difference (> 70% coefficient of similarity) were considered a potentially related cluster and PFGE patterns showing more than 6 bands difference (< 70% coefficient of similarity) were judged to be unrelated isolates.

Among the sixteen O5:H- STEC isolates investigated fifteen PFGE band patterns were observed. Among the seventeen O91:H- *E. coli* isolates investigated fifteen PFGE band patterns were observed. Seventeen band profiles were observed among the eighteen O128:H2 STEC isolates investigated. Most of the ovine STEC isolates of the same serotype seemed to be genetically heterogeneous by PFGE, even though they possessed the same virulence profiles. Large varieties of PFGE patterns have been reported among both STEC O157 and non-O157 STEC isolates (Eklund *et al.*, 2001; Khan *et al.*, 2002; Welinder-Olsson *et al.*, 2002). In this study, isolates from the same farm produced closely related patterns or identical patterns (O5G1-G3, O91B1-B3, O128F1-F3 and O128G1-G2) and are probably subtypes of the same strain. The variation in the patterns (fewer than 6 bands difference) may have occurred due to one or two independent genetic events (Tenover *et al.*, 1995). However, ovine O128:H2 STEC isolates 442, 796 and 1138 from the same farm produced banding patterns distinct from each other by more than 6 bands and clustered in different groups suggesting that these are unrelated isolates. A similar observation was made for two ovine STEC isolates of serotype O5:H- (531 and 497) from the same farm which produced unrelated banding profiles. Furthermore, isolate M527 from a sheep belonging to the same farm as isolates M537 and M570 produced unrelated patterns (exhibiting more than 6 band difference). Interestingly, ovine O5:H- STEC isolate pair VN6, 1264 and O128:H2 STEC isolate pair 700, 1138 exhibited 70% and 88% coefficient similarity respectively, suggesting that these were related isolates. However, these isolates were obtained from different farms. Movement of sheep between different farms may have caused cross-farm transmission of closely related strains of the same serotype. However, no evidence exists to support this hypothesis for these particular animals.

One of the aims of the study was to determine whether the non-O157 STEC isolates from patients with diarrhoea or HUS were related to those from sheep even though they were not epidemiologically related. None of the isolates from humans produced closely related PFGE patterns with the ovine isolates and exhibited a high level of heterogeneity (<70% coefficient similarity). However, a bovine O5:H- STEC isolates and a human O5:H- STEC isolates recovered from a patient with HUS in New Zealand produced closely related profiles (3 bands difference; 83% coefficient similarity) suggesting one of these isolates may have been derived from the other by a single genetic event (Tenover *et al.*, 1995). Furthermore, these two isolates shared identical virulence factor profiles, possessing stx_1 (stx_{1c} subtype), *eae* (Int-β subtype) and *ehxA*. The export of contaminated beef may be a reason for the incidence of closely related subtype of O5:H- serotype found in a different country. However, STEC serotype O5:H- is primarily recovered from sheep and less frequently from cattle and it has been hypothesised that particular STEC serotypes preferably inhabit different ruminant species (Hornitzky *et al.*, submitted).

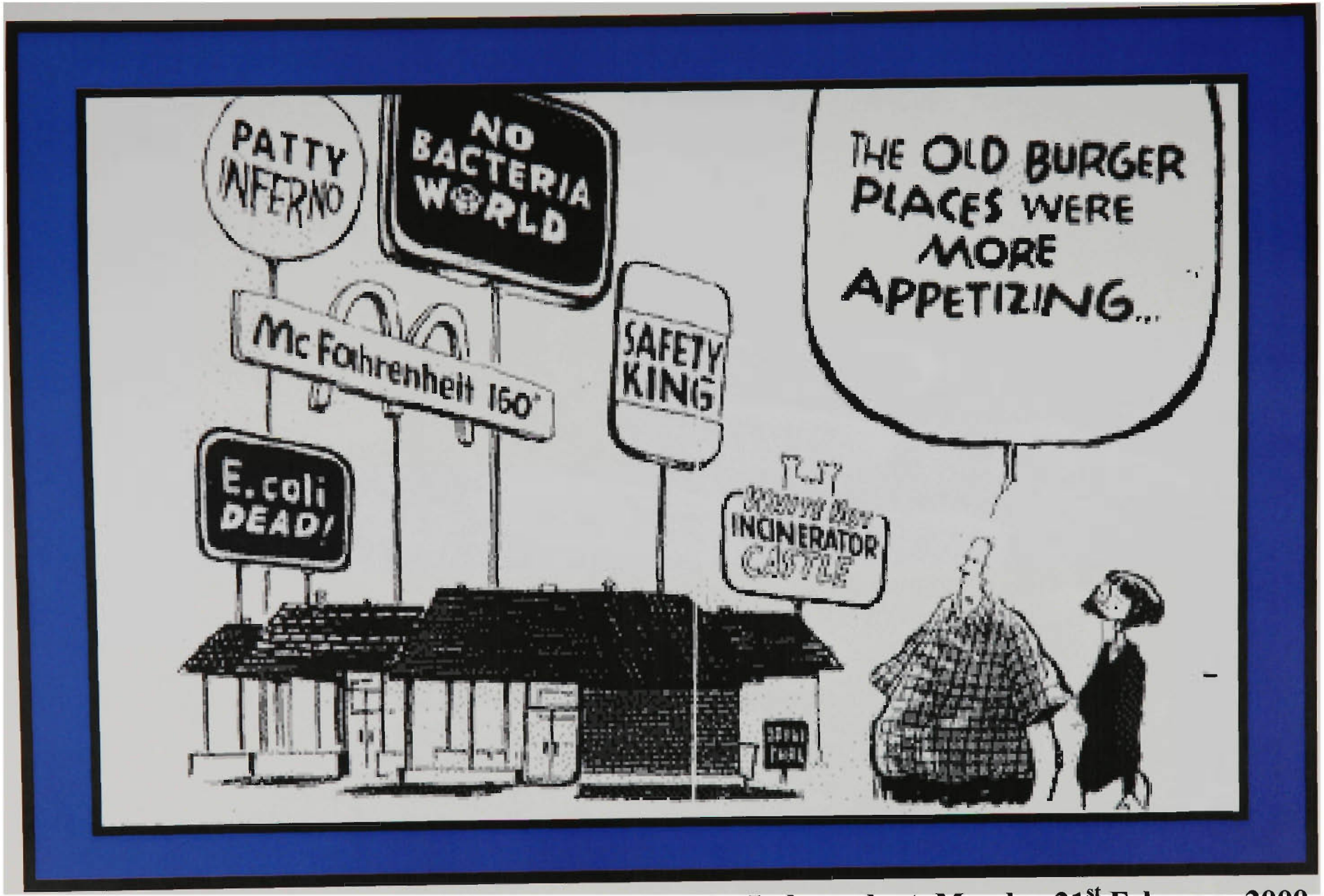
STEC of serotype O91:H- (H5) isolated from a patient with diarrhoea linked to the Garibaldi outbreak in South Australia produced an identical PFGE pattern with a STEC isolate (K13) recovered from a mettwurst sausage incriminated as the vehicle of transmission in the Garibaldi outbreak. Further, both the isolates possessed identical virulence profiles and *stx* subtypes. STEC serotype O111:H- was associated with the outbreak of HC and HUS in South Australia which was linked to the consumption of contaminated mettwurst sausage in which 23 children developed HUS with one mortality (Paton *et al.*, 1996). A variety of serogroups including O111:H- were isolated from patients

and the incriminated food source, indicating multiple strain infection (Paton *et al.*, 1996). This is further supported by the recent report by Kulkarni *et al.* (2002) who identified antibody responses to 34 different serogroups in the HUS patients associated with the outbreak. The isolation of O91:H- STEC isolates from the sausage and from a patient with diarrhoea linked to the outbreak, possessing identical virulence profile and PFGE patterns strengthens the involvement of several STEC serotypes in the outbreak. Further, it demonstrates the efficiency of PFGE as an epidemiological tool for tracing STEC infections. The contents of mettwurst contain a variety of meat including beef, sheep and pork and therefore it is not surprising to have isolated a O91:H- serotype, since it is a common ovine serotype. Human STEC isolates H4 and H2 produced identical patterns (100% coefficient similarity). These two isolates were derived from the same patient with HUS. However, they differed in their ability to ferment sorbitol (Karl Bettelheim, personal communication) and possessed different virulence factor combinations.

Analysis by REP and ERIC PCR generates species and strain specific DNA fingerprints of Gram-negative enteric bacteria (Versalovic *et al.*, 1991). Repetitive sequences in the bacterial genome are useful targets for DNA-based typing due to their restricted length and their widespread occurrence, although little is known about their function (Versalovic *et al.*, 1991). ERIC PCR has been successfully applied to genetically fingerprint STEC serogroup O157 (Adwan *et al.*, 2002). However, there have been no reports on the use of REP and ERIC PCR to fingerprint non-O157 STEC strains. REP and ERIC PCR banding patterns observed in this study were less complicated than those obtained by PFGE, but less discriminatory. They did not characterise the strains into as many groups as seen by PFGE. However, the major advantages of the PCR-based fingerprint methods (ERIC-PCR, REP-

PCR and arbitrarily primed [AP]-PCR) are technical simplicity, wide availability of equipment and reagents and rapid achievability.

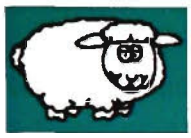
The data obtained in this study did not establish any clonal relationship between ovine and human STEC isolates of the same serotype by PFGE. However, it is known that epidemiologically unrelated isolates show unique PFGE profiles demonstrating great clonal diversity (Zhang *et al.*, 2000; Pradel *et al.*, 2001; Welinder-Olsson *et al.*, 2002). Further, horizontal gene transfer of mobile genetic elements such as pathogenecity islands, transposon and plasmids can occur at high rates among *E. coli* which could significantly alter PFGE patterns among a particular clonal lineage (de la Cruz and Davies, 2000; Dougan *et al.*, 2001). This study identifies PFGE as a useful molecular typing method for discriminating non-O157 STEC strains compared to REP and ERIC PCR based molecular typing, even though PFGE is more expensive and time-consuming. Further, in addition to DNA fingerprinting by PFGE, other typing techniques such as *stx* subtyping, *eae* subtyping and phage typing should also be used in epidemiological studies. Judging from the vast majority of non-O157 STEC strains isolated from animals and humans (www.microbionet.com.au), standardisation of the PFGE method and computer-based submission of the genomic profiles of non-O157 STEC may facilitate in discriminative and rapid comparison of non-O157 STEC strains among laboratories worldwide and may assist in clarifying the role of non-O157 STEC of ovine origin in human infections.



Adapted from The Independent, Monday 21st February 2000

Chapter 6

General Discussion and Conclusions



6 – General Discussion and Conclusions

There have been many studies examining the prevalence and characteristics of STEC recovered from cattle (Blanco *et al.*, 1996; Blanco *et al.*, 1997; Orden *et al.*, 1998; Midgley *et al.*, 1999; Pradel *et al.*, 2000; Bertin *et al.*, 2001; Cobbold and Desmarchelier, 2001; Midgley and Desmarchelier, 2001; Kobayashi *et al.*, 2001). Few studies have extensively examined the presence and virulence factor profiles of STEC in sheep, especially in Australia, which is one of the largest sheep producing countries in the world (Fegan and Desmarchelier, 1999; Bettelheim *et al.*, 2000; Djordjevic *et al.*, 2001). Although these studies identified the STEC virulence factor (*stx*$_1$, *stx*$_2$, *eae* and *ehxA*) combinations of STEC recovered from sheep, none of these studies have characterised the subtypes of these virulence factors present in the STEC populations recovered from sheep in Australia. Only two studies have so far reported the characterisation of *stx*$_1$ among ovine STEC and both these studies were conducted in Germany (Koch *et al.*, 2001; Zhang *et al.*, 2002). Furthermore, the characterisation of *stx*$_2$ and *eae* present in STEC of ovine origin has not been reported in the literature.

The overall aim of this thesis was to characterise the major STEC virulence factors (*stx*$_1$, *stx*$_2$ and *eae*) among a serologically diverse collection of STEC strains primarily recovered from sheep in Australia and to compare these factors with STEC isolates of the same serotypes recovered from symptomatic and asymptomatic humans. Non-STEC isolates recovered from sheep and humans were also included in the intimin typing study because a considerable number of *E. coli* isolates recovered from sheep and humans possessing intimin did not contain Shiga toxin genes. To further assist in determining the relationship

between ovine and human STEC, fingerprint analysis was carried out on the common ovine STEC serotypes O5:H-, O91:H- and O128:H2 recovered from both ovine and human sources.

The association of stx_1 and stx_2 gene subtypes with STEC strains from sheep and human sources were examined in Chapters 2 and 3. A PCR-RFLP system was developed to differentiate stx_{1c} from the other three common stx_1 subtype and was subsequently used to characterise stx_1 from 237 STEC strains recovered from sheep and humans. The stx_1 subtyping data revealed the predominance of stx_{1c} subtype (133 of 203 ovine isolates; 65.5%) which was present in 40 different serotypes including O5:H-, O75:H8, O123:H- and O128:H2 which are frequently isolated from ovine sources and rarely from bovine sources (Beutin *et al.*, 1997; Kudva *et al.*, 1999; Bettelheim *et al.*, 2000; Djordjevic *et al.*, 2001; Hornitzky *et al.*, 2001). However, isolates of serotype O91:H-, another commonly reported ovine STEC serotype, predominantly possessed a common stx_1 subtype (26 of 31; 83.8%) although 5 isolates possessed the stx_{1c} subtype. Furthermore, some serotypes, particularly O75:H8 (14 of 21 isolates) were shown to simultaneously possess both stx_1 and stx_{1c} subtypes. Interestingly, none of the ovine STEC strains that belonged to the classical EHEC serogroups (O26, O103 and O157) were found to carry the stx_{1c} subtype. Koch *et al.* (2001), who examined the presence of stx_{1c} subtype in STEC strains recovered from humans and animals in Germany, identified stx_{1c} as the predominant subtype in sheep. However, this study only investigated 76 STEC isolates from animals, of which 48 were from sheep and belonged to 6 different serotypes (O5:H-, O91:H-, O125:H-, O146:H21, O128:H2 and OX3:H8). With the exception of serotypes O125:H- and O146:H21, the rest of the serotypes investigated by Koch *et al.* (2001) were part of the stx_1 subtyping study in

this thesis, which also included an additional 36 different serotypes from sheep. Therefore the stx_1 subtyping study presented in this thesis is the most comprehensive study so far performed on characterising stx_1 in a serologically diverse collection of STEC recovered from sheep.

Stx2 producing isolates are more commonly associated with serious disease than isolates producing Stx1 or Stx1 and Stx2 (Kleanthous *et al.*, 1990; Boerlin *et al.*, 1999; Friedrich *et al.*, 2002). Further, it has been shown that the stx_2 genotype augments the ability of STEC to cause serious human diseases (Ostroff *et al.*, 1989; Boerlin *et al.*, 1999). Fourteen different stx_2 variants have been described from humans with clinical symptoms and from animals (refer to Table 1.4). Among the 14 stx_2 variants, stx_{2c} possessing STEC isolates are usually associated with serious human illness such as HUS, whilst STEC isolates possessing stx_{2d} or stx_{2e} variants are commonly recovered from patients with milder infections such as diarrhoea (Friedrich *et al.*, 2002). It has been shown that many STEC of ovine origin contain stx_2 and express the toxin (Djordjevic *et al.*, 2001). However, no reports have identified the stx_2 subtype in STEC of ovine origin.

Previously reported stx_2 subtyping PCR-RFLP systems (Bastian *et al.*, 1998; Pierard *et al.*, 1998) were used to characterise the stx_2 gene from 167 stx_2-containing STEC strains primarily from sheep and humans. The predominant stx_2 variants identified in this study in ovine STEC isolates that also contain *ehxA* and/or *eae* were the stx_{2d} subtypes $stx_{2d\text{-}Ount}$, $stx_{2d\text{-}O111}$ and $stx_{2d\text{-}OX3a}$. One hundred and nineteen of 146 (81.5%) ovine STEC possessed $stx_{2d\text{-}Ount}$ and were predominantly of serotypes O75:H-/H8/H40, O91:H-, O123:H-, O128:H2 and OR:H2; 17 of the remaining 27 were of serotype O5:H- and possessed $stx_{2d\text{-}}$

O111/OX3a subtypes. Only 4 of 146 (2.9%) ovine STEC possessed *stx*$_{2vha}$ (all serotype O157:H-/H21 isolates) and one isolate (serotype O5:H-) possessed *stx*$_2$.

In contrast, *stx*$_{1c}$ and *stx*$_{2d}$ subtypes are rarely detected in STEC recovered from cattle (Koch *et al.*, 2001; Brett *et al.*, 2003). None of the bovine STEC strains examined by Koch *et al.* (2001) were positive for an *stx*$_1$ gene or carried the *stx*$_{1c}$ variant. Among the bovine STEC isolates examined by Brett *et al.* (submitted) five of 81 (6.2%) and 5 of 193 (2.6%) possessed *stx*$_{1c}$ and *stx*$_{2d}$ subtypes respectively. In a recent study by Bertin *et al.* (2001), only 14 of 167 STEC strains recovered from healthy cattle possessed an *stx*$_{2d}$ subtype. However, the serotype(s) of these strains were not reported. Furthermore, STEC serotypes commonly recovered from ovine sources are less frequently isolated from cattle (Beutin *et al.*, 1993; Beutin *et al.*, 1997; Hornitzky *et al.*, 2001). The observations made in this study and other studies (Montenegro *et al.*, 1990; Djordjevic *et al.*, 2001; Hornitzky *et al.*, 2001; Brett *et al.*, 2003) suggest that genetically and serologically different populations of STEC inhabit the gastro-intestinal tract of cattle and sheep. Furthermore, serotypes (eg. O157:H7/H-) which are recovered from both cattle and sheep suggests that some serotypes display a broader host range. Irrespective of host source, O157 isolates have never been shown to possess either *stx*$_{1c}$ or *stx*$_{2d}$ genes and always possess either the common *stx*$_1$ and/or *stx*$_2$/*stx*$_{2vh}$ subtypes (Brett *et al.*, 2003; Brett *et al.*, submitted).

In addition to producing Stx, STEC may contain additional virulence factors such as intimin encoded by *eae*, one of the constituent genes of LEE, which is responsible for inducing A/E lesions in intestinal epithelial cells (McDaniel *et al.*, 1995; Frankel *et al.*, 1998). Intimin is a well characterised outer membrane protein expressed by most STEC

strains. Based on amino acid sequence divergence, ten intimin subtypes have been reported (α1, α2, β, γ, θ, δ, ε, ζ, ι, λ). There is a paucity of information describing intimin subtypes from *E. coli* recovered from ruminants. Oswald *et al.* (2000) characterised intimin from 22 STEC isolates recovered from cattle. However, studies have not been conducted to characterise the intimin subtypes from *E. coli* of ovine origin. A multiplex PCR assay (Reid *et al.*, 1999) and intimin subtype-specific PCR assays (Adu-Bobie *et al.*, 1998; Oswald *et al.*, 2000) have been developed to facilitate identification of intimin subtypes. However, no single assay has been developed to characterise all of the reported intimin subtypes.

In this thesis, a typing system was developed to type intimin from 134 of 153 *E. coli* strains from ovine and human sources (Chapter 3). This is the first system described that is capable of identifying all ten reported intimin variants in a single PCR-RFLP assay. With the exception of Int-λ, all nine intimin subtypes were identified in the 134 *E. coli* isolates examined in this study. In addition, the typing scheme facilitated the identification of two previously unreported intimin subtypes designated Int-ε2 and Int-ι2, which were identified in ovine *E. coli* isolates of serotypes O2-related:H19 and OR:H-. Intimin β was the most commonly identified subtype (58 of 153; 37.9%) among *E. coli* isolates recovered from both sheep and humans, and was found to be associated with the greatest diversity of serotypes (n=16). Using the typing system described in this thesis, all ten previously reported intimin types were identified in a study of intimin subtypes in 79 *E. coli* isolates recovered from cattle (Brett *et al.*, unpublished results). Therefore the typing system reported in this study is capable of characterising intimin from *E. coli* from a broad range of serotypes and from various sources. Phylogenetic analysis using the Phylip package

(http://www.bioweb.pasteur.fr) of all available Int_{280} sequences confirmed the previous division of intimin into the six groups (α, β, γ, δ, ε and θ) (Adu-Bobie *et al.*, 1998, Oswald *et al.*, 2000; Tarr and Whittam, 2002). In addition, the intimin subtypes ζ, ι and λ submitted to GenBank, also resolved as distinct groups. Phylogenetic analysis confirmed the close relationship of the two new subtypes ε2 and ι2 reported in this thesis, with intimin ε and ι respectively. Further, this study is the first comprehensive study to characterise and identify intimin subtypes in *E. coli* recovered from sheep.

In contrast to stx_1 and stx_2, host specificity was not observed among intimin types. However, notable serotype specificity was observed. For example, intimin β was observed in all *E. coli* isolates of serotype O26:H11 recovered from cattle (Brett *et al.*, personal communication), sheep and humans. Interestingly, intimins from 19 of 105 (18%) ovine *E. coli* strains representing 16 different serotypes could not be typed by the PCR-RFLP system indicating a potentially larger variety of intimin variants in STEC derived from ovine faeces. Further studies need to be carried out to characterise these intimin variants to determine if they represent novel subtype(s) of intimin or if they belong to divergent subset(s) of previously reported intimin subtype(s).

Although STEC strains belonging to serotype O157:H7 are by far the predominant serotype associated with human disease worldwide (Paton and Paton, 1998), more than 100 non-O157 STEC strains have been reported to be associated with severe human illness (www.microbionet.com.au). In some geographic areas including Australia, non-O157 strains are more commonly isolated from patients with diarrhoea or HUS than are O157

STEC strains (Elliot *et al.*, 2001). Furthermore, humans are probably more frequently exposed to non-O157 STEC from food and environmental sources since meat producing animals, especially ruminants, excrete STEC of which only a small percentage may be O157 *E. coli* (Bettelheim *et al.*, 2000; Djordjevic *et al.*, 2001; Meyer-Broseta *et al.*, 2001; Brett *et al.*, submitted; Hornitzky *et al.*, in press). However, the significance of non-O157 STEC strains in human infection is less well understood than that of the STEC O157:H7 strains and is underestimated because of diagnostic limitations (Karch *et al.*, 1999). A variety of non-O157 STEC serogroups (O26, O91, O123, O128, O111, O113 and O160) were identified from patients and from the source material (mettwurst sausage) during a food poisoning outbreak in Adelaide, Australia (Paton *et al.*, 1996). A recent study examining the sera from the HUS patients involved in this outbreak revealed antibody responses to 34 (including O157) serogroups indicating mixed infection. Patients with severe renal failure were shown to develop antibodies to a larger number of serogroups than those with mild infection (Kulkarni *et al.*, 2002). This and other studies (Goldwater and Bettelheim, 1994; Goldwater and Bettelheim, 2000) emphasize the significance of non-O157 STEC strains in human infections and the importance of screening for multiple STEC serotypes during sporadic and epidemic STEC outbreaks.

Another aspect of this thesis was to compare the virulence factor type of STEC isolated from sheep with virulence factor profiles of STEC of the same serotype recovered from symptomatic and asymptomatic humans to examine the role (if any) of ovine STEC in human pathogenesis. Human STEC isolates possessing the same serotypes as STEC isolates recovered from sheep were shown to harbour the same stx_1 and stx_2 types. Of 34 isolates from humans, twelve (35.3%) isolates possessed the stx_{1c} subtype and belonged to

serotypes O5:H-, O128:H2, O123:H- and OX3:H8, which are the common serotypes associated with STEC recovered from sheep (Bettelheim *et al.*, 2000; Djordjevic *et al.*, 2001). All twelve isolates possessing stx_{1c} were recovered from patients with HUS or diarrhoea. Subtyping of stx_2 from 21 STEC isolates recovered from humans revealed the presence of stx_{2d} subtype in eleven (52.4%) STEC strains, belonging to serotypes O5:H-, O91:H-, O123:H-, O128:H2 and OX3:H8; all which are commonly associated with sheep (Bettelheim *et al.*, 2000; Djordjevic *et al.*, 2001). Interestingly several STEC from HUS patients with serotypes not commonly associated with sheep did not possess either stx_{1c} or stx_{2d} subtypes. However, two human STEC isolates with serotype O91:H-, a common ovine serotype, recovered from asymptomatic patients contained an stx_1 subtype. These O91:H- isolates are atypical compared with other common ovine STEC serotypes in that they possess a common stx_1 subtype.

In a recent study, Zhang *et al.* (2002) suggested that STEC strains harbouring the stx_{1c} subtype may be associated with either mild disease or with asymptomatic carriage. This may have been due to the absence of *eae* in all of the stx_{1c} possessing human STEC strains investigated. However, in this thesis, *eae* was identified in a human STEC strain of serotype O5:H- recovered from a patient with HUS as well as in two ovine isolates of serotypes O106:HR and O158:HR. All three isolates possessed a stx_{1c} variant. In a study investigating the clinical significance of STEC harbouring stx_2 variants, stx_{2d} was not detected in 268 STEC isolates from patients with HUS (Friedrich *et al.*, 2002). The stx_{2d} variant was identified in 41 of 262 (15.6%) and 21 of 96 (21.8%) STEC strains from patients with diarrhoea who did not develop HUS and asymptomatic individuals respectively (Friedrich *et al.*, 2002). All stx_{2d} positive isolates were found to be *eae*

negative. Therefore, the authors suggested that STEC harbouring the stx_{2d} variant and are negative for *eae* might cause milder disease with a minimal risk of developing HUS. These data are consistent with studies by Pierard *et al.* (1998) showing that stx_{2d} positive isolates are less frequently associated with HUS and do not possess *eae*. In this thesis, stx_{2d} was identified in two STEC strains of serotypes O5:H- and OX3:H8 recovered from patients with HUS and neither isolate possessed *eae*. However, whether or not these human STEC isolates possessing stx_{1c} and stx_{2d} identified in this study are directly involved with the development of HUS cannot be confirmed but should not be discounted.

Genetic fingerprinting using PFGE, REP PCR and ERIC PCR were carried out on ovine and human STEC isolates of the same serotype to determine the possibility of a clonal relationship. PFGE proved to be a useful molecular typing tool for discriminating non-O157 STEC strains compared to REP and ERIC PCR. PFGE fingerprints produced by STEC strains isolated from various sources may provide significant assistance during epidemiological investigations in identifying the source of STEC infections during outbreaks. In this study, none of the human STEC isolates produced closely related PFGE patterns with ovine STEC isolates and exhibited a high level of heterogeneity, even though they harboured identical stx_1 and stx_2 variants. However, a STEC isolate of serotype O91:H- isolated from a patient with diarrhoea linked to the Garibaldi outbreak in South Australia produced an identical PFGE pattern with an O91:H- STEC isolate recovered from a mettwurst sausage incriminated as the vehicle of transmission in the Garibaldi outbreak. This shows that PFGE is a useful epidemiological tool for tracing human infections. Even though the majority of human and ovine STEC isolates of the same serotypes did not produce identical or closely related PFGE patterns, the clonal relationship between the

isolates could not be ruled out because mobile genetic elements (pathogenecity islands, transposon, plasmid and gene cassettes) can be introduced into particular *E. coli* clonal types via horizontal gene transfer which could alter PFGE patterns (Cruz and Davies, 2000; Dougan *et al.*, 2001). Therefore the genetic heterogeneity observed among ovine and human STEC isolates in this study may have been due to either serotypes being clonally divergent or outcome of the introduction of mobile genetic elements into their genomes. However, future studies need to be carried out using multi locus gel electrophoresis technique to further clarify the extend of genetic heterogeneity among these isolates.

In conclusion, this thesis provides a detailed characterisation of STEC virulence factors (*stx*$_1$, *stx*$_2$ and *eae*) among a broad range of STEC serotypes primarily recovered from the normal intestinal microflora of healthy sheep in Australia. STEC of ovine origin primarily contain *stx*$_{1c}$ and *stx*$_{2d}$ subtypes. STEC isolates recovered from symptomatic and asymptomatic humans that belonging to the same common serotypes as ovine STEC also possess the same Stx variants. However, it is believed that human infections by STEC strains harbouring *stx*$_{1c}$ and/or *stx*$_{2d}$ variants are usually asymptomatic or induce mild diarrhoeal infections with a minimal risk of developing HUS. Therefore, sheep can be considered to be a reservoir of pathogenic STEC strains that primarily cause mild infections in humans. However, the role of sheep as reservoirs for STEC strains causing mild infections such as diarrhoea should not be underestimated.

The intimin typing system developed in this thesis will facilitate more efficient identification of intimin subtypes from *E. coli* isolated from various sources. The significance of various intimin types other than α and γ in relation to tissue tropism is

currently not known. Therefore future studies need to be carried out to determine the significance of the different intimin subtypes detected among *E. coli* isolates recovered from sheep, in human pathogenesis. In conjunction with *stx* and *eae* typing, the application of PFGE fingerprinting procedures in epidemiological investigations could significantly contribute to the identification of sources of STEC strains during epidemic and sporadic outbreaks.

The work presented in this thesis provides a basis for future studies in determining the pathogenicity of STEC of ovine origin to humans. Although most STEC of ovine origin possess enterohaemolysin, which is believed to be a marker for virulence, it is not known why these STEC strains do not cause severe disease in humans. Further studies need to be carried out to screen and characterise other potential virulence factors such as, *ure* (Heimer *et al.*, 2002), *pil* (Srimanote *et al.*, 2002), *saa* (Paton *et al.*, 2001), *efa*1 (Nicholls *et al.*, 2000), *espP* (Brunder *et al.*, 1997), *katP* (Brunder *et al.*, 1996) and *astA* (Savarino *et al.*, 1991) in STEC recovered from sheep to further clarify the role of these STEC in human pathogenesis.

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Appendix

APPENDIX A – LIST OF *E. coli* ISOLATES USED IN THIS THESIS

Isolate Reference No.	Serotype	Source	Virulence Profile				stx_1 subtyping	stx_2 subtyping	*eae* subtyping	Genotyping
			stx_1	stx_2	*eae*	*ehxA*				
CNC155	O2 related :H19	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
293	O2:H29	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
NC967	O3:H8	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
VN2	O5:H-	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
VN6	O5:H-	Sheep faeces	+	+	-	+	Yes [a]	Yes [a]	N/A	Yes
VN7	O5:H-	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
VN11	O5:H-	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
VN18	O5:H-	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
VN19	O5:H-	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
VN23	O5:H-	Sheep faeces	+	+	-	+	Yes [a]	Yes [a]	N/A	Yes
VN62	O5:H-	Sheep faeces	+	+	-	+	Yes	No	N/A	No
156	O5:H-	Sheep faeces	+	+	-	+	No	Yes	N/A	No
242	O5:H-	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
246	O5:H-	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
421	O5:H-	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
427	O5:H-	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
439	O5:H-	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
495	O5:H-	Sheep faeces	+	+	-	+	No	Yes	N/A	No

497	O5:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**Yes**
531	O5:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**Yes**
576	O5:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
582	O5:H-	Sheep faeces	-	+	-	+	**N/A**	**Yes**	**N/A**	**No**
831	O5:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
878	O5:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
614	O5:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
695	O5:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
760	O5:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
773	O5:H-	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes** [a]	**N/A**	**Yes**
790	O5:H-	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
831	O5:H-	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
878	O5:H-	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
978	O5:H-	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
1046	O5:H-	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
1056	O5:H-	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
1264	O5:H-	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes** [a]	**N/A**	**Yes**
1496	O5:H-	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes** [a]	**N/A**	**Yes**
NC1082	O5:H-	Sheep faeces	+	+	-	-	**Yes**	**No**	**N/A**	**No**
CS106	O5:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**Yes**
CS147	O5:H-	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes**	**N/A**	**Yes**
CS310	O5:H-	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes**	**N/A**	**Yes**

CS468	O5:H-	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes** [a]	**N/A**	**Yes**
H1	O5:H-	Human	+	-	+	+	**Yes**	**N/A**	**Yes**	**Yes**
H2	O5:H-	Human	+	-	-	+	**Yes**	**N/A**	**N/A**	**Yes**
H3	O5:H-	Human	+	+	-	+	**Yes**	**Yes**	**N/A**	**Yes**
H4	O5:H-	Human	+	-	-	+	**Yes**	**N/A**	**N/A**	**Yes**
310	O5:H-	Cattle faeces	+	-	+	+	**Yes** [a,b]	**N/A**	**Yes** [a,b]	**Yes**
M274	O5:H11	Sheep faeces	-	-	+	+	**N/A**	**N/A**	**Yes**	**No**
M290	O5:H11	Sheep faeces	-	-	+	+	**N/A**	**N/A**	**Yes**	**No**
NC874	O5:H11	Sheep faeces	-	-	+	+	**N/A**	**N/A**	**Yes**	**No**
NC590	O5:H11	Sheep faeces	-	-	+	+	**N/A**	**N/A**	**Yes**	**No**
CNC142	O5:Hnt	Sheep faeces	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
CS124	O5:HR	Sheep faeces	+	+	+	+	**Yes**	**No**	**Yes**	**No**
865	O6:H-	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
V10	O6:H-	Sheep faeces	+	-	-	+	**Yes**	**N/A**	**N/A**	**No**
Swiss2891	O7:H-	Human	-	+	+	+	**N/A**	**Yes**	**Yes**	**No**
NC695	O7:H8	Sheep faeces	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
Swiss2954	O8:H8	Human	+	-	-	-	**Yes**	**N/A**	**N/A**	**No**
Swiss3873	O8:H14	Human	-	+	-	-	**N/A**	**Yes**	**N/A**	**No**
160	O8:Hnt	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
Swiss1156	O15:H-	Human	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
H39	O15:H2	Human	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
M285	O15:H2	Sheep faeces	-	-	+	+	**N/A**	**N/A**	**Yes**	**No**

665	O21:H21	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
450	O26:H-	Sheep faeces	+	-	+	+	**Yes**	**N/A**	**Yes**	**No**
452	O26:H-	Sheep faeces	+	-	+	+	**Yes**	**N/A**	**Yes**	**No**
Swiss3235	O26:H-	Human	-	+	+	-	**N/A**	**Yes**	**Yes**	**No**
Swiss3722	O26:H-	Human	-	+	+	-	**N/A**	**Yes**	**Yes**	**No**
A6	O26:H-	Human	+	-	+	+	**Yes**	**N/A**	**Yes**	**No**
C5	O26:H-	Human	+	-	+	+	**Yes**	**N/A**	**Yes**	**No**
C6	O26:H-	Human	+	-	+	+	**Yes**	**N/A**	**Yes**	**No**
F1	O26:H-	Human	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
F4	O26:H-	Human	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
F2	O26:H-	Human	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
H20	O26:H-	Human	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
H44	O26:H-	Human	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
359	O26:H11	Sheep faeces	+	-	+	+	**No**	**N/A**	**Yes**	**No**
451	O26:H11	Sheep faeces	+	-	+	+	**Yes**	**N/A**	**Yes**	**No**
509	O26:H11	Sheep faeces	+	-	+	+	**Yes**	**N/A**	**Yes**	**No**
825	O26:H11	Sheep faeces	+	-	+	+	**Yes**	**N/A**	**Yes**	**No**
905	O26:H11	Sheep faeces	+	-	+	+	**Yes**	**N/A**	**Yes**	**No**
NC658	O26:H11	Sheep faeces	-	-	+	+	**N/A**	**N/A**	**Yes**	**No**
NC684	O26:H11	Sheep faeces	-	-	+	+	**N/A**	**N/A**	**Yes**	**No**
NC713	O26:H11	Sheep faeces	-	-	+	+	**N/A**	**N/A**	**Yes**	**No**
NC755	O26:H11	Sheep faeces	-	-	+	+	**N/A**	**N/A**	**Yes**	**No**

B1	O26:H11	Human	+	-	+	-	**Yes**	**N/A**	**Yes**	**No**
B2	O26:H11	Human	+	-	+	-	**Yes**	**N/A**	**Yes**	**No**
B3	O26:H11	Human	+	-	+	+	**No**	**N/A**	**Yes**	**No**
B4	O26:H11	Human	+	-	+	+	**Yes**	**N/A**	**Yes**	**No**
B4A	O26:H11	Human	+	-	+	+	**Yes**	**N/A**	**No**	**No**
B5	O26:H11	Human	+	-	+	+	**Yes**	**N/A**	**Yes**	**No**
B6	O26:H11	Human	+	-	+	+	**Yes**	**N/A**	**Yes**	**No**
D4	O26:H11	Human	+	-	+	+	**Yes**	**N/A**	**Yes**	**No**
F3	O26:H11	Human	+	-	+	-	**Yes**	**N/A**	**Yes**	**No**
Swiss2079	O26:H11	Human	+	-	+	+	**Yes**	**N/A**	**Yes**	**No**
Swiss3238	O26:H11	Human	+	-	+	-	**Yes**	**N/A**	**Yes**	**No**
NC50	O28:H31	Sheep faeces	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
CNC105	O37:H-	Sheep faeces	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
CNC108	O37:H-	Sheep faeces	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
CNC115	O37:H-	Sheep faeces	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
CS139	O37:H10	Sheep faeces	+	+	+	-	**No**	**No**	**Yes**	**No**
H22	O55:H6	Human	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
CNC144	O55:H12	Sheep faeces	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
CS180	O55:H20	Sheep faeces	+	+	+	+	**Yes**	**No**	**Yes**	**No**
CNC133	O69:H8	Sheep faeces	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
V12	O69:H8	Sheep faeces	+	-	-	-	**Yes**	**N/A**	**N/A**	**No**
131	O75:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**

NC901	O75:H-	Sheep faeces	+	+	-	-	**Yes**	**No**	**N/A**	**No**
488	O75:H8	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
500	O75:H8	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
562	O75:H8	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
651	O75:H8	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
829	O75:H8	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
852	O75:H8	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
855	O75:H8	Sheep faeces	+	-	-	+	**Yes**	**N/A**	**N/A**	**No**
859	O75:H8	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
884	O75:H8	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
890	O75:H8	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
899	O75:H8	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
660	O75:H8	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
1037	O75:H8	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
1043	O75:H8	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
1068	O75:H8	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
NC900	O75:H8	Sheep faeces	+	+	-	-	**Yes**	**No**	**N/A**	**No**
NC905	O75:H8	Sheep faeces	+	+	-	-	**Yes**	**No**	**N/A**	**No**
NC1084	O75:H8	Sheep faeces	+	+	-	-	**Yes**	**No**	**N/A**	**No**
NC1087	O75:H8	Sheep faeces	+	-	-	-	**Yes**	**N/A**	**N/A**	**No**
CS66	O75:H8	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
CS90	O75:H8	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**

CS117	O75:H8	Sheep faeces	+	+	-	+	No	Yes	N/A	No
CS141	O75:H8	Sheep faeces	+	+	-	+	No	Yes	N/A	No
CS254	O75:H8	Sheep faeces	+	+	-	+	No	Yes	N/A	No
CS280	O75:H8	Sheep faeces	+	+	-	+	No	Yes	N/A	No
CNC70	O77:H12	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
CNC77	O77:H12	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
227	O75:H40	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
V1	O75:H40	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
513	O77:H4	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
477	O77:H-	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
V9	O81:H26	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
VN1	O84:H-	Sheep faeces	+	-	+	+	No	N/A	Yes	No
259	O84:H-	Sheep faeces	+	-	+	+	Yes	N/A	Yes	No
CNC94	O84:H2	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
CNC117	O84:H2	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC911	O84:H25	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC952	O84:H25	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
VN21	O85:H49	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
VN23	O85:H49	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
VN24	O85:H49	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
VN50	O85:H49	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
153	O85:H49	Sheep faeces	-	+	+	-	N/A	No	Yes	No

H24	O86:H-	Human	-	-	+	-	N/A	N/A	Yes	No
CS129	O88:H8	Sheep faeces	+	+	+	-	Yes	No	Yes	No
VN20	O91:H-	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
VN26	O91:H-	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
VN46	O91:H-	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
VN49	O91:H-	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
VN52	O91:H-	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
VN56	O91:H-	Sheep faeces	+	+	-	+	Yes	Yes [a]	N/A	Yes
VN58	O91:H-	Sheep faeces	+	+	-	+	Yes	No	N/A	No
VN75	O91:H-	Sheep faeces	+	+	-	+	Yes	No	N/A	No
VN126	O91:H-	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
76	O91:H-	Sheep faeces	+	+	-	+	No	Yes	N/A	No
VN79	O91:H-	Sheep faeces	+	+	-	+	Yes	No	N/A	No
VN80	O91:H-	Sheep faeces	+	+	-	+	Yes	No	N/A	No
338	O91:H-	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
568	O91:H-	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
849	O91:H-	Sheep faeces	+	+	-	+	Yes	Yes	N/A	Yes
867	O91:H-	Sheep faeces	+	+	-	+	No	Yes	N/A	No
881	O91:H-	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
912	O91:H-	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
917	O91:H-	Sheep faeces	+	+	-	+	Yes	Yes	N/A	Yes
536	O91:H-	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No

659	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
726	O91:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**Yes**
731	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
755	O91:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes** [a]	**N/A**	**Yes**
767	O91:H-	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
1034	O91:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes** [a]	**N/A**	**Yes**
1038	O91:H-	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
1056	O91:H-	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
NC208	O91:H-	Sheep faeces	+	+	-	-	**Yes**	**No**	**N/A**	**No**
NC66	O91:H-	Sheep faeces	+	+	-	-	**Yes**	**No**	**N/A**	**No**
NC894	O91:H-	Sheep faeces	+	+	-	-	**Yes**	**No**	**N/A**	**No**
NC964	O91:H-	Sheep faeces	+	+	-	-	**Yes**	**No**	**N/A**	**No**
CNC84	O91:H-	Sheep faeces	+	+	-	-	**Yes**	**No**	**N/A**	**No**
CS21	O91:H-	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes**	**N/A**	**Yes**
CS26	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS36	O91:H-	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes**	**N/A**	**Yes**
CS46	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS56	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS70	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS71	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS116	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS124	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**

CS134	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS154	O91:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
CS164	O91:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
CS173	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS186	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS190	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS217	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS244	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS256	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS263	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS271	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS272	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS285	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS289	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS300	O91:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
M501	O91:H-	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes** [a]	**N/A**	**Yes**
H5	O91:H-	Human	+	+	-	-	**Yes**	**Yes**	**N/A**	**Yes**
H6	O91:H-	Human	+	+	-	-	**Yes**	**Yes**	**N/A**	**Yes**
H17	O91:H-	Human	-	-	-	-	**N/A**	**N/A**	**N/A**	**Yes**
K3	O91:H-	Lamb carcass	+	+	-	+	**No**	**Yes**	**N/A**	**No**
K4	O91:H-	Lamb carcass	+	+	-	+	**No**	**Yes**	**N/A**	**No**
K5	O91:H-	Lamb carcass	+	+	-	+	**No**	**Yes**	**N/A**	**No**

K6	O91:H-	Lamb carcass	+	+	-	+	**Yes** [a]	**Yes**	**N/A**	**Yes**
K7	O91:H-	Lamb carcass	+	+	-	+	**No**	**Yes**	**N/A**	**No**
K8	O91:H-	Lamb carcass	+	+	-	+	**No**	**Yes**	**N/A**	**No**
K9	O91:H-	Lamb carcass	+	+	-	+	**No**	**Yes**	**N/A**	**No**
K11	O91:H-	Lamb carcass	+	+	-	+	**No**	**Yes**	**N/A**	**No**
K12	O91:H-	Lamb carcass	+	+	-	+	**No**	**Yes**	**N/A**	**No**
K10	O91:H-	Lamb carcass	-	+	-	-	**N/A**	**Yes**	**N/A**	**No**
K2	O91:H-	Lamb meat	+	+	-	+	**No**	**Yes**	**N/A**	**No**
K15	O91:H-	Lamb meat	+	+	-	+	**No**	**Yes**	**N/A**	**No**
K18	O91:H-	Lamb meat	+	+	-	+	**No**	**Yes**	**N/A**	**No**
K22	O91:H-	Lamb meat	+	+	-	+	**No**	**Yes**	**N/A**	**No**
K23	O91:H-	Lamb meat	+	+	-	+	**No**	**Yes**	**N/A**	**No**
K24	O91:H-	Lamb meat	+	+	-	+	**No**	**Yes**	**N/A**	**No**
K17	O91:H-	Lamb meat	+	+	-	-	**No**	**Yes**	**N/A**	**No**
K19	O91:H-	Lamb meat	+	+	-	-	**No**	**Yes**	**N/A**	**No**
K20	O91:H-	Lamb meat	+	+	-	-	**No**	**Yes**	**N/A**	**No**
K14	O91:H-	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes**	**N/A**	**Yes**
K16	O91:H-	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes**	**N/A**	**Yes**
K13	O91:H-	Mettwurst sausage	+	+	-	-	**Yes** [a]	**Yes**	**N/A**	**Yes**
639	O91:H-	Cattle faeces	+	+	-	+	**Yes** [a,b]	**Yes** [a,b]	**N/A**	**Yes**
756	O91:H2	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
H7	O91:H10	Human	-	+	-	-	**N/A**	**Yes**	**N/A**	**No**

K21	O91:H21	Human	-	+	-	+	N/A	Yes	N/A	No
1639	O98:H-	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
285	O103:H2	Sheep faeces	+	-	+	+	Yes	N/A	Yes	No
368	O103:H2	Sheep faeces	+	-	+	+	No	N/A	Yes	No
NC830	O103:H2	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC866	O103:H2	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC884	O103:H2	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC943	O103:H2	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC732	O103:H2	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC798	O103:H2	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC940	O103:H2	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
N657	O103:H2	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
Swiss762	O103:H2	Human	+	-	+	+	No	N/A	Yes	No
Swiss4033	O103:H2	Human	+	-	+	+	Yes	N/A	Yes	No
828	O103:H38	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
763	O106:H18	Sheep faeces	+	+	-	+	Yes	No	N/A	No
CNC134	O106:HR	Sheep faeces	+	-	+	-	Yes	N/A	Yes	No
CNC135	O106:HR	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
NC863	O109:H-	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC895	O109:H-	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC716	O109:H-	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC849	O109:H-	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No

H26	O111:H-	Human	-	-	+	-	N/A	N/A	Yes	No
H27	O111:H-	Human	-	-	+	-	N/A	N/A	Yes	No
CS89	O112ab:H2	Sheep faeces	+	+	+	+	Yes	No	Yes	No
CNC44	O112ab:H2	Sheep faeces	+	+	-	-	Yes	No	N/A	No
CNC48	O112ab:H2	Sheep faeces	+	+	-	-	Yes	No	N/A	No
V2	O112ab:H2	Sheep faeces	+	-	-	-	Yes	N/A	N/A	No
Swiss1641	O117:H7	Human	+	-	-	-	Yes	N/A	N/A	No
Swiss3552	O117:H7	Human	+	-	-	-	Yes	N/A	N/A	No
Swiss3373	O118:H16	Human	+	-	+	+	Yes	N/A	Yes	No
512	O121:H2	Sheep faeces	+	+	-	+	Yes	No	N/A	No
CNC181	O121:H19	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC318	O121:H19	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
Swiss838	O121:H19	Human	-	+	+	+	N/A	Yes	Yes	No
Swiss3623	O121:H19	Human	-	+	+	-	N/A	Yes	Yes	No
VN69	O123:H-	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
93	O123:H-	Sheep faeces	+	+	-	+	No	Yes	N/A	No
159	O123:H-	Sheep faeces	+	+	-	+	No	Yes	N/A	No
265	O123:H-	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
273	O123:H-	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
353	O123:H-	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
367	O123:H-	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
415	O123:H-	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No

430	O123:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
423	O123:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
436	O123:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
335	O123:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
553	O123:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
565	O123:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
580	O123:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
665	O123:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
858	O123:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
870	O123:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
874	O123:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
887	O123:H-	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
770	O123:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
781	O123:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
CS96	O123:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
CS123	O123:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
CS129	O123:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
CS184	O123:H-	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
H14	O123:H-	Human	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
999	O123:H11	Sheep faeces	+	-	-	+	**Yes**	**N/A**	**N/A**	**No**
H41	O125:H6	Human	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**
H32	O126:H2	Human	-	-	+	-	**N/A**	**N/A**	**Yes**	**No**

H33	O127:H-	Human	-	-	+	-	N/A	N/A	Yes	No
H34	O127:H-	Human	-	-	+	-	N/A	N/A	Yes	No
VN5	O128:H2	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
VN8	O128:H2	Sheep faeces	+	+	-	+	Yes	Yes [a]	N/A	Yes
VN16	O128:H2	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
VN47	O128:H2	Sheep faeces	+	+	-	+	Yes	Yes [a]	N/A	Yes
VN51	O128:H2	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
VN71	O128:H2	Sheep faeces	+	+	-	+	Yes	No	N/A	No
206	O128:H2	Sheep faeces	+	+	-	+	No	Yes	N/A	No
442	O128:H2	Sheep faeces	+	+	-	+	Yes	Yes	N/A	Yes
443	O128:H2	Sheep faeces	+	+	-	+	Yes	No	N/A	No
481	O128:H2	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
510	O128:H2	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
519	O128:H2	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
604	O128:H2	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
764	O128:H2	Sheep faeces	+	+	-	+	Yes	No	N/A	No
796	O128:H2	Sheep faeces	+	+	-	+	Yes [a]	Yes [a]	N/A	Yes
824	O128:H2	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
922	O128:H2	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
938	O128:H2	Sheep faeces	+	+	-	+	Yes	No	N/A	No
993	O128:H2	Sheep faeces	+	+	-	+	Yes	No	N/A	No
700	O128:H2	Sheep faeces	+	+	-	+	Yes	Yes	N/A	Yes

705	O128:H2	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**No**
1012	O128:H2	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
1018	O128:H2	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
1020	O128:H2	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
1029	O128:H2	Sheep faeces	+	+	-	+	**Yes**	**No**	**N/A**	**No**
1138	O128:H2	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes** [a]	**N/A**	**Yes**
CS193	O128:H2	Sheep faeces	+	+	-	+	**Yes**	**Yes**	**N/A**	**Yes**
CS238	O128:H2	Sheep faeces	+	+	-	+	**No**	**Yes**	**N/A**	**No**
CS241	O128:H2	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes** [a]	**N/A**	**Yes**
CS462	O128:H2	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes** [a]	**N/A**	**Yes**
M527	O128:H2	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes** [a]	**N/A**	**Yes**
M537	O128:H2	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes** [a]	**N/A**	**Yes**
M570	O128:H2	Sheep faeces	+	+	-	+	**Yes** [a]	**Yes** [a]	**N/A**	**Yes**
NC987	O128:H2	Sheep faeces	+	+	-	-	**Yes**	**No**	**N/A**	**No**
NC1078	O128:H2	Sheep faeces	+	+	-	-	**Yes**	**No**	**N/A**	**No**
NC1088	O128:H2	Sheep faeces	+	-	-	-	**Yes**	**N/A**	**N/A**	**No**
H8	O128:H2	Human	+	+	-	+	**Yes**	**Yes**	**N/A**	**Yes**
H9	O128:H2	Human	+	+	-	+	**Yes**	**Yes**	**N/A**	**Yes**
H10	O128:H2	Human	+	+	-	+	**Yes**	**Yes**	**N/A**	**Yes**
H12	O128:H2	Human	+	+	-	+	**Yes**	**Yes**	**N/A**	**Yes**
H11	O128:H2	Human	+	+	-	-	**Yes**	**Yes**	**N/A**	**Yes**
H13	O128:H2	Human	+	+	-	-	**Yes**	**Yes**	**N/A**	**Yes**

H38	O128:H2	Human	-	-	+	-	N/A	N/A	Yes	No
H19	O128:H2	Human	-	-	+	-	N/A	N/A	Yes	No
H35	O128:H2	Human	-	-	+	-	N/A	N/A	Yes	No
H46	O128:H2	Human	-	-	+	-	N/A	N/A	Yes	No
CSS41	O128:H-/H2	Sheep faeces	+	-	-	-	Yes	N/A	N/A	No
959	O128:H2/H8	Sheep faeces	+	+	-	+	Yes	No	N/A	No
556	O128:H-	Sheep faeces	+	+	-	+	No	Yes	N/A	No
656	O128:H-	Sheep faeces	+	+	-	+	No	Yes	N/A	No
501	O128:Hnt	Sheep faeces	+	+	-	+	Yes	No	N/A	No
NC357	O145:H-	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC656	O145:H-	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
Swiss2458	O145:H-	Human	-	+	+	-	N/A	Yes	Yes	No
Swiss3212	O145:H-	Human	-	+	+	-	N/A	Yes	Yes	No
576	O152:H21	Sheep faeces	+	+	-	+	Yes	No	N/A	No
419	O153:H-	Sheep faeces	+	+	-	+	No	Yes	N/A	No
914	O153:H-	Sheep faeces	+	+	-	+	No	Yes	N/A	No
V13	O153:H-	Sheep faeces	+	-	-	-	Yes	N/A	N/A	No
M337	O153:H8	Sheep faeces	+	+	-	-	Yes	No	N/A	No
H40	O153:H11/H21	Human	-	-	+	-	N/A	N/A	Yes	No
579	O153:H25	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
609	O153:H25	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
933	O153:H25	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No

936	O153:H25	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
NC819	O153:H25	Sheep faeces	+	-	-	-	Yes	N/A	N/A	No
753	O154:HR	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
H37	O156:H1	Human	-	-	+	-	N/A	N/A	Yes	No
VN59	O157:H-	Sheep faeces	+	+	+	+	No	No	Yes	No
72	O157:H-	Sheep faeces	+	+	-	+	Yes	No	N/A	No
152	O157:H-	Sheep faeces	-	+	+	+	N/A	Yes	Yes	No
461	O157:H-	Sheep faeces	+	+	+	+	Yes	Yes	Yes	No
586	O157:H-	Sheep faeces	+	+	+	+	Yes	Yes	Yes	No
587	O157:H-	Sheep faeces	+	+	-	+	Yes	No	N/A	No
588	O157:H-	Sheep faeces	+	+	+	-	No	No	Yes	No
919	O157:H-	Sheep faeces	-	+	+	+	N/A	No	Yes	No
O157	O157:H-	Human	-	+	+	+	N/A	No	Yes	No
NC677	O157:H11	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC836	O157:H11	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC908	O157:H11	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC955	O157:H11	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
464	O157:H21	Sheep faeces	+	+	+	+	Yes	Yes	Yes	No
CS74	O158:HR	Sheep faeces	+	-	+	+	Yes	N/A	Yes	No
CNC69	O158:HR	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
CNC132	O162:H10	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
V3	O163:H19	Sheep faeces	+	-	-	-	Yes	N/A	N/A	No

NC1079	O166:H49	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC96	O168:H21	Sheep faeces	+	-	-	-	Yes	N/A	N/A	No
1642	O172:H1	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC961	Ont:H-	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
V5	Ont:H-	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
NC953	Ont:H-	Sheep faeces	+	+	-	-	Yes	No	N/A	No
M284	Ont:H-	Sheep faeces	+	+	-	-	Yes	No	N/A	No
CNC81	Ont:H-	Sheep faeces	+	-	-	-	Yes	N/A	N/A	No
Ont:H-	Ont:H-	Human	+	-	+	+	Yes	N/A	Yes	No
CNC75	Ont:H6	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
CS126	Ont:H8	Sheep faeces	+	+	+	-	Yes	No	Yes	No
CNC107	Ont:H8	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
CNC174	Ont:H8	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
V7	Ont:H8	Sheep faeces	+	+	-	-	Yes	No	N/A	No
NC1130	Ont:H19	Sheep faeces	+	+	-	-	Yes	No	N/A	No
NC914	Ont:H25	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC934	Ont:H25	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC949	Ont:H25	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
CNC177	Ont:H25	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
179	Ont:H25	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC412	Ont:H25	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC687	Ont:H25	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No

NC706	Ont:H25	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC787	Ont:H25	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC816	Ont:H25	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
NC967	Ont:H25	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
NC960	Ont:H25	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
NC635	Ont:H25	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
CS136	Ont:H49	Sheep faeces	+	+	+	-	Yes	N/A	Yes	No
CNC106	Ont:HR	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
622	Ont:HR	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
639	Ont:HR	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
683	Ont:HR	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
686	Ont:HR	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
742	Ont:HR	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
747	Ont:HR	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
784	Ont:HR	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
CNC46	Ont:Hnt	Sheep faeces	+	+	-	-	Yes	N/A	N/A	No
H36	Ont:Hnt	Human	+	-	+	+	Yes	N/A	Yes	No
NC38	OR:H-	Sheep faeces	-	-	+	-	N/A	N/A	Yes	No
1633	OR:H-	Sheep faeces	+	+	+	+	N/A	N/A	Yes	No
241	OR:H-	Sheep faeces	+	+	-	+	Yes	N/A	N/A	No
580	OR:H-	Sheep faeces	+	+	-	+	Yes	N/A	N/A	No
V4	OR:H-	Sheep faeces	+	+	-	-	Yes	N/A	N/A	No

401	OR:H2	Sheep faeces	+	+	-	+	N/A	Yes	N/A	No
873	OR:H2	Sheep faeces	+	+	-	+	N/A	Yes	N/A	No
723	OR:H2	Sheep faeces	+	+	-	+	N/A	Yes	N/A	No
754	OR:H2	Sheep faeces	+	+	-	+	Yes	Yes	N/A	No
V6	OR:H2	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
CNC116	OR:H2	Sheep faeces	-	-	+	+	N/A	N/A	Yes	N/A
658	OR:H4	Sheep faeces	+	+	-	+	Yes	N/A	N/A	No
774	OR:H4	Sheep faeces	+	+	-	+	Yes	N/A	N/A	No
NC931	OR:Hnt	Sheep faeces	-	-	+	+	N/A	N/A	Yes	No
737	OR:HR	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
M301	OX3:H2	Sheep faeces	+	-	-	-	Yes	N/A	N/A	No
NC68	OX3:H8	Sheep faeces	+	+	-	-	Yes	N/A	N/A	No
Swiss167	OX3:H8	Human	+	+	-	-	Yes	Yes	N/A	No
751	OX3:HR	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
626	OX3:HR	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No
1015	OX3:HR	Sheep faeces	+	-	-	+	Yes	N/A	N/A	No

[a] isolates used only in Chapter 5
[b] isolates subtyped by Kim Brett at Elizabeth Macarthur Agricultural Institute, Australia

APPENDIX B– CLUSTAL W ALIGNMENT OF Int_{280} AMINO ACID SEQUENCES (Refer to section 4.2.6)

```
                1         11        21        31        41        51        61        71        81        91
AF530554        ASITEIKADKTTAKANGSDAITYTVKVMKNNQPEVNHSVTFSTNFGNLGGNSQTQIVQTDKDGKATVK-LTSGSEGSAVVSAKVSEVNTEVKASEVKFFS
AF116899        ASITEIKADKTTAKANGSDAITYIVKVMKNNQPEANHSVTFSTNFGNLGGNSNTQIVKTDKDGRATVK-LTSGVAGNAVVSAKVSEVNTEVKAPEVKFFS
AF530553        ASITEIKASKTTAKADGVDAILYTVKVMKNGVPEKGQVVAFSTNLGKLN----LQTVETNKDGLASVT-LTSVSVGKAVVSAKVSEAGSVINADAVNFFA
AJ308551        ASITEIKADKTTAVANGKDAVTYTVKVMKNGLPEKGHVVTFSTDLGKLN----LQTVATDKDGFASVT-LTSDSVGKAVVSAKVSEAGSVVNADAVNFFA
AF530557        ASITEIKADKTTAVANGKDAIKYTVKVMKSGQPVKGYDVTFLTTAGNLS----KTKELTDKDGYATVN-LTSNAAGKAVVSAKVSDVNTEVKASEVEFFT
AF439538        ASITEIKADKTTAVANGKDAIKYTVKVMKNGQPVKGYDVTFLTTAGNLS----KTKELTDKDGYATVN-LTSNAAGKAVVSAKVSDVNSEVKASEVEFFT
AJ275091        ASITEIKADKTTAKADGSDAITYTVRVMKEGAPVVDQKVTFSKDFGTLN----KTEATTDQNGYATVK-LSSNTPGKAIVSAKVSGVGTEVKATTVEFFA
U60002          ASITEIKADKTTAKADGSDAITYTVRVMKEGAPVVDQKVTFSKDFGTLN----KTEATTDQNGYATVK-LSSNTPGKAIVSAKVSGVGTEVKATTVEFFA
AF453441        ASITEIKADKTTAKADGSDAITYTVRVMKEGAPVVDQKVTFSKDFGTLN----KTEATTDQNGYATVK-LSSNTPGKAIVSAKVSGVGTEVKATTVEFFA
AF200363        ASITEIKADKTTAKADGSDAITYTVRVMKEGAPVVDQKVTFSKDFGTLN----KTEATTDQNGYATVK-LSSNTPGKAIVSAKVSGVGTEVKATTVEFFA
AF130315        ASITEIKADKTTAKADGSDAITYTVRVMKEGAPVVDQKVTFSKDFGTLN----KTEATTDQNGYATVK-LSSNTPGKAIVSAKVSGVGTEVKATTVEFFA
AF099073        ASITEIKADKTTAKADGSDAITYTVRVMKEGAPVVDQKVTFSKDFGTLN----KTEATTDQNGYATVK-LSSNTPGKAIVSAKVSGVGTEVKATTVEFFA
AF099072        ASITEIKADKTTAKADGSDAITYTVRVMKEGAPVVDQKVTFSKDFGTLN----KTEATTDQNGYATVK-LSSNTPGKAIVSAKVSGVGTEVKATTVEFFA
AF081187        ASITEIKADKTTAKADGSDAITYTVRVMKEGAPVVDQKVTFSKDFGTLN----KTEATTDQNGYATVK-LSSNTPGKAIVSAKVSGVGTEVKATTVEFFA
AF081186        ASITEIKADKTTAKADGSDAITYTVRVMKEGAPVVDQKVTFSKDFGTLN----KTEATTDQNGYATVK-LSSNTPGKAIVSAKVSGVGTEVKATTVEFFA
U59502          ASITEIKADKTTAKADGSDAITYTVRVMKEGAPVVDQKVTFSKDFGTLN----KTEATTDQNGYATVK-LSSNTPGKAIVSAKVSGVGTEVKATTVEFFA
AF253560        ASITEIKADKTTAKADGSDAITYTVRVMKEGAPVVDQKVTFSKDFGTLN----KTEATTDQNGYATVK-LSSNTPGKAIVSAKVSGVGTEVKATTVEFFA
AF065628        ASITEIKADKTTAKADGSDAITYTVRVMKEGAPEVDQKVTFSKDFGTLN----KTEATTDQNGYATVK-LSSNTPGKAIVSAKVSGVGTEVKATTVEFFA
AF530556        ASITEIKADKTTAKANGSDAVTYTVKVMKGGTPVSGQKVTFSKDFGTLD----KTEATTDQNGYATVK-LSSSTPGKAIVSAKVSDVDTEVKATTVEFFT
AB040740        ASITEIKVDKTIATADNKDTIEYTVKVMKGGNPISGQKVTFSKDFGTLN----KTEATTDQNGYATVK-LSSGTPGKAIVSAKVSEVNTEVKAATGEFFA
L11691          ASITEIKVDKTIATADNKDTIEYTVKVMKGGNPISGQKVTFSKDFGTLN----KTEATTDQNGYATVK-LSSGTPGKAIVSAKVSEVNTEVKAATVEFFA
AJ308552        ASITEIKADKTTAVANGQDAITYTVKVMKNGQPLSGEEVTFFTDFGALD----KTKVTTDQSGYATVK-LSSSTSGKAIVRAKVSDVDTEVKAAAVEFFA
Y13112          ASITEIKADKTTAVANGQDAITYTVKVMKNGQPLSGEEVTFFTDFGALD----KTKVTTDQSGYATVK-LSSSTSGKAIVRAKVSDVDTEVKAAAVEFFA
U66102          ASITEIKADKTTAVANNQDAITYTVKVMENGQPLSGEEVTFFTDFGALD----KTKVTTDQSGYATVKNLSSSTSGKAIVRAKVSDVDTEVKAAAVEFFA
AF530555        ASITEIKADKTTAVANGKDAITYTVKVMKNGQPVSGEEVTFTKTLGTLS----KPTEKTDANGYAKVT-LTSATQGKSLVSARVSDVAVDVKAPEVEFFT
AF022236        ASITEIKADKTTAVANGQDAITYTVKVMKGDKPVSNQEVTFTTTLGKLS----NSTEKTDTNGYAKVT-LTSTTPGKSLVSARVSDVAVDVKAPEVEFFT
AF449416        ASITEINADKKTAKANGSDAITYTVKVMKDGKPLSAQDVTFTATLGTLS----KSTEKTDANGYAKVT-LTSKTTGKSLVSASISGSAIDVKAPEVEFFT
AF449417        ASITEINADKKTAKANGSDAITYTVKVMKDGKPLSAQDVTFTATLGTLS----KSTEKTDANGYAKVT-LTSKTTGKSLVSASISGSAIDVKAPEVEFFT
AJ271407        ASITEINADKKTAKANGSDAITYTVKVMKDGKPLSAQDVTFTATLGTLS----KSTEKTDANGYAKVT-LTSKTTGKSLVSARISGSAIDVKAPEVEFFT
AJ275089        ASITEINADKKTAKANGSDAITYTVKVMKDGKPLSAQDVTFTATLGTLS----KSTEKTDANGYAKVT-LTSKTTGKSLVSARISGSAIDVKAPEVEFFT
AJ298279        ASITEINADKKTAKANGSDAITYTVKVMKDGKPLSAQDVTFTATLGTLS----KSTEKTDANGYAKVT-LTSKTTGKSLVSARISGSAIDVKAPEVEFFT
AF449414        ASITEIKADKTTAVANGNDAVTYTVKVMKEGQPVQGHSVAFTTNFGMFNGKSQTQNATTGSDGRATIT-LTSSSAGKATVSATVSGG-NDVKAPEVTFFD
AF449418        ASITGIKADKTTAVANGNDAVTYTVKVMKEGQPVQGHSVAFTTNFGMFNGKSQTQNATTGSDGRATIT-LTSSSAGKATVSATVSGG-NDVKAPEVTFFD
AF449415        ASITEIKADKTTAVANGNDAVTYTVKVMKEGQPVQGHSVAFTTNFGMFNGKSQTQNATTGSDGRATIT-LTSSSAGKATVSATVSGG-NDVKAPEVTFFD
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APPENDIX B– Clustal W of Int_{280} amino acid sequences

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AF449419    ASITEIKADKTTAVANGNDAVTYTVKVMKEGQPVQGHSVAFTTNFGMFNGKSQTQNATTGSDGRATIT-LTSSSAGKATVSATVSGG-NDVKAPEVTFFD
AF449420    ASITEIKADKTTAVANGNDAVTYTVKVMKEGQPVQGHSVAFTTNFGMFNGKSQTQNATTGSDGRATIT-LTSSSAGKATVSATVSGG-NDVKAPEVTFFD
AJ132982    ASITEIKADKTTAVANGNDAVTYTVKVMKEGQPVQGHSVAFTTNFGMFNGKSQTQNATTGSDGRATIT-LTSSSAGKATVSATVSGG-NDVKAPEVTFFD
AF025311    ASITEIKADKTTAVANGNDAVTYTVKVMKEGQPVHGHSVAFTTNFGMFNGKSQTQNATTGSDGRATIT-LTSSSAGKATVSATVSGG-NDVKAPEVTFFD
AF253561    GSITEIKADKTTAVANGNDAVTYTVKVMKEGQPVQGHSVAFTTNFGMFNGKSQTQNATTGSDGRATIT-LTSSSAGKATVSATVSGG-NDVKAPEVTFFD
X60439      ASITEIKADKTTAVANGKDAIKYTVKVMKNGQPVNNQSVTFSTNFGMFNGKSQTQ-ATTGNDGRATIT-LTSSSAGKATVSATVSDG-AEVKATEVTFFD
Z11541      ASITEIKADKTTAVANGKDAIKYTVKVMKNGQPVNNQSVTFSTNFGMFNGKSQTQ-ATTGNDGRATIT-LTSSSAGKATVSATVSDG-AEVKATEVTFFD
AF081182    ASITEIKADKTTAVANGKDAIKYTVKVMKNGQPVNNQSVTFSTNFGMFNGKSQTQ-ATTGNDGRATIT-LTSSSAGKATVSATVSDG-AEVKATEVTFFD
AF081183    ASITEIKADKTTAVANGKDAIKYTVKVMKNGQPVNNQSVTFSTNFGMFNGKSQTQ-ATTGNDGRATIT-LTSSSAGKATVSATVSDG-AEVKATEVTFFD
AF081184    ASITEIKADKTTAVANGKDAIKYTVKVMKNGQPVNNQSVTFSTNFGMFNGKSQTQ-ATTGNDGRATIT-LTSSSAGKATVSATVSDG-AEVKATEVTFFD
AF081185    ASITEIKADKTTAVANGKDAIKYTVKVMKNGQPVNNQSVTFSTNFGMFNGKSQTQ-ATTGNDGRATIT-LTSSSAGKATVSATVSDG-AEVKATEVTFFD

                      101       111       121       131       141       151       161       171       181       191
AF530554    VLSIGNN-VNIIGTSAD-GALPNIWLQYGQFKLTAKGGDGKYKWHSKDTSVASVDASTGQVTLLKKGTTTIEVVSGDNQTATYTINQPENIITVE-T
AF116899    VLSIDSN-VSIIGTSAN-GALPNIWLRYGQFKLTAKGGDGKYQWRSQDPSVASVDALTGRVTLLKKGTTTIEVVSGDNQTAMYTINTPTKFISVE-TQNK
AF530553    TLNIDKN-VEIVGTKVS-GELPNIWLQYGQVKLNANGGNGGYTWSSDNPNIASIDSNTGIITLNKKGTAVIKVVSGDKQIATYTIKTPQEIVSLD-K
AJ308551    TLSIDNN-VEIVGTKVR-GELPNIWLRYGQVKLNANGGNGGYSWSSDNPDIASIDANTGIITLNKKGTTVIKVISGDKQIATYTIKTPQEIVSLD-NSVK
AF530557    ELSINKN-VEVLGTKVS-GELPDVWLQYGQIKLNVNGGNDKYSWSSSNPNIASIDASSGIITLKEKGEAVIKVVSGDKQTATYTISTPKKIVSVN-SDSR
AF439538    ELSINKN-VEVLGTKAS-GELPDVWLQYGQIKLNVNGGNDKYSWSSSNPNIASIDASSGIITLKEKGEAVIKVVSGDKQTATYTISTPKKIVSVN-SGSR
AJ275091    PLSIDGDKVTVIGTGIT-GALPKNWLQYGQVKLQATGGNGKYTWKSSNTKIASVDN-SGVITLNEKGSATITVVSGDNQSATYTINAPGSIVIAVDKNTR
U60002      PLSIDGDKVTVIGTGIT-GALPKNWLQYGQVKLQATGGNGKYTWKSSNTKIASVDN-SGVITLNEKGSATITVVSGDNQSATYTINAPGSIVIAVDKNTR
AF453441    PLSIDGDKVTVIGTGIT-GALPKNWLQYGQVKLQATGGNGKYTWKSSNTKIASVDN-SGVITLNEKGSATITVVSGDNQSATYTINAPGSIVIAVDKNTR
AF200363    PLSIDGDKVTVIGTGIT-GALPKNWLQYGQVKLQATGGNGKYTWKSSNTKIASVDN-SGVITLNEKGSATITVVSGDNQSATYTINAPGSIVIAVDKNTR
AF130315    PLSIDGDKVTVIGTGIT-GALPKNWLQYGQVKLQATGGNGKYTWKSSNTKIASVDN-SGVITLNEKGSATITVVSGDNQSATYTINAPGSIVIAVDKNTR
AF099073    PLSIDGDKVTVIGTGIT-GALPKNWLQYGQVKLQATGGNGKYTWKSSNTKIASVDN-SGVITLNEKGSATITVVSGDNQSATYTINAPGSIVIAVDKNTR
AF099072    PLSIDGDKVTVIGTGIT-GALPKNWLQYGQVKLQATGGNGKYTWKSSNTKIASVDN-SGVITLNEKGSATITVVSGDNQSATYTINAPGSIVIAVDKNTR
AF081187    PLSIDGDKVTVIGTGIT-GALPKNWLQYGQVKLQATGGNGKYTWKSSNTKIASVDN-SGVITLNEKGSATITVVSGDNQSATYTINAPGSIVIAVDKNTR
AF081186    PLSIDGDKVTVIGTGIT-GALPKNWLQYGQVKLQATGGNGKYTWKSSNTKIASVDN-SGVITLNEKGSATITVVSGDNQSATYTINAPGSIVIAVDKNTR
U59502      PLSIDGDKVTVIGTGIT-GALPKNWLQYGQVKLQATGGNGKYTWKSSNTKIASVDN-SGVITLNEKGSATITVVSGDNQSATYTINAPGSIVIAVDKNTR
AF253560    PLSIDGDKVTVIGTGIT-GALPKNWLQYGQVKLQATGGNGRYTWKSSNTKIASVDN-SGVITLNEKGSATITVVSGDNQSATYTINAPGSIVIAVDKNTR
AF065628    PLSIDGDKVTVIGTGIT-GALPTNWLQYGQVKLQATGGNGKYTWKSSNTKIASVDN-SGVITLNEKGSATITVVSGDNQSATYTINAPGSIVIAVDKNTR
AF530556    PLSIDGNKVTVIGTGVT-GSLPNNWLQYGKVKLQAAGGNGKYTWKSSDTKIASVDS-TGVITLNEKGSATITVVSGDNQSATYTINAPSSIVIAVDKNTR
AB040740    PLSIDGNKVTVIGTGVT-GSLPKNWLQYGQVKLQATGGNGKYTWKSSNTKIASVDN-SGVITLNEKGSATITVVSGDNQSATYTINTPDNIIIAVDKINR
L11691      PLSIDGNKVTVIGTGVT-GSLPKNWLQYGQVKLQATGGNGKYTWKSSNTKIASVDN-SGVITLNEKGSATITVVSGDNQSATYTINTPDNIIIAVDKINR
AJ308552    SLSIDSNKVTVIGTGVT-GSLPKNWLQYGQAKLQATGGNGKYTWKSSDTKIASVDS-SGVITLNGKGSTIITVVSGDNQSATYTISTPDKIVIAVDKINR
Y13112      SLSIDSNKVTVIGTGVT-GSLPKNWLQYGQAKLQATGGNGKYTWKSSDTKIASVDS-SGVITLNGKGSTIITVVSGDNQSATYTISTPDKIVIAVDKINR
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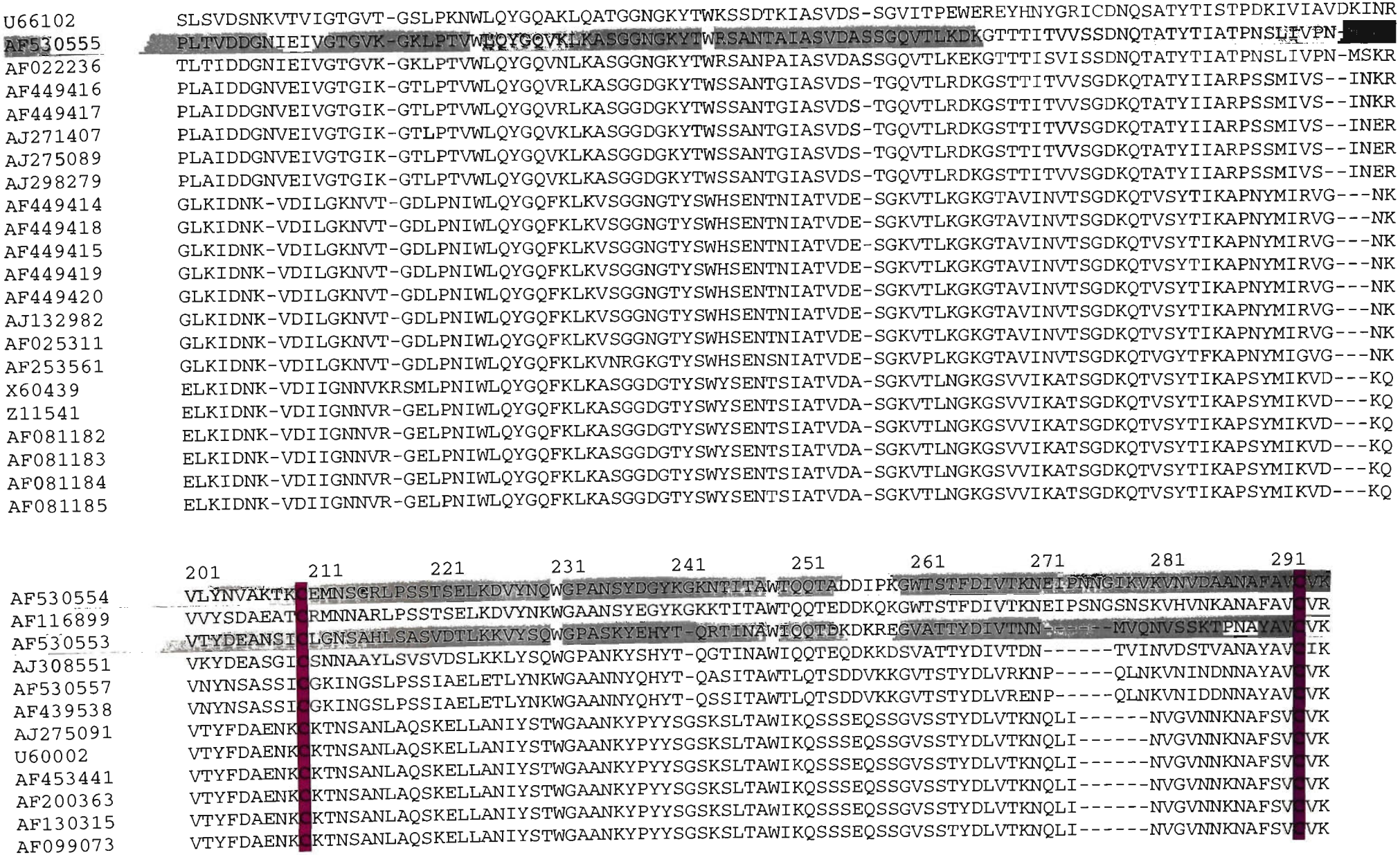
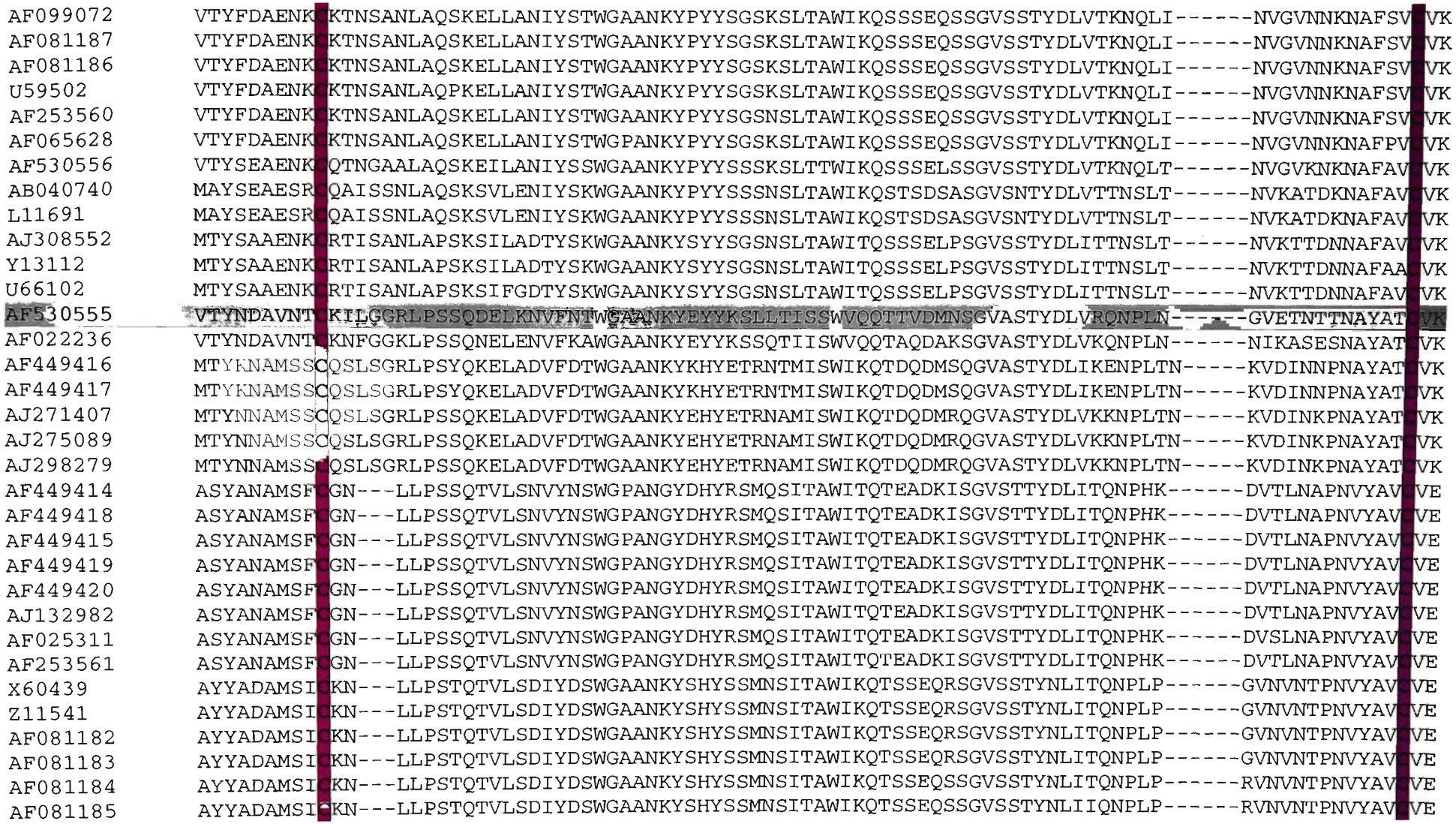
APPENDIX B– Clustal W of Int_{280} amino acid sequences



APPENDIX B– Clustal W of Int_{280} amino acid sequences



The two cysteine (C) residues necessary for the formation of disulfide bond and the binding activity are highlighted in pink;
The four conserved tryptophan (W) residues are highlighted in **yellow**.