Molecular epidemiology of Shiga toxin-producing *Escherichia coli* (STEC) O157 and non-O157 STEC in calves in the North Island of New Zealand

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by

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) are common inhabitants of the ruminant gastrointestinal tract and have emerged as important zoonotic pathogens of global public health concern. Four studies were carried out in the North Island of New Zealand to understand the epidemiology of STEC O157 and non-O157 STEC in calves.

The aim of the first study was to determine population structure, transmission dynamics and spatial relationship between genotypes of two zoonotic pathogens, STEC and *Campylobacter jejuni*, found on farms in a defined catchment area in the Waikato region. Pooled faecal samples (n=72) obtained from calves grazing in the catchment were analysed by RT-PCR for *E. coli* O26, O103, O111, O145 and O157. The number of samples positive for O26 (30/72) was high compared to O103 (7/72), O145 (3/72) and O157 (2/72) while no samples were positive for O111. Using immuno-magnetic separation 18 O26, two O103 and a single O145 isolate were recovered from RT-PCR positive samples. Fifty-three *C. jejuni* isolates were recovered from 72 pooled faecal samples. *E. coli* O26 and *C. jejuni* isolates were genotyped using pulsed field gel electrophoresis (PFGE) and multilocus sequence typing respectively. All O26 isolates could be divided into three clusters on the basis of PFGE using *XbaI*. These results indicated that *E. coli* O26 isolates recovered from calves on the same farm were generally more similar than isolates recovered from different farms in the catchment. There were a total of 13 different sequence types (STs) of *C. jejuni* isolated from the cattle and most of the molecular variation (approximately 75%) resided between animals within farms. This study suggested a high level of within farm transmission, and limited between-farm transmission indicating direct contact between animals or sharing of personnel or equipment could be important routes of transmission and good biosecurity measures may be helpful in reducing localised transmission.

The aim of the second study was to determine the distribution of virulence genes (*stx1*, *stx2*, *eae* and *ehxA*) in *E. coli* isolates from dairy calves less than a week of age (bobby calves). Sampling was carried out by systematic collection of recto-anal mucosal swab (RAMS) samples from bobby calves slaughtered at two abattoirs in the North Island of New Zealand. The samples were inoculated onto tryptone bile X-glucuronide (TBX) and sorbitol MacConkey agar (CT-SMAC). Blue and white colonies (one each) and purple and grey colonies (one each) were selected at random from TBX and CT-SMAC plates respectively. In total 975 *E. coli* isolates obtained from the two media were analysed by multiplex PCR to detect *stx1*, *stx2*, *eae* and *ehxA* genes. The most common combination of virulence markers were *eae*, *ehxA* (n=35) followed by *eae* (n=9). Only eight STEC were identified of which four were *stx2*, *eae*, *ehxA*-positive (3 O157:H7 and 1 O68:H24), three were *stx1*, *eae*, *ehxA* (2 O26 and 1 O71:HR) and there was one *stx2*-only isolate (ONT:HNM). All the isolates could be divided into 15 clusters with >70% similarity using *XbaI* by PFGE. These findings indicate that STEC of public health significance such as O157 and O26 are present in bobby calves and may represent an important source of human infection in New Zealand.

In the third study samples obtained in the second study were processed to determine the occurrence and spatial distribution of *E. coli* O26, O103, O111 and O145 in bobby calves in the North Island of New Zealand. The association of IgG concentration, weight, sex and breed with occurrence of O26, O103, O111 and O145 as determined by direct RT-PCR on RAMS enrichments was also investigated. Using RT-PCR 134/299 (44.8%) of RAMS were positive for O26, 68/299 (22.7%) for O103 and 47/299 (15.7%) for O145. No RAMS samples were RT-PCR positive with O111-specific primers. The success of isolation by culture of *E.*

coli O26 (49/134 isolates) was higher from RT-PCR positive samples as compared to O103 (4/68 isolates) and O145 (5/47 isolates). Using a logistic regression model no association was observed between PCR prevalence and the variables IgG, sex or breed of the calves. However, calves positive for O26 were more likely to be positive for O103 and vice versa (P=0.01) and similar association was found between calves positive for O145 and O103 (P=0.03). O26 isolates could be grouped into four clusters (A-D) of $>70\%$ similarity using *XbaI* PFGE. K function analysis did not indicate any evidence of spatial clustering of farms positive for O26, O103 or O145. This study indicted that O26 is more prevalent in bobby calves as compared to O103 and O145 and colostrum feeding may not be helpful in reducing the carriage of *E. coli* O26, O103 and O145.

In the fourth study *E. coli* isolates (n=137) obtained from previous studies were genotyped using PFGE and allelic profiling (based on 8 genes). The *eae* and *ehxA* genes of *E. coli* isolates were subtyped using PCR-restriction fragment length polymorphism (RFLP) analysis. Endonuclease digestion of *ehxA* PCR products with *TaqI* from 121 *ehxA* positive *E. coli* isolates resulted in six *ehxA* subtypes (A-F). Endonuclease digestion with *HhaI* or *HaeIII* enzymes of the 129 *eae* positive isolates indicated that 82 were β followed by ϵ (n=34), γ (n=11) and δ/κ (n=2). An association between *eae* subtype and *E. coli* serogroup was also observed. All O26 isolates were subtype β, all O103 isolates were subtype ϵ and all O157 isolates were γ. *E. coli* isolates were also analysed for plasmid-associated allels *espP*, *etpD*, *katP* and α-*hly* genes. Of 137 isolates 93 (67.8%) were positive for *espP*, 32 for *etpD* (23.3%), 76 for *katP* (55.4%) and nine for the α-*hly* (6.5%) gene. The genotyping ability of allelic profiling was compared with PFGE profiling using PERMANOVA and multidimensional scaling and the results indicated that isolates having similar allelic profiles had similar PFGE profiles and tended to cluster together. The results also indicted that *eae* and *ehxA* profiles could explain a high population of the variance in PFGE profiles. These results may provide basis for the development of new genotyping method for *E. coli* isolates, having high discriminatory power and being easier to perform. The *E. coli* serogroups and *eae* and *ehxA* subtypes observed in these serogroups have also been observed from human *E. coli* isolates indicating the public health significance of these isolates.

These studies provide useful information about epidemiology of O157 and non-O157 in calves in the North Island of New Zealand and indicate that calves may be an asymptomatic reservoir of STEC and a possible source of infection for humans. Better understanding of the population structure and transmission of STEC would help in devising appropriate control strategies. The implementation of these control measures could reduce the prevalence of STEC in calves and thus reduce transmission to humans.

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1 Chapter 1

1.1 Literature review

The German scientist, Theodor Escheric isolated *Escherichia coli* (*E. coli*) for the first time in 1885 from the intestinal flora of infants. He called them Bacterium coli commune (Escherich, 1885). Later, this name was changed to *E. coli* in his honour. The importance of this organism among microbiologists and the general public developed after the recognition that certain strains of *E. coli* are associated with diarrhoea. John Bray in 1945 first described a pathotype of *E. coli* strains that were responsible for causing a disease syndrome known as infantile diarrhoea (Bray, 1945). Since the first report of Shiga toxin-producing *E. coli* (STEC) infection in humans in 1983 (Riley et al., 1983) many outbreaks of STEC have been recorded (Besser et al., 1993; Brooks et al., 2005; Michino et al., 1999; Sharp et al., 1994; Waters et al., 1994). It has been estimated that STEC causes approximately 0.1 million illnesses in the USA each year (Mead et al., 1999). The morbidity (3,222 cases) and the mortality (39 deaths) associated with the most recent European enteroaggregative enterohaemorrhagic *E. coli* (EAEHEC) O104 outbreak (Frank et al., 2011) have once again highlighted the importance of STEC to public health. Food, especially of cattle origin, and direct contact with cattle are considered important routes of STEC transmission to humans (Armstrong et al., 1996; Bettelheim, 2007).

The most common STECs associated with disease in humans belong to serogroups O26, O45, O103, O111, O121, O145 and O157 (Bettelheim, 2007; Mainil and Daube, 2005). The USA has a zero tolerance policy for STEC O157 and some non-O157 STECs in meat (the common serogroups: O26, O45, O103, O111, O121 and O145) (Food Safety and Inspection Service, 2011a,b). The dairy and meat industries make a significant contribution to the New Zealand economy (Cavanagh, 2003). Therefore, these changes may have serious implications for the New Zealand economy due to their impact on international trade. Information about the epidemiology of STEC in New Zealand cattle is scarce. Therefore, more knowledge is required about the epidemiology of STEC in cattle to satisfy the importing countries that meat exported by New Zealand is free from STEC and also to devise appropriate on farm and pre-slaughter control strategies. The objectives of this thesis are;

1. To study the molecular epidemiology of STEC in calves in the North Island of New Zealand.
2. To examine the hypothesis that carriage of STEC is associated with the level of IgG in the serum.
3. To study the diversity and relatedness of STEC and *Campylobacter jejuni* between farms in a dairy catchment and test hypothesis related to transmission of STEC and *C. jejuni* within and between farms.
4. To examine the hypothesis that the genotyping ability of allelic profiling is similar to pulse field gel electrophoresis (PFGE).

Therefore, the focus of this review is to understand the present knowledge about the prevalence, transmission and control of STEC in cattle and also to highlight the areas where more research is

needed. As the population structure and transmission dynamics of *C. jejuni* in addition to STEC are discussed in chapter 2, *C. jejuni* is also included in the literature review.

1.2 Pathotypes of *E. coli*

Diarrhoeagenic *E. coli* can be differentiated into pathotypes on the basis of their clinical presentation, age groups affected and associated virulence factors. There are six distinct clinical groups of diarrhoeagenic *E. coli*, namely STEC, enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (Kaper et al., 2004). EPEC were described for the first time in 1940 (Parry and Palmer, 2002) and were given this name in 1955 (Neter et al., 1955). They mainly cause acute diarrhoea in children less than six months of age (Levine and Edelman, 1984). EPEC lack Shiga toxin (*stx*) gene but contain *E. coli* attaching and effacing (*eae*) gene and are able to form attachment and effacement (AE) lesions on cultured epithelial cells in-vivo (Kaper, 1996). EPEC can be further divided into two subgroups; typical EPEC (tEPEC) and atypical EPEC (aEPEC). tEPEC contain bundle forming pilli (*bfp*) and are normally recovered from human diarrhoeal cases whereas aEPEC lack *bfp* and are commonly recovered from ruminants and may also be associated with human diarrhoeal disease (Trabulsi et al., 2002).

EAEC was described for the first time in 1987 and is considered to be an emerging group. It has a unique aggregative pattern (stacked brick pattern) of adherence to HEp-2 epithelial cells (human cancerous cells from the larynx) in culture (Nataro, 2005). A large outbreak due to EAEC involving 2,697 children was reported in Japan in 1993 (Itoh et al., 1997). It is also thought that outbreaks or cases due to EAEC are under-diagnosed, either because most public health workers are unaware of EAEC or due to limited diagnostic facilities in laboratories for EAEC (Huang et al., 2006). However, the virulence of EAEC is not well defined at the molecular level. Therefore, further investigations are required to understand the virulence of this group (Harrington et al., 2006; Huang et al., 2006).

Among the diarrhoeagenic *E. coli*, ETEC are considered as the most common cause of diarrhoea especially in developing countries (World Health Organization, 1999). They mostly causes diarrhoea in infants less than two years of age (Qadri et al., 2000; Rao et al., 2003). ETEC produce a range of different enterotoxins; some of these are heat stable and others are heat labile enterotoxins. The clinical disease caused by ETEC is known as traveler's diarrhoea (Qadri et al., 2005). ETEC are also considered an important cause of diarrhoea in calves (Nagy and Fekete, 1999).

EIEC were diagnosed and described in 1960s (Kaper, 2005). They cause watery diarrhoea in humans by invading gut epithelial cells (Nataro and Kaper, 1998). EIEC and *Shigella* are similar clinically and diagnostically and share some virulence factors (Escobar-Paramo et al., 2003; Parsot, 2005). EIEC cases have been reported from all over the world and are endemic in underdeveloped countries such as Chile (Faundez et al., 1988), Thailand (Echeverria et al., 1992) and Brazil (Blake et al., 1993) but are rare in developed countries (Beutin et al., 1997; Prats and Llovet, 1995). This difference may be due to high quality sanitation and high socio-economic levels in

developed countries.

DAEC are named due to their ability to form diffuse adherence pattern on epithelial HEp-2 and HeLa cells (cervical cancer cells) (Nataro et al., 1985; Scaletsky et al., 1984). Conflicting information is available about the involvement of DAEC in acute infantile diarrhoea. For example, Giron et al. (1991) and Jallat et al. (1993) reported a significant association between acute diarrhoea cases and isolation of DAEC whereas other studies did not find this association (Cravioto et al., 1991; Gomes et al., 1989; Magalhaes et al., 1992).

In 1982, another pathotype of *E. coli* known as STEC was found to be the cause of human disease (Riley et al., 1983). The first reports of haemorrhagic colitis (HC) due to *E. coli* O157:H7 (Riley et al., 1983) led to the recognition of STEC (Levine, 1987). STEC include those strains which are responsible for causing either HC or haemorrhagic uraemic syndrome (HUS). Initially, Levine (1987) identified two STEC strains (O157:H7 and O26:H11). However, Tzipori et al. (1988) extended the group by adding other serotypes like O4:H-, O45:H2, O111:H- and O145:H-. Today all the strains of *E. coli*, from humans and animals, which produce Shiga toxin (Stx) are known as STEC or verocytotoxin producing *E. coli* (VTEC).

E. coli O26, O91, O103, O111, O113, O121, O145 and O157 are considered important serogroups of STEC due to their frequent involvement in causation of disease in humans (Karmali et al., 2003). The frequency, distribution and virulence of these strains are different in different parts of the world. Therefore, there are different schemes of classification of STEC on the basis of qualitative and quantitative differences in disease association. The most common and simplest classification divides STEC into two groups i.e., STEC O157 and STEC non-O157. Another classification (Karmali et al., 2003) divided STEC into five seropathotypes (SPT) (A through E). This classification is based upon the frequency of occurrence of specific serotypes in human disease and their association with HUS and with disease outbreaks (Table 1.1).

1.3 History of Shiga toxin-producing *E. coli*

The production of verocytotoxin (VT) by *E. coli* was reported for the first time by Konowalchuk et al. (1977). They observed the death of vero cells (Green monkey kidney cells) due to cytotoxin produced by different strains of *E. coli*. These strains were isolated from humans suffering from diarrhoea and piglets suffering from post-weaning oedema disease (ED). They also reported that this cytotoxin was heat labile but antigenically different from other heat labile enterotoxins. The effect of this toxin on vero cells (which appeared round but shriveled) was also different from heat labile enterotoxins (which caused cells to become enlarged, thick walled, refractile and possessing several filamentous tendrils). O'brien et al. (1982) reported the same effect of cytotoxins (appearing round but shriveled) produced by various strains of *E. coli* isolated from humans. They also reported that the effect of these cytotoxins could be neutralized by an immune serum produced against Stx of *S. dysenteriae* type-1. Therefore, the name Shiga-like toxin (SLT) was given to this cytotoxin (Calderwood et al., 1996). In 1982, the *E. coli* O157:H7 serotype was found to be the cause of HC in humans (Riley et al., 1983). Due to the involvement of serotype *E. coli* O157:H7

with HC and HUS the term enterohaemorrhagic *E. coli* (EHEC) has been used for Stx-producing O157:H7.

1.4 Epidemiology of STEC

The epidemiology of STEC is complex and not completely understood. Cattle are considered to be the main reservoir of infection. *E. coli* O157 was isolated for the first time from cattle faeces in 1987 (Chapman et al., 1989). There have been more than 400 serotypes of STEC isolated from healthy cattle (Bettelheim, 2003b; Beutin et al., 1993; Blanco et al., 2004b; Karmali et al., 2010). More than 100 non-O157 serotypes have been reported to be pathogenic for humans (World Health Organization, 1998) and some outbreaks due to these serotypes have been recorded (Caprioli et al., 1994; Mariani-Kurkdjian et al., 1993; Morabito et al., 1998) but still *E. coli* O157 are considered to be more important due to their association with HC and HUS. The underestimated importance of non-O157 *E. coli* may be due to very few laboratories looking for the presence of non-O157 as causative organism. However, the importance of non-O157 *E. coli* is increasing with passage of time and researchers have started looking into the epidemiology of these organisms (Caprioli et al., 2005; Mathusa et al., 2010).

1.4.1 Prevalence of STEC in cattle

The studies giving the rate of prevalence of STEC in cattle in different countries of the world are summarised in Table 1.2. These studies show that the prevalence of STEC ranged from 13% to 70% in dairy and beef cattle. Faecal samples, rectal swabs or a combination of both were collected to determine the rate of prevalence in nine, four and two studies respectively. There was little difference in the range of prevalence of STEC in the studies where faecal samples (13-70%) or rectal swabs (28-70%) were used for screening purposes. However, one study (Meichtri et al., 2004) compared the prevalence rate obtained from faecal samples and rectal swabs. They reported a higher prevalence of STEC using rectal swabs (87.7%) as compared to faecal samples (34.3%) but they used different enrichment media for each sampling and the samples were collected in different months of the year. In most of the studies PCR for *stx* was used as a screening method for STEC while in some studies isolation of STEC was used as a screening method. The prevalence of STEC was higher in the studies where PCR (13-70%) was used as compared to when only culturing of isolates (16-41%) was performed. This could be due to PCR being a more sensitive method than isolation (Grys et al., 2009). The difference in prevalence in different studies might also be due to difference in sample sizes, management practices and environmental factors. Table 1.2 also shows the distribution of *stx1*, *stx2* and *stx1* and *stx2* in different studies. However, some of these studies (Leomil et al., 2003; Leung et al., 2001; Pradel et al., 2000) also provide information about the distribution of *eae* which encodes the intimin (section 1.7.1) and *ehxA* which encodes the enterohaemolysin (section 1.7.3) in the isolates. This information might be biased because only samples which were positive for *stx1*, *stx2* or both were subjected to isolation. There could be other isolates and samples which would have been positive for *eae*, *ehxA* or both.

1.4.2 Prevalence of STEC in New Zealand cattle

The information about the prevalence of STEC in New Zealand cattle is very limited. In 1997 a study was conducted to determine the prevalence of *E. coli* O157 in New Zealand cattle (Buncic and Avery, 1997). Cattle faecal samples (n=371) were collected from a slaughter plant in Waikato region. Only two (0.53%) samples were positive for *E. coli* O157. However, this study was focused on *E. coli* O157 only and it did not provide any information about non-O157 STEC. Another study estimated the prevalence of STEC (including non-O157 STEC) in cattle (n=187) and sheep (n=132) in the lower North Island of New Zealand. The prevalence of STEC was higher in sheep (65.9%) as compared to cattle (27.2%) (Cookson et al., 2006b). In another study Cookson et al. (2006c) provided information about the prevalence of STEC and EPEC isolated from recto-anal mucosal swabs from pasture fed cattle and sheep in the lower North Island of New Zealand. They reported that 43% of the animals were positive for STEC and 33% for EPEC. However, these studies did not specifically look for important serotypes of *E. coli* such as *E. coli* O26, O103, O111, O145 and O157 which were frequently reported to be involved in causing disease in humans.

The study of literature clearly shows that information about the epidemiology of *E. coli* O157 and non-O157 in New Zealand cattle is scarce and further studies are required to fill in this knowledge gap.

1.4.3 Prevalence of *E. coli* O26, O103, O111, O145 and O157 in cattle

Most human cases of STEC are caused by the O157, O26, O103, O111 and O145 and cattle are considered to be the main reservoir of these groups (Bettelheim, 2007; Karmali et al., 2010). A large number of surveys have been conducted to estimate the prevalence of *E. coli* O157 in cattle in different countries (Meyer-Broseta et al., 2001). The cattle surveys conducted in America and Europe reported that the herd prevalence of *E. coli* O157 in cattle ranged from less than 1% to almost 10% (Blanco et al., 1993; Clarke et al., 1988; Martin et al., 1994). However, recent studies conducted in the UK reported a higher prevalence of *E. coli* O157 in English cattle (38.7%) (Paiba et al., 2003). This could be due to the use of selective enrichment followed by immuno-magnetic separation (IMS) whereas other studies attempted culturing of *E. coli* O157 without using IMS.

Several studies have also been conducted to estimate the within-herd prevalence of *E. coli* O157 in cattle. A study conducted in the USA reported less than 1.5% within herd prevalence of *E. coli* O157 in animals less than eight weeks of age (Hancock et al., 1994). A higher prevalence of *E. coli* O157 was also reported in calves (56%) as compared to non-lactating adult cows (40%) in England (Mechie et al., 1997).

There are few studies about the prevalence of STEC O26, O103, O111 and O145 in cattle. For example, Pearce et al. (2006) conducted a national survey to determine the prevalence of STEC O26, O103, O111 and O145 in faeces of Scottish cattle. They reported that the weighted mean prevalences of STEC O26, O103, and O145 in faecal pats were 4.6%, 2.7% and 0.7% respectively. They could not detect any STEC O111 in the faecal pats. They also reported that 28.9% of O26

isolates carried the *stx1*, *eae* and *ehxA* gene profile. This profile is commonly found in the O26 strains isolated from human disease cases (Schmidt et al., 1999a; Zhang et al., 2000b). Similarly, Bonardi et al. (2007) studied the prevalence of STEC O26, O103, O111 and O145 in Italian cattle using IMS. Faecal samples were collected from 182 cattle from slaughter plants. The prevalence of O157 and O26 was 3% and 0.5% respectively. They could not isolate O103, O111 or O145 in faecal samples.

Lee et al. (2008) studied the occurrence of O26 and O111 in 442 beef and dairy calves (257 calves with diarrhoea and 185 without diarrhoea) less than 16 weeks of age in Korea. They reported that 14.4% and 7.6% of 257 calves with diarrhoea were tested positive for O26 and O111 respectively while 7.6% and 5.9% of 185 calves without diarrhoea were tested positive for O26 and O111 respectively. These results show the potential of O26 and O111 to cause diarrhoea in calves.

1.4.4 Transmission of STEC between cattle

Horizontal transmission of STEC between calves plays an important role in the epidemiology of STEC. Different studies have identified horizontal transmission of STEC between calves. For example, Besser et al. (2001) experimentally challenged four of a group of 17 calves with STEC O157. They observed that all the calves started excreting the same strain of STEC O157 within 21 days post-challenge. The similarity between the challenged and isolated strain was confirmed by using PFGE. Similarly, there is also evidence about the transmission of STEC from the farm environment to the calves (Lahti et al., 2003). Of 31 calves negative for STEC, six started shedding STEC one day after introduction into the farm. STEC was also isolated from surface samples taken from feed passages, water bowls and floors of pens on the day of arrival of the calves. The PFGE profile of most of the isolates from environmental samples and calves were indistinguishable.

Water is considered to be an important source of horizontal transmission of STEC and it has been isolated from cattle drinking water (Hancock et al., 1994; Shere et al., 1998; van Donkersgoed et al., 2001). There are some studies which provide evidence about the association between shedding of STEC by calves and contaminated drinking water (Hancock et al., 1998; Sargeant et al., 2003; Shere et al., 1998). Saliva of the infected animals may also play an important role in horizontal transmission of STEC through feed and water (Buchko et al., 2000; Shere et al., 1998).

Most of the longitudinal studies have suggested that maintenance of STEC in cattle herds requires continuous re-inoculation of individual animals (Rahn et al., 1997). The environment is considered to be an important source for re-inoculation of STEC to cattle. Liu et al. (2007) used mathematical modeling to show the importance of the environment as source of infection in Scottish cattle. They estimated that 80% of STEC transmission between calves was from environmental sources and 20% from direct calf to calf transmission. A previous study has reported similar findings (Liu et al., 2005).

There are some studies which provide information about the duration of survival of STEC in cattle faeces (Wang et al., 1996) and slurry (McGee et al., 2001). Wang et al. (1996) showed that STEC can survive in faeces at 37 °C for 49 days. McGee et al. (2001) reported the survival of STEC

in slurry for 12 weeks. The survival of STEC in faeces and slurry indicate the importance of the environment in transmission of STEC.

1.4.5 Seasonality

The effect of season on prevalence of STEC in cattle has been observed by many researchers. Most studies reported the highest prevalence of STEC O157 in summer as compared to other seasons (Hancock et al., 1997; Heuvelink et al., 1998b; Mechie et al., 1997; Schouten et al., 2004). For example, Heuvelink et al. (1998b) reported a summer prevalence of STEC O157 in Dutch dairy cattle of 15% as compared to 5.9% winter. The effect of seasonality and genetic diversity of STEC O157 was also observed by some researchers. Aslam et al. (2010) reported higher diversity of STEC O157 strains in summer and autumn as compared to winter. This may be due to the fact that some strains can survive better in winter as compared to other months (Bach et al., 2005). However, there are some studies which showed that prevalence of *E. coli* O157 was higher in winter as compared to summer (Alam and Zurek, 2006; Ogden et al., 2004; Synge et al., 2003). For example, prevalence of *E. coli* O157 in Scottish beef cattle has been estimated to be 11.2% in winter and 7.5% in summer (Ogden et al., 2004).

The studies related to prevalence of non-O157 in cattle in different seasons provide variable findings. Some studies show that the prevalence and shedding of non-O157 STEC was higher in summer as compared to winter. Pearce et al. (2006) showed that the percentage of Scottish farms with cattle positive for O26 was higher in summer (33%) as compared to winter (17%). Similar findings were reported by Fukushima and Seki (2004) and Widiasih et al. (2004a) in Japanese cattle. However, the highest prevalence of non-O157 STEC in winter (19.9%) was observed by Cobeljic et al. (2005) in Serbia as compared to summer (9.8%). Similarly, Leung et al. (2001) reported the highest prevalence of non-O157 and O157 STEC in winter as compared to summer in cattle in Hong Kong. But the study conducted by Barkocy-Gallagher et al. (2003) in American cattle showed highest prevalence of non-O157 STEC in cattle in spring (17.7%) and autumn (22.2%) as compared to summer (10.2%) and winter (10.3%).

The exact reason for the difference in prevalence of STEC in different seasons is not known. However, some studies provide possible explanations for higher prevalences of STEC in summer or winter. For example Mechie et al. (1997) assumed that *E. coli* O157 can survive better in warm and moist environments, therefore pasture will be contaminated with higher concentration of *E. coli* O157 in summer and due to grazing of animals in summer the likelihood of *E. coli* O157 infection increases. According to Ogden et al. (2004) cattle are housed in winter so they are in close contact with each other which results in an increase animal to animal transmission.

1.4.6 Risk Factors

A number of studies have been conducted to examine the risk factors for carriage and shedding of STEC O157 and non-O157 by cattle. The most common risk factors studied have been herd size,

animal age and management practices.

The literature provides conflicting information about herd size as a risk factor. A study conducted in Sweden indicated that an increase in herd size from 20 to 100 animals resulted in an increase in risk of obtaining positive samples for *E. coli* O157 (OR=3.5 by multivariate analysis; CI=0.9-12.8) within herd (Eriksson et al., 2005). A study conducted on Scottish beef cattle also reported that farms with 12-28 animals were more likely to be positive than those with <12 animals (log-odds ratio 0.68; P=0.003) (Gunn et al., 2007). A certain number of animals are required for circulation of infection (Anderson and May, 1991) and the probability of total loss of infection is higher in small group of animals as compared to large group. Furthermore, the probability of movement and contact of calves and young heifers within large herds resulting in O157 transmission was higher than in small herds. However, other studies could not find an association between herd size and shedding of *E. coli* O157 (Garber et al., 1995; Nielsen et al., 2002).

The age of the animals is an important risk factor for the prevalence of *E. coli* O157. The prevalence of *E. coli* O157 is higher in weaned calves as compared to un-weaned calves (Meyer-Broseta et al., 2001). A case-control study conducted by Garber et al. (1995) in the USA indicated that the calves were three times more likely to shed *E. coli* O157 after weaning than before weaning (P<0.005). Danish study showed that the calves between 2-6 months of age were most likely to be infected while the calves <2 months and >6 months of age were less likely to be infected with *E. coli* O157 (Nielsen et al 2002). Another study reported that the prevalence of *E. coli* O157 was higher in calves between 2-12 months of age than in calves <2 months and >12 months of age (Paiba et al., 2003). Other studies also reported similar findings (Eriksson et al., 2005; Gunn et al., 2007; Wilson and Bettelheim, 1980). This age related difference in prevalence of STEC may be associated with the development of rumen. The rumen of adult calves is well developed and there is high concentration of volatile fatty acids and low pH which inhibits the growth of STEC (Rasmussen et al., 1993). This may also be due to differences in nutrition, immunity and management practices. For example, a study showed that the shedding of *E. coli* O157 was higher in calves that were fed straw compared to those that were not fed straw (OR = 2.29, 95% CI=1.2-5.5) (Ellis-Iversen et al., 2007). Another study reported similar findings (Rugbjerg et al., 2003).

Berg et al. (2004) evaluated the effect of barley-based and corn-based finishing rations on shedding of *E. coli* O157 in faeces. The prevalence of *E. coli* O157 was higher in barley fed animals (2.4%) as compared to corn fed animals (1.3%). Animals that were fed barley were more likely to excrete *E. coli* O157 in higher concentrations (3.3 log CFU/g) in faeces as compared to animals that were fed corn based finishing rations (3.0 log CFU/g) (P<0.01). This has been supported by other studies (Buchko et al., 2000; Dargatz et al., 1997). Fermentation of barley takes place in the rumen while that of corn in the large intestine (Orskov, 1986). Therefore, it is suspected that lower pH and excessive production of inhibitory volatile fatty acids due to fermentation of corn in the large intestine make it unsuitable for growth of *E. coli* O157.

The effects of other management practices on excretion of *E. coli* O157 in faeces have also been reported. Rugbjerg et al. (2003) found that the likelihood of shedding *E. coli* O157 was lower in calves that had suckled colostrum compared to calves that had not suckled colostrum. Another

study reported high level of antibodies in sera of calves against STEC O26, O111 and O157 that were fed colostrum with high antibody titers (Widiasih et al., 2004b). However, these calves were not challenged with STEC O26, O111 and O157 to see whether this high level of antibodies is protective or not. Other studies have reported that high concentrations of antibodies in serum may not be protective against intestinal colonisation of STEC (Snodgrass et al., 1980; van Diemen et al., 2007). Therefore, more studies are required to establish the protective efficacy of colostrum feeding against STEC.

A longitudinal study investigated the effect of animal level and management level factors on shedding of *E. coli* O157 in cattle faeces. The association between excretion and magnitude of shedding of *E. coli* O157, and animal and housing level factors were investigated. At housing level, the height of bedding and presence of the weaned group within the pen were significantly associated with increased chances of isolation of *E. coli* O157 from faeces. However, at animal level the chances of isolating *E. coli* O157 were higher from faeces of male Friesian calves and faeces containing pieces of grain (Robinson, 2004).

The impact of organic and conventional farming on prevalence of STEC in cattle has also been investigated. Faecal samples were collected from 966 cows from 60 farms with organic production (481 cows) and 60 farms with conventional production (485 cows) in Switzerland. STEC was detected in all the 120 farms. This showed that there was no significant effect of conventional or organic farming on presence of STEC infection on the farm (Kuhnert et al., 2005).

There are several studies with conflicting results about the presence of pigs as risk factor for STEC in cattle. Some studies reported the presence of pigs on the farm to be a risk factor for STEC (Eriksson et al., 2005; Gunn et al., 2007). However, a Scottish study indicated presence of pigs on farm as protective against STEC in cattle (Synge et al., 2003). *E. coli* O157 has been isolated from pigs (Chapman et al., 1997; Heuvelink et al., 1999) and colonisation of intestine with O157 and excretion in the faeces have also been reported (Booher et al., 2002) therefore, pigs have a potential to act as reservoir for *E. coli* O157. However, these conflicting findings show that the exact role of pigs as risk factors for STEC in cattle is not known and further studies are required to improve understanding in this area.

The literature indicates that there is not enough information about the risk factors for survival, shedding and transmission of STEC, therefore further information is required to bridge this gap of information.

1.5 Control of STEC in cattle

The control of STEC in cattle is an uphill task due to its complex epidemiology that is currently not fully understood. A number of control measures have been investigated including good management practices, vaccination and use of probiotics.

1.5.1 Vaccination

Different workers have tried various vaccines to control STEC in cattle. A plant cell based intimin vaccine given orally to the mice reduced the period of shedding *E. coli* O157 (Judge et al., 2004). However, the efficacy of this vaccine in cattle was not tested. Most recently McNeilly et al. (2010) developed a vaccine by combining three different bacterial proteins (intimin-531, EspA and Tir). This vaccine significantly reduced the shedding of *E. coli* O157 in experimentally infected cattle. They also suggested that addition of H7 antigen (previously demonstrated to be partially effective) to the vaccine might further enhance the efficacy of the vaccine. Similarly, Thomson et al. (2009) evaluated the efficacy of a siderophore receptor and porin (SRP) protein-based vaccine and reported that it was effective in reducing the concentration of *E. coli* O157 in cattle faeces presented for slaughter.

Potter et al. (2004) prepared a vaccine using type three secretory proteins (TTSP) namely Tir and EspA. This vaccine significantly reduced the number of *E. coli* O157 in faeces, the number of animals shedding and the duration of shedding in experimental conditions. Vaccination was also significantly (P=0.04) helpful in reducing the prevalence of *E. coli* O157 in a clinical trial in a feedlot system. Asper et al. (2007) prepared TTSP vaccine against serotypes O26:H11, O103:H2 and O111:NM. Each vaccine provided immunity against its specific serotypes but did not provide any cross protection. They suggested that serotype specificity is a major hurdle in preparing effective vaccines against non-O157 serotypes and new methods are required to prepare effective vaccines against different strains of STEC serogroups.

1.5.2 Probiotics

There are some bacteria, including non-pathogenic strains of *E. coli* which produce metabolites (e.g. colicin) which are harmful to *E. coli* O157 (Schamberger and Diez-Gonzalez, 2002). Therefore, these bacteria can be used to reduce the carriage of *E. coli* O157 in cattle. For example, Zhao et al. (1998) reported that use of probiotics in cattle before exposure to *E. coli* O157 reduced the ruminal carriage of *E. coli* O157. Similarly, Tkalcic et al. (2003) evaluated the effect of probiotics on faecal shedding of O26, O111, and O157 in calves. They reported that use of probiotics was helpful in significantly reducing ($P < 0.05$) the shedding of O157 and O111 but it did not have any effect on the shedding of *E. coli* O26.

1.5.3 Bacteriophages

Bacteriophages have been used in-vivo and in-vitro in different experimental studies to control *E. coli* O157 (Fischer et al., 2004; Kudva et al., 1999; O'Flynn et al., 2004; Tanji et al., 2005). One study investigated the effects of a cocktail of three bacteriophages against *E. coli* O157 on pieces of steak (O'Flynn et al., 2004). In seven of nine trials bacteriophages completely destroyed *E. coli* O157 on the surface of the meat. Another study evaluated the efficacy of bacteriophages against *E. coli* O157 in cattle. Of 10 steers challenged with *E. coli* O157 (10^6 CFU) five were treated with

a mixture of two phages (KH1 and SH1). Phages were applied to recto-anal junction mucosa. A significant decrease ($P<0.05$) in the number of *E. coli* O157 (10^6 CFU to $10^{1.4}$ CFU) was observed in the phage treated group as compared to the control group (10^6 CFU to $10^{2.7}$ CFU). However, this treatment could not completely clear the bacteria from steers (Sheng et al., 2006). Niu et al. (2009) investigated host range and lytic ability of phages against bovine and clinical human STEC O157:H7 isolates. Different phages have different lytic capability against various STEC O157 isolates, therefore, it was suggested that a cocktail of various phages may be a useful approach to control STEC O157 in cattle on the farm. However, this therapy was not evaluated in the farm animals (Niu et al., 2009).

The use of chemicals such as sodium chlorate (Callaway et al., 2002) and plant products such as esculin (Duncan et al., 2004) have also been reported as being helpful in reducing the shedding of *E. coli* O157 from cattle.

1.6 Epidemiology of STEC in humans

STEC has emerged as an important human pathogen in the last three decades, causing morbidity and mortality in humans. Outbreaks of STEC in humans have been reported from all over the world (Bettelheim, 2007). The most important serotypes of STEC involved in causation of disease in humans are O157, O26, O103, O111 and O145 (Beutin, 2006).

E. coli O157-associated outbreaks have been more commonly reported in North America, Scotland and England as compared to non-O157-associated outbreaks (Brooks et al., 2005; Health Protection Scotland, 2010; Jenkins et al., 2008). In North America, 85-95% of cases of HUS are associated with *E. coli* O157 (Griffin, 1995). The literature shows that the human cases of *E. coli* O157 infection have strong seasonality in Canada, England, Japan and North America. The number of cases is higher in summer, reaching to a maximum level in July or August and then becoming lower in winter (Pai et al., 1984; Parry and Palmer, 2002).

However, in parts of southern hemisphere and continental Europe non-O157 STEC (especially O26, O103, O111 and O145) are considered to be the most important cause of human outbreaks (Brooks et al., 2004, 2005; Hedican et al., 2009; Sonntag et al., 2004; van Duynhoven et al., 2008). It has also been observed that the number of human cases due to non-O157 STEC is on the rise worldwide, for example a 300% increase in the incidence of STEC serotypes O26, O111 and O103 has been reported from 2004 to 2007 in New Mexico USA (Lathrop et al., 2009). Outbreaks due to non-O157 STEC (O26, O103, O111 and O145) have been observed in Italy (Caprioli et al., 1994), Australia (Cameron et al., 1995), Germany (Morabito et al., 1998), France (Mariani-Kurkdjian et al., 1993), United States (Bergmire-Sweat et al., 2000; Tarr et al., 1996) Ireland (McMaster et al., 2001) and Japan (Padola et al., 2002).

Of these STEC serotypes O26 has prime importance in causing disease in humans in central and southern Europe (Tozzi et al., 2003; Zhang et al., 2000b). For example half of the STEC strains isolated from paediatric HUS patients between 1997 and 2000 in Germany and Austria were non-O157 (90 of 207 strains) and most commonly isolated non-O157 STEC strain was O26 (Gerber

et al., 2002). The most frequently isolated strains of O26 from diseased humans are O26:H11 and O26:H- (Bettelheim, 2003a).

The STEC serogroup O111 is also of great importance. Outbreaks due to O111 have been reported from the USA (Brooks et al., 2004), Australia (Vally et al., 2012), Japan (Tanaka et al., 1989) and Europe (Caprioli and Tozzi, 1998). Non-O157 STEC related HUS cases in the USA have been mostly due to O111 (Brooks et al., 2005). The first outbreak of STEC O111 in the USA was reported in 1999. This outbreak occurred in Texas and involved 58 teenage campers including two HUS cases. The source of outbreak could not be identified (Centres for Disease Control and Prevention, 2000). The largest O111 outbreak occurred in Oklahoma, USA in 2008 and involved 341 cases. Of these 25 cases developed HUS and one person died. A restaurant was found to be the source of the outbreak but exact mode of transmission of organism within restaurant could not be identified (Oklahoma State Department of Health, 2009).

STEC O103 has also been isolated from human cases in different countries. It is considered an emerging food-borne pathogen (Prager et al., 2002) and it can become more virulent by acquiring bacteriophages harbouring the *stx2* gene (Beutin et al., 2005; Prager et al., 2002). STEC O103 was identified as a causative organism of HUS for the first time in 1992 (Mariani-Kurkdjian et al., 1993). Outbreaks (Gomez et al., 1995; Schimmer et al., 2008) and sporadic cases (Tarr et al., 1996) due to STEC O103 have been reported. One outbreak was recorded in Japan in a family (Saito et al., 1998) and a second was in Argentina in a day care centre (Gomez et al., 1995). In both the outbreaks O103 isolates contained *stx1* toxin genes. Another outbreak of HUS due to O103:H25 was reported in Norway in 2006. This strain was positive for *stx2* and was the first reported outbreak due to a *stx2*-positive STEC O103 (Schimmer et al., 2008).

Different investigators have isolated O145 from human cases (Beutin et al., 1998; Brooks et al., 2005; Tozzi et al., 2003). STEC O145 is considered as an emerging serotype (Padola et al., 2002). The prevalence of O145 in human disease is low compared to other non-O157 serotypes (de Schrijver et al., 2008). It contributes 5-7% of all non-O157 cases in Finland, Germany (Busch et al., 2007) and the USA (Brooks et al., 2005). The first outbreak due to STEC O145 was recorded in Japan in 1984, affecting 100 children (Padola et al., 2002). Consequently sporadic cases and outbreaks caused by O145 have been observed in different parts of the world (Busch et al., 2007; de Schrijver et al., 2008). Seasonality of human cases has been observed, for example, the highest number of cases reported in summer and lowest in winter in Ireland (Carroll et al., 2005). Similarly, Hedican et al. (2009) observed the highest number of cases due to O145 in summer in Minnesota, USA.

A large outbreak of STEC was observed in Germany in May-June 2011. This outbreak involved 3,222 cases, of which 810 were HUS cases. This outbreak was caused by EAEHEC O104:H4 (Frank et al., 2011). Fenugreek sprouts have been reported to be the source of the outbreak (Bielaszewska et al., 2011). The name EAEHEC was given because in addition to *stx2* the fenugreek sprout outbreak isolates also contained genes typical of EAEC such as *attA*, *aggR*, *aap*, *aggA*, *aggC*. It is also thought that the strain might have originated from an EAEC pathotype because of high resemblance of its genome to that of EAEC strain 55989 (Brzuszkiewicz et al.,

2011). Cases due to the Fenugreek sprout outbreak strain were reported from 15 countries all with history of travel to Germany during May and June 2011. This outbreak was different from other large outbreaks in many aspects. For example, a larger number of outbreak cases (25%) developed HUS compared to previously reported outbreaks (Bell et al., 1994; Fukushima et al., 1999; Guh et al., 2010; Soderstrom et al., 2008). The majority of the HUS cases were adults rather than children. The causative organism of this outbreak was non-O157 STEC while earlier reported large STEC outbreaks were due to STEC O157. Another striking feature of this pathogen is a longer incubation period (8 days) than normal (3-4 days) (Frank et al., 2011). STEC O104:H4 has been isolated previously from HUS cases in Germany (1 case) (Mellmann et al., 2008) and South Korea (1 case) (Bae et al., 2006). However, the recent outbreak showed that virulence of the O104:H4 outbreak strain was greater than that previously observed with O157. This may be due to the unique combination of STEC and EAEC virulence factors (Brzuszkiewicz et al., 2011). EAEHEC O104 has never been reported from human cases in New Zealand, however, STEC O104:H-/H7 has been isolated from sheep meat in New Zealand (Brett et al., 2003).

1.6.1 Epidemiology of STEC in humans in New Zealand

The first human cases relating to STEC in New Zealand were reported in 1980. The causative STEC serotypes for those cases were O26 and O39 (Wilson and Bettelheim, 1980). However, the first human case of *E. coli* O157 in New Zealand was reported in 1993 (Wright et al., 1993). In 2011, 153 cases of STEC infection were recorded in New Zealand. Of these 139 cases were due to STEC O157 and 14 due to non-O157 STEC. The highest rate of STEC was recorded in Waikato (8.2 cases per 100,000 population; 30 cases) and Taranaki (8.2 cases per 100,000 population; 9 cases) followed by Northland (5.1 cases per 100,000 population; 8 cases). The rate of STEC infection in New Zealand has increased from 1.3 cases per 100,000 population in 1998 to 3.5 cases per 100,000 population in 2011 (Figure 1.1) (Environmental Science and Research, 2011). This suggests an increasing trend of STEC infection in New Zealand. This rate of infection is higher than that in the UK which is 1.28 cases per 100,000 population (Health Protection Agency, 2010) and much higher than that in the USA (0.9 cases per 100,000 population) (Centres for Disease Control and Prevention, 2011). The highest rate of STEC in New Zealand was reported in children between 1-4 years old (23.8 cases per 100,000 population; 60 cases) followed by children less than one year old (14.4 cases per 100,000 population; 9 cases). ESR data also suggest that STEC associated infection is equal for female (3.8 cases per 100,000 population; 85 cases) and male (3.1 cases per 100,000 population; 68 cases). However, disease was more common in people of European and Other ethnicity (4.3 cases per 100,000 population; 131 cases) as compared to Maori ethnicity (2 cases per 100,000 population; 13 cases) (Environmental Science and Research, 2011). There is a possibility of underestimating the rate of infection in the New Zealand population. For example people affected by mild cases of disease might not consult the doctor, testing may not be carried out in early stages of infection and some laboratories look for STEC O157 only overlooking non-O157 STEC (Baker et al., 1999). The increasing trend of STEC in New Zealand suggests that more efforts are required to look into the epidemiology of this disease.

1.6.2 Incubation period

The usual incubation period of *E. coli* O157 is 3-4 days. For example, the incubation periods recorded for Michigan and Oregon outbreaks in 1982 were 3.8 and 3.9 days respectively (Karmali, 1989). However, incubation periods as short as one day and as long as eight days have also been observed (Griffin, 1995; Ryan et al., 1986). Dundas and Todd (2000) observed the association of HUS and incubation period and reported that there were more chances of development of HUS in patients with short incubation periods.

1.6.3 Dose response

The relationship between dose and infection of *E. coli* O157 in healthy volunteers is unlikely to be determined experimentally. Lack of treatment and complications of infection make these studies unethical. However, some workers have tried to estimate the dose response relationship for *E. coli* O157. Their studies showed that an infectious dose for *E. coli* O157 is very low. For example, Tilden et al. (1996) estimated the most probable number of *E. coli* O157 (0.4 organism per gram) in positive salami samples collected from a plant. This number was used to calculate the number of *E. coli* O157 in a piece of salami consumed by the patient. They estimated that less than 50 bacteria were sufficient to cause disease in humans. A study conducted by Tuttle et al. (1999) suggested that the infectious dose may be fewer than 700 organisms. Similarly, Willshaw et al. (1994) found that in an outbreak linked with beef burgers, less than 700 organisms were enough to cause the disease. Strachan et al. (2001) also reported a low infectious dose (4-24 organisms) in an environmental outbreak of *E. coli* O157 in humans. Studies on transmission of *E. coli* O157 via person to person (Belongia et al., 1993) and transmission by water (Swerdlow et al., 1992) also suggest a low infectious dose.

1.6.4 Clinical manifestation

STEC can cause various clinical manifestations. It can cause asymptomatic infection, non- bloody diarrhoea, bloody diarrhoea, HUS, thrombotic thrombocytopenic purpura (TTP) and death (Griffin et al., 1988). Most STEC infections start with abdominal cramps and non-bloody diarrhoea. This non-bloody diarrhoea can become bloody three to four days after the onset of disease. Sometimes the bloody diarrhoea is so severe that the stool from patients with *E. coli* O157 may be described as all blood and no stool (Riley et al., 1983). Nausea and vomiting can also be observed in some patients. However, fever may be absent or of low grade (Griffin et al., 1988). The clinical signs of STEC infection usually disappear in about one week. It is very hard to distinguish between the illness caused by STEC O157 and non-O157 STEC. However it has been observed that non-O157 STEC cause watery diarrhoea more often than bloody diarrhoea (Jelacic et al., 2003; Werber et al., 2004). A prospective study conducted in Japan showed a significant difference in clinical signs associated with O157 and non-O157 infection (Pai et al., 1988). They reported that patients infected with non-O157 had a longer duration of diarrhoea (9.1 days) as compared to the patients

infected with O157 (5.7 days). They also reported that bloody diarrhoea was more common in patients infected with STEC O157 (97%) as compared to patients infected with non-O157 STEC (42%).

1.6.5 Vehicles of STEC transmission

STEC is transmitted to humans from various sources. The largest outbreak of *E. coli* O157 in the USA was reported in 1993. It resulted in more than 500 cases and four deaths. Hamburgers were reported to be the source of the outbreak (Bell et al., 1994). Due to association of STEC with hamburgers it was also called the hamburger bug (Aspan and Eriksson, 2010). However, a variety of foods have been found to be responsible for STEC outbreaks. These include under-cooked minced beef (Armstrong et al., 1996), lettuce (Ackers et al., 1998) and yoghurt (Morgan et al., 1993). Contaminated drinking water (Swerdlow et al., 1992) and other drinks including apple cider (Zhao et al., 1993) and unpasteurized milk (Borczyk et al., 1987) have also been reported as likely sources of STEC transmission to humans. Person to person transmission (Belongia et al., 1993; Kohli et al., 1994) and direct transmission from animals to humans (Aspan and Eriksson, 2010; Renwick et al., 1993) have also been well documented.

Environmental contamination with animal faeces is also a possible source of human infection. Strachan et al. (2001) reported an outbreak of *E. coli* O157:H7 in scouts after camping. Of the 226 scouts 18 were confirmed infected with *E. coli* O157:H7. The vehicle of transmission of *E. coli* O157 to scouts was thought to be the camping field previously used for sheep grazing. PFGE also showed that clinical and environmental isolates were same.

1.7 Mechanism of virulence and pathogenicity

The pathogenesis of STEC is a complex and multistep process that depends upon the interaction between host and bacterial factors. The virulence factors in STEC have mostly been studied in *E. coli* O157. *E. coli* O157 possesses some important virulence factors, the most important factors of STEC being intimin, production of Stx and plasmid-associated virulence factors.

1.7.1 *E. coli* attachment and effacement gene

Enteric bacteria causing AE lesions are known as attaching-effacing *E. coli* (AEEC). AEEC strains associated with gastroenteritis in humans are generally divided into two groups; EPEC and STEC (Wales et al., 2005). The locus of enterocyte effacement (LEE) is a large chromosomal pathogenecity island which harbors the *eae* gene responsible for AE lesions (Kaper et al., 1998). The *eae* gene encodes intimin, an outer membrane protein of which there are at least 20 types/subtypes (Adu-Bobie et al., 1998; Blanco et al., 2004a,b,c, 2005, 2006a; Gannon et al., 1993; Garrido et al., 2006; Oswald et al., 2000; Tarr and Whittam, 2002; Zhang et al., 2002). Several studies have indicated that different *E. coli* serogroups posses a specific intimin type. For example, serogroup O26

often possesses intimin type β, serogroup O103 intimin type θ or ϵ and serogroups O145 and O157 intimin type γ (Blanco et al., 2004c; Cookson et al., 2007a; Kozub-Witkowski et al., 2008; Posse et al., 2007). Host specificity and tissue tropism displayed by various STEC may be influenced by different *eae* types (Fitzhenry et al., 2002, 2003; Phillips and Frankel, 2000; Phillips et al., 2000). Naylor et al. (2003) found recto-anal mucosal junction as primary site for attachment of STEC O157 (γ *eae* subtype) in experimentally inoculated cattle. However, the attachment site for other *eae* types is not known. The contact of STEC or EPEC with eukaryotic cells leads to the activation of LEE genes including *espA*, *espB*, *espC*, *espD* (*E. coli* secretory protein) and *tir* (translocation intimin receptor) (Jarvis et al., 1995; Kenny et al., 1997; Perna et al., 1998). *espA* encodes EspA protein which forms a tube-like structure known as translocon which acts as a channel for transportation of EspB, EspC, EspD and Tir (Knutton et al., 1998). Tir is inserted into the eukaryotic cell membrane where it acts as a receptor for intimin (Kenny et al., 1997). Host cells cytoskeletal structure undergoes certain changes such as formation of cup like pedestals due to accumulation of filamentous actin at site of Tir attachment (Knutton et al., 1989; Moon et al., 1983). Intimin then attaches to Tir to form AE lesions (Donnenberg and Kaper, 1991; Jerse et al., 1990).

1.7.2 Shiga toxins

The most important virulence marker of STEC is *stx* and most of the HUS and HC cases are associated with the strains of *E. coli* that produce Stx (Karmali et al., 2010; Paton and Paton, 1998b; Tarr et al., 2005). There are two types of Stx, namely Stx1 and Stx2 (Cohen et al., 1987; Lingwood et al., 1987). Stx2 has 55% homology with Stx1 (Sandvig, 2001). Stx1 can be further divided into three subgroups (Stx1, Stx1c and Stx1d) and Stx2 into eight subgroups on the basis of difference in number of amino acids (Friedrich et al., 2002; Sandvig, 2001). Stx1 and Stx2 consist of two subunits known as A (Active) and B (Binding) subunits. The B subunit first binds to the receptor with the A subunit entering the cell and inhibiting protein synthesis (Obrig et al., 1987). The receptor for attachment of Stx is a glycolipid globotriaosyl ceramide (Gb3) present on the surface of susceptible cells. It has been observed that the STEC producing Stx2 or both Stx1 and Stx2 are more frequently involved in the causation of disease than STEC producing Stx1 only (Boerlin et al., 1999; Scotland et al., 1987; Thomas et al., 1993). Boerlin et al. (1999) observed that *stx2* negative isolates are five time less likely to be associated with severe disease than *stx2* positive isolates. Biologically active Stx1 and Stx2 can cross the intestinal epithelial monolayer and penetrate the epithelium (Hurley et al., 1999) and can cause damage to colonic blood vessels. Endothelial cells are considered to be the primary site of attachment of Stx (Karmali and Goglio, 1994; Karmali et al., 1994). In-vitro studies have shown that human renal endothelial cells were 100 to 1000 times more sensitive to Stx than human vein umbilical endothelial cells which may be due to presence of higher number of receptors (50 time higher than human vein umbilical endothelial cells) for Stx on human renal endothelial cells (Obrig et al., 1993).

1.7.3 Plasmid Associated virulence factors

Plasmid O157 (pO157) is a highly conserved plasmid present in *E. coli* O157. pO157 of two *E. coli* O157 outbreak strains have been sequenced and its size ranged from 92-104kb. The pO157 has heterogenous composition and is composed of different mobile genetic elements such as prophages, transposon and insertion sequence elements (Burland et al., 1998; Makino et al., 1998). The pO157 contains various virulence genes involved in the pathogenesis of EHEC. These are enterohaemolysin (*ehxA*) (Schmidt et al., 1994), catalase peroxidase (*katP*) (Brunder et al., 1996), serine protease (*espP*) (Brunder et al., 1997) and type II secretion system (T2SS) (*etp*) (Schmidt et al., 1997).

Plasmid encoded *ehxA* is an important virulence marker which can cause haemolysis of washed sheep erythrocytes (Beutin et al., 1988, 1989; Schmidt and Karch, 1996; Taneike et al., 2002). The exact role of *ehxA* in disease mechanism is not clearly understood. However, in-vitro studies have shown an increased level of the proinflammatory cytokine interleukin-1β from human monocytes in response to eneterohaemolysin from STEC O128:H12 (Taneike et al., 2002).

katP encoding catalase peroxidase (KatP) was identified for the first time from pO157. KatP is homologous to bacterial bifunctional catalase peroxidases (Brunder et al., 1996). It has been suggested that KatP plays a role in neutralizing the cytotoxic oxidants produced by macrophages and neutrophils to damage bacteria. The role of KatP has been studied in *Salmonella typhimurium* (Kagaya et al., 1992) and *Shigella flexneri* (Franzon et al., 1990). However, further studies are required to understand the role of KatP in EHEC virulence.

pO157 also contains a gene known as extracellular serine protease (*espP*) which encodes for EspP. *espP* has 1300 open reading frames (ORFs) and encodes 1300 amino acids. The exact role of this enzyme in pathogenesis is unknown. However, it is thought that mucosal haemorrhages in HC patients are due to break-down of pepsin A and coagulation factor V by EspP. Further, antibodies against EspP have been detected in sera of patients infected with EHEC (Brunder et al., 1997).

pO157 has 13 ORFs frames named from *etpC* to *etpO* which encodes for EtpC to EtpO proteins. These proteins are similar to T2SS of Gram negative bacteria. The analysis of 30 *E. coli* O157 EHEC strains, 30 non-O157 EHEC strains from human patients and 30 STEC strains from cattle faeces revealed that the *etp* gene cluster was present in all *E. coli* O157 EHEC strains, 60% of non-O157 EHEC strains and 10% of STEC strains from cattle faecal samples (Schmidt et al., 1997). The role of this gene cluster in disease mechanisms is not known and further studies are required to gain a better understanding of this gene cluster.

1.8 Importance of STEC for the New Zealand economy

The meat and dairy industries are key contributors to the New Zealand economy and contributed NZ$10.8 billion to the economy in 2001 (Cavanagh, 2003). It is the requirement of meat importing countries, especially the USA, that the meat should be free from *E. coli* O157 and six non-O157 STEC (O26, O103, O45, O111, O121 and O145) (Food Safety and Inspection Service, 2011a).

Therefore, a better understanding of epidemiology of *E. coli* O157 and non-O157 is required to avoid possible losses to the red meat and dairy industries of New Zealand.

1.9 Molecular epidemiology

A better understanding of molecular epidemiology of disease is helpful in devising disease control strategies. A number of techniques have been used to study the molecular epidemiology of STEC. These include phage typing (Ahmed et al., 1987), multilocus enzyme analysis (Whittam and Wilson, 1988), multilocus sequence typing (MLST) (Liu et al., 2009), plasmid profiling and toxin genotyping (Ostroff et al., 1989), macrorestriction fingerprinting with PFGE (MRF-PFGE) (Bohm and Karch, 1992) and bacteriophage lambda-generated restriction fragment length polymorphism (l-RFLP) (Samadpour et al., 1993).

1.9.1 Molecular epidemiology of *E. coli* O157

PFGE is widely used for genetic subtyping of *E. coli* O157. This method is considered to have high discriminatory power and reproducibility (Barrett et al., 1994; Izumiya et al., 1997).

Many studies have used PFGE for outbreak investigation. Willshaw et al. (2001) used PFGE in outbreak investigations and linked a sandwich bar outbreak to a secondary outbreak. It has also been used to link sporadic cases to diffuse outbreaks (Bender et al., 1997). It has also been used in investigating the source of human disease for example; Akiba et al. (1999) investigated 77 *E. coli* O157 isolates from Japanese cattle using *XbaI* PFGE and found that three human isolates were similar to seven cattle isolates indicating that cattle could be the likely source of outbreak in humans. Heuvelink et al. (1998a) compared 63 cattle and sheep isolates with 33 human isolates of *E. coli* O157 using PFGE and found that 90.9% of the human isolates were similar to cattle and sheep isolates. PFGE has also been used to study the diversity of *E. coli* O157 isolates from different sources and from different countries. Akiba et al. (2000a) compared cattle isolates from Japan (n=91) and the USA (n=415) using PFGE, phage typing, and location of *stx* gene and found that three isolates from Japanese cattle had high similarity (number of band differences were four or fewer with *XbaI*, *BlnI* and *SpeI*) with cattle isolates from the USA. *E. coli* O157 isolates (n=73) from human patients in Argentina, Australia and New Zealand have been analysed by PFGE (Leotta et al., 2008). There were 46 different patterns by *XbaI* PFGE and no common *XbaI* PFGE pattern was observed in the isolates recovered from three countries indicating little spread of O157 between these countries.

PFGE has also been used to study the ecology of *E. coli* O157 in cattle. Akiba et al. (2000b) experimentally inoculated two calves with *E. coli* O157 isolates and the third calf was used as an un-inoculated control. A few weeks after inoculation 401 isolates were recovered from these calves. Of the 401 isolates, 147 isolates were different from the inoculated isolates by PFGE. However, the control calf remained culture negative throughout the experiment. This suggested that there could be high level of clonal turnover in naturally infected animals. Cobbold and Des-

marchelier (2001) analysed isolates from three different farms by using PFGE and observed four distinct restriction patterns. Less restriction pattern variability in *E. coli* O157 isolates was observed between farms. A survey conducted in 1994 to estimate the prevalence and sources of serotypes of *E. coli* O157 in Wisconsin dairy cattle used PFGE to study the restriction pattern of *E. coli* O157 isolates. The isolates recovered from the same farms had similar restriction patterns and the isolates from different farms had different restriction patterns (Faith et al., 1996). Similar observations were made by Shere et al. (1998) where *E. coli* O157 isolates (n=294) recovered from naturally infected cattle and environment on two farms in Scotland were typed by PFGE. There were 19 PFGE subtypes of *E. coli* O157 in two farms which showed that there was a high genetic variation among *E. coli* O157 isolates (Vali et al., 2004).

A study conducted in England and Wales to observe genetic variability among *E. coli* O157 isolates from bovine also used PFGE and reported 57 PFGE types among 228 *E. coli* O157 isolates. Most of the PFGE types were found on individual farms (Liebana et al., 2003). In another study, 371 *E. coli* O157 isolates from 11 different farms were typed using PFGE. Cleavage with *XbaI* and *NotI* resulted in 81 and 23 subtypes of *E. coli* O157 respectively. Significant genetic diversity among *E. coli* O157 isolates on most farms was observed (Rice et al., 1999). Similarly, 107 *E. coli* O157 isolates obtained from range cattle, water sources and wild life faecal samples were analysed by PFGE. Seven subtypes from cattle and water sources were similar to each other while one subtype from wild life was similar to subtypes of cattle and water sources (Renter et al., 2003).

stx-encoding bacteriophage insertion (SBI) site typing has also been used to study the genetic diversity of *E. coli* O157 isolates (Besser et al., 2007; Shaikh and Tarr, 2003). Shaikh and Tarr (2003) reported three different clusters of *E. coli* O157 clinical isolates identified by this method. In another study, SBI typing was used to study the genetic diversity of *E. coli* O157 in cattle and humans (Besser et al., 2007). It was observed that 95% of clinical isolates and 51.3% of bovine isolates belonged to cluster 1, 2, or 3. In addition, 13 genotypes of *E. coli* O157 isolates from humans and cattle were also reported. The study showed that there was broad genetic diversity of *E. coli* O157 in bovine reservoir. Therefore, further studies are required on *E. coli* O157 isolates from cattle to have better understanding of *E. coli* O157 SBI genotypes in cattle.

Manning et al. (2008) genotyped 528 *E. coli* O157 isolates by detecting SNPs using real time PCR (RT-PCR) to study the genetic diversity and variability in virulence of *E. coli* O157 isolates. The primers were designed using 96 SNP loci. They reported that *E. coli* O157 isolates could be classified into 9 clades. The isolates in clade 8 were reported to be more virulent and their frequency in HUS cases had increased over the last five years.

1.9.2 Molecular epidemiology of non-O157 STEC

Different molecular techniques such as multilocus enzyme electrophoresis (MEE) (Whittam et al., 1993), random amplified polymorphic DNA (RADP) (Peixoto et al., 2001) and PFGE have been used for molecular characterisation of non-O157 STEC. However, PFGE is considered to be the most sensitive and discriminatory typing method for non-O157 STEC (Zhang et al., 2000b).

PFGE has been used for molecular characterisation of non-O157 STEC in cattle. For example, in Scottish study seven PFGE patterns for O26 isolates were observed but two were more dominant as compared to other patterns. Similarly, O103 isolates comprised of 10 PFGE patterns. However, PFGE patterns of O145 isolates were indistinguishable (Pearce et al., 2004). PFGE has also been used to study the genetic heterogeneity between O26:H11 isolates (129) obtained from Norwegian sheep and found 63 distinct PFGE profiles (Sekse et al., 2011). The genetic heterogeneity of Brazilian STEC and non-STEC isolates from human and environmental sources was studied using PFGE. The serogroups of non-STEC isolates were similar to STEC isolates. There were 46 and 30 PFGE patterns among STEC and non-STEC isolates respectively. The PFGE patterns of STEC isolates were substantially different from non-STEC isolates. Moreover, there was more similarity among *stx*-positive and *stx*-negative O26 isolates in comparison to other serogroups (Vaz et al., 2006).

A study conducted in Scottish beef cattle used PFGE to identify the association between antibiotic resistant *E. coli* O26 (297), O103 (152) and O145 isolates (13) and their PFGE patterns. There was more genetic diversity among O103 (27 PFGE patterns) isolates as compared to O26 (13 PFGE patterns) and O145 (6 PFGE patterns) isolates. Some *E. coli* O26 antibiotic resistant phenotypes were associated with distinct PFGE patterns (Vali et al., 2007).

PFGE has also been used for non-O157 outbreak investigations. Most recently an outbreak due to *E. coli* O145 occurred in different states of the USA. *E. coli* O145 was isolated from the stool of the patients and the PFGE pattern in all the isolates from different patients was same indicating the same source of infection. Later epidemiological and molecular studies (same PFGE pattern as the isolates from patients) indicated that shredded romaine lettuce from a processing facility was the source of infection (Centers for Disease Control and Prevention, 2010).

Ribotyping and PFGE were used to determine the genetic relatedness of epidemiologically unrelated STEC O111 isolates from different countries (Morabito et al., 1999). The results of the study showed that the STEC O111 isolates belonged to two different clonal lineages. The first group comprised of non motile, *eae* and *ehxA* positive isolates while second group comprised of O111:H2 epidemic strain isolated from HUS cases in France.

1.9.3 Molecular Evolution

E. coli are normally present in the intestine of man and animals including cattle as commensals. However, some *E. coli* strains have become pathogenic (Kaper, 2005) by acquiring virulence factors such as LEE, prophages and plasmid encoded factors (Boyd and Brussow, 2002; Porwollik and McClelland, 2003; Schmidt and Hensel, 2004). Bacteria can acquire new properties by two different mechanisms i.e. vertical and horizontal transmission (Sokurenko et al., 1999). It has been reported that approximately half of the genome of *E. coli* O157:H7 EDL 9333 is likely to consist of prophage remnants (Perna et al., 2001) thereof indicating that prophages have played an important role in evolution and divergence of *E. coli* O157 (Hayashi et al., 2001; Ohnishi et al., 2001).

Whittam et al. (1988) made the first attempt to study the evolution of *E. coli* O157 using MEE. This technique detects differences in proteins due to changes in the sequence of amino acids by measuring differences in the mobility of enzymes during electrophoresis. Differences in the strains are directly related to differences in mobility of enzymes. For example, analysis of various STEC O157 and non-O157 strains revealed that O157 strains grouped together indicating close genetic association. Another study analysed 1300 *E. coli* isolates of 16 serotypes, including O26:H11, O55:H6, O55:H7, O111:H2, O128:H2 and O157:H7 using MEE to observe the genetic relatedness. Close genetic relatedness was observed between O157:H7 and O55:H7 clones. On the basis of these findings it was proposed that O157:H7 evolved from O55:H7 (Whittam et al., 1993).

Feng et al. (1998) analysed *E. coli* isolates (163) including *E. coli* O157:H7 (78) from contaminated food and HUS patients, *E. coli* O157:H- (4) from HUS patients from Germany, non-motile O157 from the USA and Japan (42), O157 strains having H type other than H7 (10) and different O:H serotypes (33) using MEE to study the evolution of *E. coli* O157. On the basis of their finding a model was proposed for evolution of *E. coli* O157 (Figure 1.2). This model proposed the evolution of O157:H7 from *E. coli* O55:H7 first by acquiring *stx2* gene and then *rfbE* gene. This O157:H7 clone (sorbitol fermenting and β-glucuronidase positive) then diverged into two clones, O157:H7 (non-sorbitol fermenting and β-glucuronidase positive) and O157:H- (sorbitol fermenting and β-glucuronidase positive). This model also proposed that *stx2* gene was acquired before the acquisition of other virulence genes such as *stx1* and pO157.

Zhang et al. (2006) estimated the evolution and evolutionary time frame of *E. coli* O157 using comparative genome sequencing (CGS) microarrays. Chromosomal genes and the pO157 of 11 O157 isolates from human outbreak cases were analysed. The results indicated that Sakai and atypical *E. coli* O157 493/89 (sorbitol fermenting and β-glucuronidase positive) evolved from a common ancestor between 51.1 and 58.3 thousand years ago. Similarly, divergence of Sakai and G5101 (non-sorbitol fermenting and β-glucuronidase positive) from a common ancestor took place between 39.5 and 39.7 thousand years ago. Although *E. coli* O157 have existed for thousands of years the recent emergence as human pathogens may be due to changes in ecology, virulence, human susceptibility and bacterial adaptation.

Leopold et al. (2009) used single nucleotide polymorphism (SNP) analysis to study the evolution of *E. coli* O157:H7. SNPs within ORFs making up the backbone in seven previously sequenced *E. coli* O157:H7, O157:H- and O55:H7 were identified. The evolutionary steps of *E. coli* O157:H7 are shown in Figure 1.3. Due to the existence of cluster 1 for several millennia it was thought that members of this cluster have radiated on multiple branches from its founder. However, the results of the study indicated only a major and a minor branch (between the red and green symbols in Figure 1.3). This finding provides evidence that evolution of *E. coli* O157:H7 is not random. It was also estimated that cluster 2 emerged 2300 years after cluster 1 and synonymous radial SNPs of O157 Sakai and EDL 933 indicated four to six century intervals between the founding of cluster 3 to the present.

More than 100 non-O157 STEC have been identified from human cases (World Health Organization, 1998). Many of the non-O157 STEC such as O26 and O103 posses the same virulence genes

as O157 (*stx* and *eae*) and produce the same clinical manifestations in humans as STEC O157. Therefore, evolution of non-O157 STEC might be similar to STEC O157. However, the evolution of non-O157 STEC is not well understood and differences in evolution of non-O157 STEC from STEC O157 have been observed. For example, LEE has different insertion sites in STEC O26 and STEC O111 compared to STEC O157 (Wieler et al., 1997). Various non-O157 STEC possess different intimin subtypes e.g. STEC O26 posses intimin type β and STEC O103 possessed intimin type ϵ which is different from STEC O157 intimin type γ (Blanco et al 2004c; Cookson et al 2007; Kozub-Witkowski et al 2008). These differences indicate that sources of intimin may be different for STEC O26, O103 and O157 (Law, 2000). Further studies are required to get better understanding of differences in evolution between different non-O157 STEC and differences from STEC O157.

1.10 *Campylobacter*

Campylobacter is the most common food-borne zoonotic pathogen in the developed world (Oberhelman and Taylor, 2000). The most common clinical symptoms of campylobacteriosis include diarrhoea, fever and abdominal cramps (Blaser, 1997). Generally the incubation period is from 1-3 days but it can be as long as seven days (Skirrow, 1994). Although death due to campylobacteriosis is rare it can lead to various complications including Guillain-Barre syndrome (Mishu and Blaser, 1993). Seasonal peaks of campylobacteriosis have been observed in summer in various countries including the UK, Nordic countries, New Zealand and Australia (Hudson et al., 1999; Meldrum et al., 2005; Nylen et al., 2002).

Campylobacteriosis is the most frequently notifiable infectious disease in New Zealand. In 2011 the rate of infection in New Zealand was 151.9 cases per 100,000 population (6692 cases). The highest rates of campylobacteriosis in New Zealand were recorded in South Canterbury (223.5 cases per 100,000 population; 126 cases) and Wairarapa (219.3 cases per 100,000 population; 89 cases). The highest rate of infection was observed in children 1-4 years of age (289.4 cases per 100,000 population; 729 cases). The highest rate of infection has been observed in European or Other ethnic group (175.5 cases per 100,000 population; 5350 cases) (Environmental Science and Research, 2011).

Campylobacter has been isolated from various hosts including cattle, sheep (Stanley et al., 1998a,b), pigs (Weijtens et al., 1999), poultry (Wallace et al., 1997) and wild birds including ducks, gulls, starlings, pukeko (French et al., 2011b), sparrow (Chuma et al., 2000) and wildfowl (Fallacara et al., 2001). The most common sources of transmission of *Campylobacter* to humans are chicken, red meat, milk, water and contact with farm animals (Friedman et al., 2000; Kapperud et al., 2003).

1.10.1 Cattle as a reservoir of *Campylobacter*

In New Zealand cattle is considered the second most common source of transmission of *Campylobacter* to humans after poultry. A source attribution study in New Zealand indicated that cattle

contributed 18% (95% CI=10-26%) of human cases of campylobacteriosis (Mullner et al., 2009b). Several studies have reported the prevalence of *C. jejuni* in cattle ranging from 0.8% to 97% (Giacoboni et al., 1993; Rosef et al., 1983). A study conducted in feedlot cattle in Alberta, Canada reported high faecal prevalence of *Campylobacter* (87%; 2420/2776) in freshly voided faecal samples. In this study sampling was not random as only those farmers who were willing to participate were selected for the study. This study could not find a significant difference between the prevalence of *C. jejuni* in summer (70%, 1210/1376; 95% CI=67-72%) and winter (64%, 1210/1400; 95% CI=58-70%) (Hannon et al., 2009). A study of 15 dairy and beef farms in Washington State found 43.2% (297/686) overall prevalence of *Campylobacter*. Samples were collected directly from the rectum and from faecal pats on the ground. The prevalence of *C. jejuni* was 34.1% (234/686) (Bae et al., 2005). A Norwegian study reported low prevalence of *C. jejuni* and *C. coli* in cattle. Of 254 rectal swabs collected from cows two (0.8%) were positive for *Campylobacter* spp. However, the samples were stored at $4\,^{\circ}\mathrm{C}$ for 2-3 days before analysis (Rosef et al., 1983) and this could have contributed to the low prevalence of *Campylobacter* spp. in this study (Deguevara et al., 1989).

Kwan et al. (2008) conducted a longitudinal study to estimate the prevalence of *C. jejuni*. Faecal samples (1208) were collected from Cheshire, UK from five farms over an 11 month period. The overall prevalence of *Campylobacter* was 50.8% (434/1208). The prevalence was high in summer and spring (62.2%) as compared to autumn and winter (37.7%). Ellis-Iversen et al. (2009) conducted cross-sectional study to estimate the prevalence of *C. jejuni* and *C. coli* in cattle from England and Wales. Faecal samples were collected from dairy and beef cattle (n=235). The prevalence of *C. jejuni* (60/235; 25.5%) was higher than *C. coli* (5/235; 2.12%). The sampling was not random as the samples were collected from farms previously classified as STEC O157 positive farms. A Danish study found a 22.6% (77/332) prevalence of *Campylobacter* in dairy cows. The prevalence of *Campylobacter* was higher in calves $<$4 months (45/107; 42.1%) of age than adult cows (11/120; 9.2%) The sampling was carried out from August to October 1999 and faecal samples were collected per rectum from 332 cattle. Samples were enriched in Preston broth for 18-24 hours at $41.5\,^{\circ}\mathrm{C}$ before inoculating onto mCCDA agar (Modified Charcoal Cefoperazone Deoxycholate agar) (Nielsen, 2002). A Japanese study also reported the difference in prevalence of *Campylobacter* in different age groups. Faecal samples were collected from calves less than one year of age (n=34) and also older than one year (n=60). The prevalence of *Campylobacter* was higher in calves less than one year of age (97%; 33/34) compared to calves more than one year of age (46.7%; 28/60) (Giacoboni et al., 1993). Meanger and Marshall (1989) collected 273 RAMS from dairy cows in summer, autumn and winter seasons from dairy farms at Massey University, New Zealand. The overall prevalence of *Campylobacter* was 22.3% (61/273). The prevalence of *Campylobacter* was higher in summer (24%; 17/72) and autumn (31%; 33/106) as compared to winter (12%; 11/95). RAMS were collected from 52 milking cows at the number 4 dairy unit, Massey University Palmerston North between 5^{th} April and 25^{th} May 2002. Samples were enriched in Bolton broth for 48 hours at $42\,^{\circ}\mathrm{C}$ and inoculated onto mCCDA plates for isolation of *C. jejuni*. The prevalence of *C. jejuni* was 54% (28/52) (Adhikari et al., 2004). Use of enrichment of faecal samples before culturing may be the reason for the higher prevalence of *Campylobacter*

in cattle in this study compared to study of Meanger and Marshall (1989).

1.10.2 Role of water in *Campylobacter* transmission

Water is considered an important source of transmission of *Campylobacter* and many water-borne *Campylobacter* outbreaks have been reported (Clark et al., 2003; Kuusi et al., 2005; Richardson et al., 2007). *Campylobacter* have been isolated from rivers and water-ways (Bolton et al., 1987; Carter et al., 2009; Jones, 2001) and transmission of *Campylobacter* from water to livestock and vice versa have been suggested due to isolation of identical biotypes of *Campylobacter* from water and cattle (Stanley et al., 1998a). Main sources of contamination of rivers with *Campylobacter* are effluent from treatment plants and surface run-off water. Different studies have reported rates of isolation of *Campylobacter* from different rivers ranging from 37% in central Washington to 82% in Germany (Carter et al., 1987; Stelzer and Jacob, 1991). These variations may be due to differences in catchment-specific factors (topography, soil type and land use) and culture methods for *Campylobacter*. Differences in population of *Campylobacter* in streams have also been reported e.g. mainly *C. jejuni* and *C. coli* were present in a stream passing through pasture grazed by cattle (Stanley et al., 1998a) and sheep (Stanley et al., 1998b) whereas the population of *Campylobacter* in streams draining duck ponds mainly included *C. jejuni*, *C. lari* and urease-positive thermophilic campylobacters (Jones, 2001). Seasonal variation has also been reported in rate of isolation of *Campylobacter* from river water (Obiri-Danso and Jones, 1999). Obiri-Danso and Jones (1999) collected 288 water samples (144 each site) from two different sites on the River Lune in England. The total rate of isolation of *Campylobacter* was 82.9% (239/288). The rate and number of isolation of *Campylobacter* was higher in winter, autumn and spring as compared to summer on both the sites of the River Lune.

Campylobacter has been isolated from rivers and streams in New Zealand (Carter et al., 2009; Devane et al., 2005). The prevalence of *Campylobacter* from Ashburton River water samples was 55.2% (162/293) (Devane et al., 2005). A similar finding was reported by Savill et al. (2001). Various sequence types have been reported from New Zealand rivers for example, Carter et al. (2009) characterised 244 *C. jejuni* isolates from three rivers in New Zealand (Taieri River, Ashburton River and Manawatu River) using MLST and identified 88 sequence types (STs). Most of the STs identified have also been reported from wild birds (French et al., 2009) and seven STs were unique to New Zealand (Carter et al., 2009).

1.11 Conclusion

STEC causes different clinical manifestations in people ranging from diarrhoea to HUS and death of the patients. Cattle is not only an important reservoir of STEC but also considered an important source of transmission of STEC to humans through direct contact with infected animals or indirectly through contaminated food and water. In addition to public health importance it has great economic importance for New Zealand. The USA has a zero tolerance policy for STEC O157 and six non-O157 (O26, O45, O103, O111, O121 and O145) in the meat. Information about the

epidemiology of STEC in New Zealand is scarce, therefore more information is required about the epidemiology of STEC O157 and non-O157 (O26, O103, O111 and O145), distribution of virulence genes and their subtypes in *E. coli* isolates from calves and transmission of STEC between farms to devise appropriate control strategies. These control strategies would help in reducing the increasing number of human STEC cases in New Zealand and also to avoid possible losses to New Zealand economy.

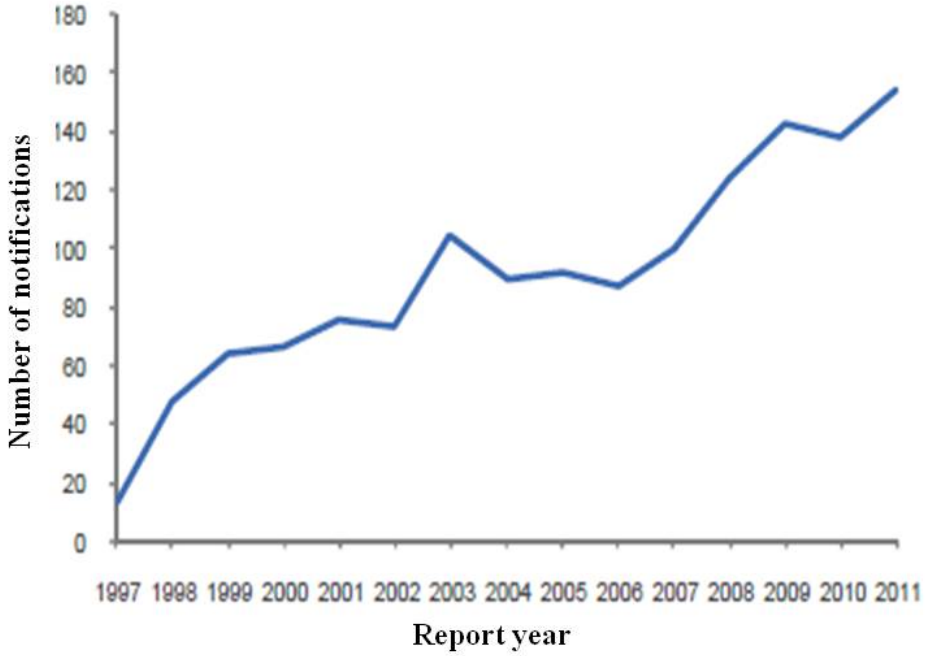


Figure 1.1: Notified cases of Shiga toxin-producing *Escherichia coli* in New Zealand from 1997-2011 (Environmental Science and Research, 2011)

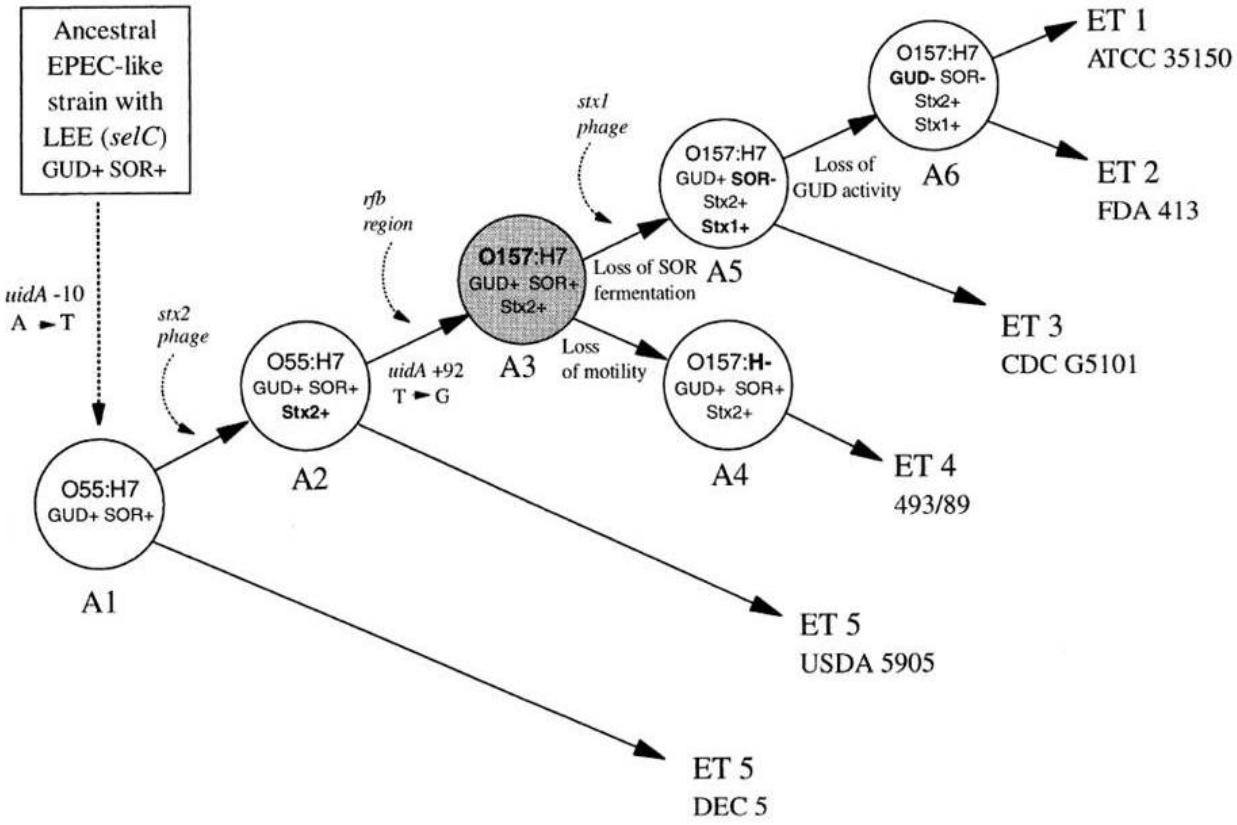


Figure 1.2: Proposed evolutionary model for emergence of *E. coli* O157:H7 complex based on mutation in *uidA*, *stx* producing, sorbitol and β-glucuronidase phenotypes, and multilocus enzyme electrophoretic profiles of *E. coli* O157:H7 and its relatives. Phenotypes of ancestor A1-A6 are shown; changes predicted to have occurred are in bold. Representative isolates are given below each electrophoretic type (ET). Strain with tracts of ancestor A3 (shaded circle) has not been reported (Feng et al., 1998)

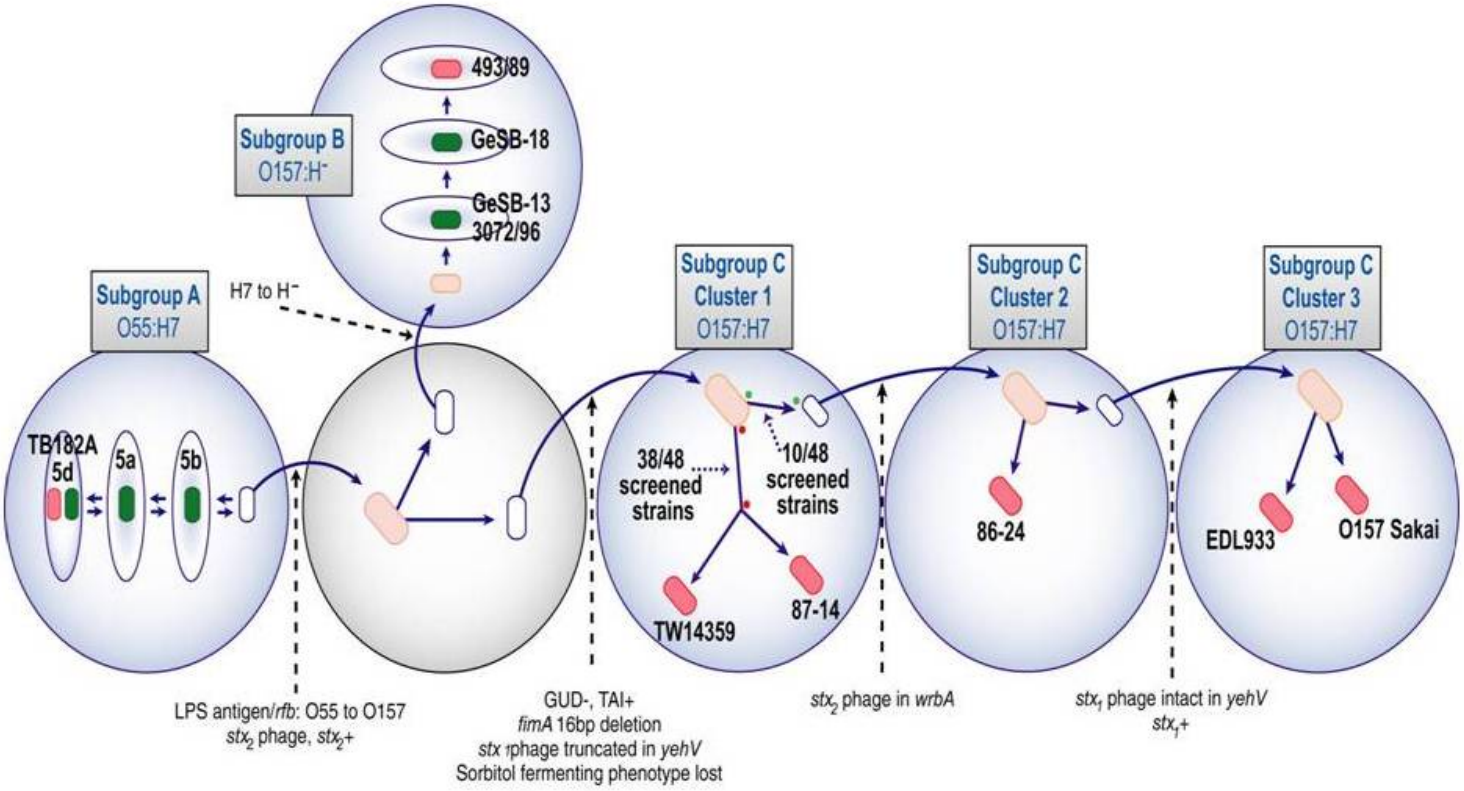


Figure 1.3: Evolutionary scenario for EHEC 1 pathogens. *E. coli* O55:H7 belongs to the most ancestral subgroup of the EHEC 1 clade (subgroup A). The gray sphere depicts a probably extinct intermediate between O55:H7 and *E. coli* expressing the O157 lipopolysaccharide, with subgroup B consisting of the sorbitol fermenting (SF) O157:H- and subgroup C consisting of *E. coli* O157:H7. Critical intraclade events are noted. Intracluster ovals represent genomically sequenced strains (dark-pink), strains used for SNP consensus sampling (green), inferred founders (pale-pink), and postulated organisms that are immediate progenitors to the next cluster or subgroup (white). Screened strains were assigned to the main branch if they had each of the three signature SNPs among the 111 shared SNPs (38/48), and the minor branch if they lacked this SNPs (10/48). Distances not drawn to scale (Leopold et al., 2009)

Table 1.1: Classification of Shiga toxin-producing *Escherichia coli* serotypes into seropathotypes (Karmali et al., 2003)

Seropathotype	Relative incidence	Frequency of involvement in outbreaks	Association with severe disease[a]	Serotypes
A	High	Common	Yes	O157:H7, O157NM[b]
B	Moderate	Uncommon	Yes	O26:H11, O103:H2, O111:NM, O121:H19, O145:NM
C	Low	Rare	Yes	O91:H21, O104:H21, O113:H21; others
D	Low	Rare	No	Multiple
E	Non human only	NA[c]	NA	Multiple

[a]HUS or HC.
[b]NM, non-motile.
[c]NA, not applicable

Table 1.2: The prevalence of Shiga toxin-producing *Escherichia coli* in cattle in different countries

Country	Year	Month	Prevalence (%)	Cattle type	Diagnostic method	*E. coli* isolates	Sample collected	*stx* types	Reference
France	2004	Jan-Aug	145/415 (34.9)	Dairy	Isolation	80	Faecel	Not mentioned	(Fremaux et al., 2006)
USA	2001-2002	Apr-Feb	396/1189 (33.3)	Beef	PCR	Unknown	Faecel	Not mentioned	(Barkocy-Gallagher et al., 2003)
France	1997-1998	Oct-Sep	330/471 (70)	Beef	PCR	186	Faecel	*stx1*=118 *stx2*=106 *stx1* & *stx2*=62	(Pradel et al., 2000)
Hong Kong	1996-1998	Aug-Dec	409/986 (41.4)	Unknown	Isolation	409	Rectal swabs & carcass swabs	*stx1*=1 *stx2*=105 *stx1* & *stx2*=302	(Leung et al., 2001)
Argentina	1995-2000	Unknown	12/75 (16%)	Beef	Isolation	15	Faecal & Rectal swabs	*stx1*=9 *stx2*=3 *stx1* & *stx2*=3	(Mercado et al., 2004)
Brazil	1996-1997	Apr-Sep	139/197 (70.5)	Diary & beef	PCR	15	Rectal swabs	*stx1*=1 *stx2*=5 *stx1* & *stx2*=9	(Cerqueira et al., 1999)
Bangladesh	2006	Jan-May	101/139 (72.6)	Beef	PCR	31	Faecal	*stx1*=15 *stx2*=13 *stx1* & *stx2*=3	(Islam et al., 2008)
USA	Unknown	Sep-Jan	17/82 (20.7)	Beef	Isolation	17	Faecal	*stx1*=4 *stx2*=5 *stx1* & *stx2*=7	(Hussein et al., 2003)
Uganda	1998	Mar-Apr	45/159 (28.3)	Beef	Isolation	47	Rectal swab	*stx1*=7 *stx2*=13 *stx1* & *stx2*=27	(Kaddu-Mulindwa et al., 2001)

Germany	1998-1999	Unknown	970/2163 (44.8)	Dairy & beef	PCR	587	Rectal swabs	*stx1*=73 *stx2*=227 *stx1* & *stx2*=287	(Geue et al., 2002)
Japan	1998-1999	Feb-Oct	258/510 (50.5)	Diary & beef	PCR	Not mentioned	Faecal	Not mentioned	(Shinagawa et al., 2000)
Argentina	1999-2000	Jul-Dec	138/200 (69)	Beef	PCR	86	Faecal & rectal swab	*stx1*=6 *stx2*=68 *stx1* & *stx2*=12	(Meichtri et al., 2004)
Scotland	2000	May-Dec	176/710 (24.7)	Beef	PCR	94	Faecal	*stx1*=29 *stx2*=48 *stx1* & *stx2*=17	(Jenkins et al., 2002)
Japan	2000-2001	Apr-Mar	227/605 (37.5)	Dairy & beef	Isolation	114	Faecal	Not mentioned	(Fukushima and Seki, 2004)
Brazil	Not mentioned	Unknown	44/344 (12.7)	Beef	PCR	24	Faecal	*stx1*=12 *stx2*=4 *stx1* & *stx2*=8	(Leomil et al., 2003)

2 Chapter 2

Diversity and relatedness of Shiga toxin-producing *Escherichia coli* and *Campylobacter jejuni* between farms in a dairy catchment

2.1 Abstract

The aim of the study was to explore the population structure of two zoonotic pathogens, Shiga toxin-producing *Escherichia coli* (STEC) and *Campylobacter jejuni* (*C. jejuni*) between 23 farms in a defined catchment area. Pooled faecal samples (72) were collected from calves grazing in the catchment and analysed by real time PCR (RT-PCR) for the presence of *Escherichia coli* (*E. coli*) serogroups O26, O103, O111, O145 and O157. RT-PCR positive samples underwent immuno-magnetic separation (IMS) and subsequent culture on (selective) media in an attempt to isolate these *E. coli* serogroups. All isolates underwent multiplex PCR for detection of Shiga toxin 1 (*stx1*), Shiga toxin 2 (*stx2*), *E. coli* attaching and effacing (*eae*) and enterohaemolysin (*ehxA*) genes. Thirty (41.6%) of the pooled faecal samples were positive by RT-PCR for *E. coli* O26. RT-PCR positive samples of *E. coli* serogroups O103 (7 of 72, 9.7%), O145 (3 of 72, 4.2%) and O157 (2 of 72, 2.7%) were detected less frequently while no samples were positive for O111. Using IMS and RT-PCR, 18 O26, two O103 and a single O145 were isolated from enrichment cultures. Of the 18 O26 isolates, 13 (72.2%) were positive for *stx1*, *eae*, *ehxA* and the remaining five (27.7%) were positive for *eae*, *ehxA*. Both of the O103 isolates and a single O145 isolate, were positive for *eae, ehxA*. The population of *C. jejuni* (53 of 72, 73.6%) isolated from the same cattle in the catchment was also compared with those recovered from the stream. Pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were used to genotype the O26 and *C. jejuni* isolates respectively. *E. coli* O26 isolates were subsequently divided into three clusters (1-3). All isolates from clusters 2 and 3 were *stx1*-positive in contrast to the five of six isolates from cluster 1 that were *stx1*-negative. Isolates recovered from cattle on the same farm were generally more similar than isolates from different farms. There was a total of 13 different sequence types (STs) of *C. jejuni* isolated from the cattle and 6/13 of these STs were also recovered from the stream running through the catchment. Although most of the molecular variation (74-75%) resided between animals within farms, there was significant clustering of genotypes both within and between farms. The pattern of genotypes of both pathogens showed a trend of within farm relatedness, and limited between farm transmission that could not be attributed to the stream network.

2.2 Introduction

Shiga toxin-producing *Escherichia coli* (STEC) and *Campylobacter* spp. are important bacterial causes of gastro-enteritis in humans worldwide (Beutin, 2006; Botteldoorn et al., 2008) and cattle are considered an important reservoir for both pathogens (Besser et al., 2005; Bettelheim, 2007)

STEC have emerged globally as important food-borne human zoonotic pathogens associated with outbreaks and sporadic cases of diarrhoea, haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (Bettelheim, 2007; Karmali et al., 2010). There are more than 100 STEC serogroups (World Health Organization, 1998) that have been associated with human disease, but five (O26, O103, O111, O145 and O157) are considered to be most important due to their association with large outbreaks and many of the HUS and HC cases in humans (Beutin, 2006). STEC-associated disease outbreaks have been reported from all over the world (Bettelheim, 2007). In New Zealand, the first STEC associated human case was reported in 1980 (Wilson and Bettelheim, 1980) and the rate of STEC infection in New Zealand has increased from 1.3 cases per 100,000 population in 1998 to 3.5 cases per 100,000 population in 2011 (Environmental Science and Research, 2011). This rate of infection is higher than that from the UK (1.28 cases per 100,000 population) (Health Protection Agency, 2010) and from the USA (0.9 cases per 100,000 population) (Centres for Disease Control and Prevention, 2011).

Cattle and sheep are considered to be the main reservoir of STEC (Bettelheim, 2007; Hancock et al., 1998). Cattle are thought to play an important role in STEC transmission as most of the human cases are associated with ingestion of contaminated food or water, or direct animal contact (Armstrong et al., 1996; Renwick et al., 1993).

C. jejuni and *C. coli* are the most important gastro-enteritis causing *Campylobacter* spp. worldwide (Allos and Blaser, 1995; Dekeyser et al., 1972; Solomon and Hoover, 1999). The disease caused by these organisms campylobacteriosis, is the most commonly notifiable infectious disease in New Zealand. In 2011, 6692 (151.9 cases per 100,000 population) human cases of campylobacteriosis were notified (Environmental Science and Research, 2011). Chicken, red meat, milk, water and contact with farm animals are considered to be the most common sources of transmission of *Campylobacter* to humans (Friedman et al., 2000; Kapperud et al., 2003) and since the reduction in human cases in New Zealand is associated with control measures in the poultry industry, ruminant sources have become relatively more important (Sears et al., 2011).

Water channels and rural streams can become contaminated with *Campylobacter* and *E. coli* by direct deposition of cattle faeces and surface and subsurface flows following rainfall or irrigation (Collins et al., 2004). In New Zealand, higher levels of faecal bacteria have been reported from rural streams including the catchment where this study was conducted (Davies-Colley et al., 2008; Wilcock et al., 2006) in which dairy farming is the major land use. One way to potentially reduce the number of human cases of zoonotic disease is to understand the population structure and dynamics of transmission of harmful microorganisms from farms to water channels. Better understanding of population structure and transmission of zoonotic pathogens would help in devising appropriate control strategies. The implementation of these control measures could reduce

environmental contamination which in turn would reduce transmission to animals and humans. This study was therefore, conducted to determine the degree of relatedness of STEC and *C. jejuni* between farms as a function of their separation and the extent to which they are connected by network of streams. Understanding the population structure of STEC and *C. jejuni* within and between communities of farms is important for the development of mitigation strategies that could help to reduce both within and between farm spread.

2.3 Materials and methods

The catchment where the study was conducted was selected because 70% of the catchment land is used for dairy farming and catchment boundaries are well defined. There are 23 farms in this catchment including 18 dairy farms, four dry stock farms and one horse farm.

Farms that shared a boundary (excluding farms separated by roads, but including those separated by a stream) were grouped together (Figure 2.1a). The region was also divided into farms adjacent to the main stream, and those in the periphery of the catchment (Figure 2.1b).

Sampling was carried out over a five month period in late spring 2009. Faecal samples were collected from 288 animals, which were either born in 2009 (Y0) or 2008 (Y1), from 20 of the farms. The three farms not sampled were: a dairy farm which had no Y0 calves but where the Y1 animals were grazed on a neighbouring farm from which they were able to be sampled; a dry stock farm that had no calves during the study period and a horse farm. Faecal sampling was done with the co-operation of farmers who separated calves into distinct age groups. Different farms were sampled at each occasion and samples were collected from a freshly voided faecal pat shed by an individual animal.

In order to increase the probability of obtaining the target STEC and *C. jejuni* for subsequent genotyping, sets of four pat samples (grouped on the basis of farm and age-class) were thoroughly mixed to form a single composite sample for microbiological analysis. In total, 72 pooled faecal samples from the 20 farms in the catchment area were transported under cold conditions to the laboratory. These samples were analysed for the presence of STEC (O26, O103, O111, O145 and O157) and *C. jejuni*. In addition, 33 water samples were collected from different sites in the main stream in the catchment and analysed for *C. jejuni*. Due to limited resources water samples were not tested for *E. coli* O26.

2.3.1 Isolation and characterisation of STEC

For STEC analysis, two grams of each composite sample was enriched in 18 ml of buffered peptone water (BPW) for 24 hours at 37 °C. Faecal PCR kits (Bioline, Auckland, New Zealand) were used for isolation of DNA from enriched faecal samples which was then tested by real time PCR (RT-PCR) for the presence of *wzx* (O26) gene (Perelle et al., 2004), *wzx* (O103) gene (Fratamico et al., 2005), *wbdI* (O111) gene (Perelle et al., 2004), *wzx1* (O145) (Fratamico et al., 2009) gene and *rfbE* (O157) gene (Perelle et al., 2004).

Each PCR reaction contained 1x reaction buffer (Invitrogen, Auckland, New Zealand), 0.1mM of each dNTP (Fermentas, Auckland, New Zealand), 0.15mM $MgCl_2$ (Invitrogen, Auckland, New Zealand), 0.03mM SYTO-9 (Invitrogen, Auckland, New Zealand), 1 unit of *Taq* DNA Polymerase (Invitrogen, Auckland, New Zealand), 2μl of template DNA and made to final volume of 20μl with sterile water. The concentration of primer in each reaction for O26, O111 and O145 was 500nM, and 100nM and 200nM for O103 and O157 respectively. The amplification was carried out in a Rotor Gene 6000 series thermal cycler (Bio Strategy, Auckland, New Zealand). For amplification of the *rfbE* from O157 an initial denaturing step of 96 °C for 5 minutes was used followed by 40 cycles of 15 seconds at 96 °C, 10 seconds at 62 °C and 10 seconds at 72 °C:, after which the PCR product was detected by thermal melt from 75 °C to 90 °C at a rate of 0.05 °C per second. Similarly, for detection of O26, O103, and O111 an initial denaturing step of 96 °C for 10 minutes was followed, by 40 cycles of 95 °C for 10 seconds, 60 °C for 30 seconds and 72 °C for 10 seconds, after which the PCR product was detected by thermal melt from 75 °C to 95 °C at a rate of 0.05 °C per second. For O145 an initial denaturing step of 94 °C for 2 minutes was followed, by 35 cycles of 94 °C for 20 seconds, 60 °C for 60 seconds and 72 °C for 60 seconds, after which the PCR product was detected by thermal melt from 75 °C to 95 °C at a rate of 0.05 °C per second. The positive and negative controls for *E. coli* O26, O103, O111, O145 and O157 were included in each run.

O26, O103, O111, O145 or O157 RT-PCR-positive samples underwent immuno-magnetic separation (IMS) for isolation of the respective serogroup. An enriched sample (1ml) was mixed with 20μl of magnetic beads (Invitrogen, Auckland, New Zealand) coated with polyclonal antibodies raised against *E. coli* O26, O103, O111, O145 or O157 lipopolysaccharide and IMS was performed according to the manufacturer's instructions. The bead suspension (100μl) was inoculated onto sorbitol MacConkey agar supplemented with cefixime (50μg/ml) and potassium tellurite (2.5mg/ml) (CT-SMAC) (Fort Richard, Auckland, New Zealand) for isolation of O157, rhamnose MacConkey agar supplemented with cefixime and potassium tellurite (CT-RMAC) (Fort Richard, Auckland, New Zealand) for isolation of O26 and sorbitol MacConkey agar (SMAC) (Fort Richard, Auckland, New Zealand) for isolation of O103, O111 and O145. The plates were incubated at 37 °C for 24 hours and observed for the presence of *E. coli* O157 colonies (grey colour/colourless colonies on CT-SMAC), *E. coli* O26 colonies (grey colour/colourless colonies on CT-RMAC) and O103, O111 and O145 colonies (pink/purple colonies on SMAC). O157 and non-O157 suspect colonies were sub-cultured on MacConkey agar. O157 suspect colonies were identified by using *E. coli* O157 latex agglutination kits (Oxoid, Auckland, New Zealand) and colonies suspected positive for O26, O103, O111 and O145 were identified by using RT-PCR. The colonies positive for *E. coli* O157 and non-O157 were analysed by multiplex PCR to detect the presence of Shiga toxin 1 (*stx1*), Shiga toxin 2 (*stx2*), *E. coli* attaching and effacing (*eae*) and enterohaemolysin (*ehxA*) genes.

Each PCR reaction contained 1x reaction buffer (Invitrogen, Auckland, New Zealand), 0.2μM of each primer (Paton and Paton, 1998a; Sharma and Dean-Nystrom, 2003), 0.1mM of each dNTP (Fermentas, Auckland, New Zealand), 0.15mM $MgCl_2$ (Invitrogen, Auckland, New Zealand), 1 unit of *Taq* DNA Polymerase (Invitrogen, Auckland, New Zealand), 2μl of DNA and made to final

volume of 25μl with sterile water.

The amplification was carried out in a Gene Amp PCR system 9700 (Applied Biosystems, Melbourne, Australia). An initial denaturing cycle of 5 minutes at 96 °C was followed by 40 cycles of, 30 seconds at 96 °C, 30 seconds at 60 °C, 30 seconds at 72 °C, followed by a final cycle of 5 minutes at 72 °C, after which the PCR products were electrophoresed through an agarose (2% w/v) gel (Invitrogen, Auckland, New Zealand) and visualised using ethidium bromide under ultraviolet illumination.

Molecular typing of each isolate was done by pulse field gel electrophoresis (PFGE) following the standard procedure described by PulseNet USA (Centres for Disease Control and Prevention, 2009). Briefly, the suspension of each isolate was prepared in cell suspension buffer (100mM tris: 100mM EDTA, pH 8.0). This suspension (400μl) was mixed with 20μl of proteinase K (20mg/ml) (Total Lab System, Auckland, New Zealand) and 400μl of SeaKem Gold agarose (1% w/v) gel (Invitrogen, Auckland, New Zealand) in Tris/EDTA buffer (10mM tris: 1mM EDTA, pH 8.0) and this mixture was immediately added to block former to make plugs. After solidification the plugs were incubated in cell lysis buffer (50mM tris: 50mM EDTA, pH 8.0 + 1% Sarcosyl) (5ml) and proteinase K (25μl of 20mg/ml) (Total Lab System, Auckland, New Zealand) mixture at 56 °C for 2 hours in shaking water bath. These plugs were washed twice with ultrapure distilled water and three times with Tris/EDTA buffer (10mM tris: 1mM EDTA, pH 8.0). Each washing was done for 15 minutes at 56 °C in shaking water bath. After washing the plugs were digested with 5μl of *XbaI* (10 units/μl) (Roche Diagnostics, Auckland, New Zealand) for 2 hours at 37 °C in a heating block. The plugs were then loaded into SeaKem Gold agarose (1% w/v) gel (Invitrogen, Auckland, New Zealand) in tris borate EDTA buffer (TBE). Electrophoresis was carried out with CHEF DR II system (Bio-Rad Laboratories, Auckland, New Zealand) at 14 °C in 0.5 x TBE, with plus ramp of initially 6.8 seconds and finally 35.4 seconds, for 20 hours at constant voltage of 6 V/cm. After electrophoresis gel was stained in ethidium bromide (0.5g/ml) for 20 minutes and visualised under short wave length ultraviolet light.

2.3.2 Isolation and characterisation of *C. jejuni*

C. jejuni analysis was done on a 1g aliquot from each composite sample using the New Zealand Reference Method (Ministry of Health, 2003). Briefly, 1g of each composite faecal sample was enriched in 9ml of Exeter broth for 48 hours at 42 °C. A loopful of enriched broth was plated onto mCCDA (Modified Charcoal Cefoperazone Deoxycholate agar) and plates were incubated at 42 °C for 48 hours in microaerobic conditions. For isolation of DNA, one suspected colony of *C. jejuni* was taken from the culture plate and mixed with 900μl of Milli-Q water and heated at 100 °C for 12 minutes. It was then cooled at 4 °C for 2 minutes and centrifuged for 5 minutes at 12,000 g. The supernatant containing the DNA was transferred to another tube and tested for the presence of *C. jejuni* by PCR (Inglis and Kalischuk, 2003). Each PCR reaction contained 10 x reaction buffer (Bioline, Auckland, New Zealand), 1μM of each primer, 0.2mM of each dNTP (NEB, Auckland, New Zealand), 3mM $MgCl_2$ (Bioline, Auckland, New Zealand), 1.25 units of *Taq* DNA Polymerase (Bioline, Auckland, New Zealand), 5μl of DNA, to final volume of 50μl

with sterile water. The amplification was carried out in Biometra Tpersonal Combi thermocycler (Whatman Biometra, Goettingen, Germany). Initial denaturation temperature was 95 °C for 15 minutes followed by 25 cycles of 30 seconds at 94 °C, 90 seconds at 58 °C and 60 seconds at 72 °C and finally, an extension at 72 °C for 10 minutes. The PCR products were electrophoresed through an agarose (1.5% w/v) gel (Invitrogen, Auckland, New Zealand) and visualised using ethidium bromide under ultraviolet illumination.

In total 33 water samples were collected from the catchment stream. *C. jejuni* was isolated from water samples using Minimum Probable Number (MPN) method. 100 ml, 10 ml and 1 ml of each water sample was taken in replicates of three. 100 ml and 10 ml water samples were filtered through 0.45μm filters. Each filter was placed in a tube containing 30 ml and 10 ml Exeter broth respectively while each 1 ml water sample was mixed with Exeter broth without filtration. These tubes were incubated at 37 °C for 24 hours and transferred to 42 °C for a further 24 hours. A loopful of inoculum from each tube was inoculated onto mCCDA plates and incubated in a gas jar in microaerobic conditions for 24 to 48 hours. The suspected *C. jejuni* colonies were identified using PCR as described previously.

Multilocus sequence typing (MLST) of *C. jejuni* isolates was performed using seven house-keeping genes: *aspA* (aspartase A), *glnA* (glutamine synthase), *gltA* (citrate synthase), *glyA* (serine hydroxymethyltransferase), *pgm* (phosphoglucomutase), *tkt* (transketolase) and *uncA* (ATP synthase alpha subunit) based on the method as outlined by (Dingle et al., 2001). Sequence data was collated and alleles assigned using the *Campylobacter* PubMLST database (http://pubmlst.org/Campylobacter/).

2.3.3 Statistical analysis

The relationship between genotypes isolated from cattle and water was examined using Minimum Spanning Trees and by calculating the proportional similarity index (PSI) (Garrett et al., 2007; Mullner et al., 2009b). PSI also known as Czekanowski index can be calculated by:

PSI = 1-0.5$\sum_i \mid p_i - q_i \mid = \sum_i min(p_i - q_i)$

Where *pi* and *qi* represent the proportion of strains belonging to type *i* from all strains typed from sources P and Q (i.e. cattle and water).

This index gives value between 0 and 1, where 1 indicate complete identity between strains from two sources and 0 indicates no similarity (Rosef et al., 1985). Bootstrap confidence intervals for PSI were calculated using method described by Garrett et al. (2007). A minimum spanning tree was created using Bionumerics Version 6.6 (www.applied-maths.com).

Population differentiation and gene flow was examined by calculating FST values and conducting an Analysis of Molecular Variance (AMOVA) (Excoffier et al., 1992; Slatkin and Barton, 1989). A global AMOVA was calculated that was the weighted average over all seven MLST loci. The population of *Campylobacter* isolated on each farm was examined to estimate the components of molecular variation and test the following hypotheses:

1. Networks of farms that share a boundary have isolates that are clustered/more similar compared to those that do not share a boundary (Groups A-E in Figure 2.1a).
2. Farms that are located either side of the main catchment stream have isolates that are clustered/more similar compared to farms that are located away from the main catchment stream (Groups X and Y in Figure 2.1b).

2.4 Results

2.4.1 Population structure and characterisation of STEC

In total, 72 pooled faecal samples collected from Y0 (n=46) and Y1 (n=23) calves were analysed by RT-PCR to detect the presence of *E. coli* O26, O103, O111, O145 and O157. The age of the animals from the three remaining samples was not recorded. Using RT-PCR, *E. coli* O26 was the most commonly detected *E. coli* serogroup (30 of 72 samples, 41.6%) as compared to O103, O145 and O157, or O111, where no samples were positive (Table 2.1). Thirty O26 positive samples obtained from 13/20 (65%) farms, resulted in 18 isolates recovered from 11 farms. Of these isolates 13 were positive for *stx1, eae, ehxA* using multiplex PCR, while the remaining five were positive for *eae, ehxA*. Similarly, samples positive for O103 and O145 originated from six and three farms respectively. Both O157 positive samples were from the same farm, but isolates were not recovered for further analysis. Both of the O103 isolates and a single O145 isolates were *eae, ehxA* positive. Most of the composite faecal samples positive for O26, O103, O145 and O157 were from Y0 calves (Table 2.1). Given the low numbers of PCR positives and isolates of other serotypes, all subsequent analysis focused on the distribution of *E. coli* O26.

The 18 *E. coli* O26 isolates from the 17 composite faecal samples could be broadly grouped into three clusters (>70% similarity) based on their PFGE profile (Figure 2.2). All isolates from clusters 2 and 3 were *stx1*-positive in contrast to the five of six isolates from cluster 1 that were *stx1*-negative. Unlike regions B, C and D which produced multiple O26 isolates, only a single isolate was identified from regions A and E. The isolate from region A was *stx* negative and present in cluster 1 while the isolate from region E was *stx* positive and present in cluster 2. Isolates recovered from cattle on the same farm were generally more similar than isolates from different farms (as indicated by different colours), although two farms had isolates from different clusters. There was no obvious association between the regions identified in Figure 2.1a or 2.1b and genotype of STEC O26, and given the small sample of isolates no further analysis were conducted.

2.4.2 Population structure and characterisation of *C. jejuni*

Fifty three of the 72 pooled faecal samples yielded *C. jejuni* isolates. Samples from Y0 calves (n=46) provided 38 (82.6%) *C. jejuni* isolates whereas samples from Y1 calves (n=23) provided 12 (52%) isolates. Similarly, the water samples yielded 109 *C. jejuni* isolates. The distribution of sequence types (STs) from cattle and catchment stream are shown in Table 2.2. The diversity of

genotypes of *C. jejuni* (taxonomic richness) was much higher in the catchment stream compared to the cattle population. Figure 2.3 shows the rarefaction curves with 95% CIs for the cattle and water samples. For a given sample size (e.g. 50 samples) the number of genotypes observed was higher in the water samples (17) compared to the cattle samples (13). There was a total of 13 different STs isolated from the cattle, and of these six (46%) were also recovered from the catchment stream. All of the STs common to cattle and water have been associated with human clinical cases in New Zealand (Mullner et al., 2009b). Conversely there was much greater diversity in the water isolates; a total of 23 different STs were isolated meaning that only 26% of the water STs were recovered from cattle in the catchment. Most of the water isolates (61/109) were associated with wildlife sources in New Zealand (ducks, starling, pukeko and takahe) (French et al., 2011b) and these are rarely associated with human disease e.g. ST associated with pukeko and takahe, ST 2381 (22 isolates) and ducks and starling, ST 1324 (1 isolate) and ST 2378 (2 isolates).

The area of overlap of the two genotype distributions (PSI) was low-moderate, PSI=0.21 (95% bootstrapped confidence intervals=0.13-0.29). This is greater than the estimated overlap between water isolates and those recovered from human cases (PSI=0.18), but lower than the PSI for most other source pairwise comparisons (e.g. the PSI for cattle compared to sheep isolates is 0.50 and cattle compared to human isolates is 0.34 (Garrett et al., 2007; Mullner et al., 2009b).

The structure of the population of *Campylobacter* isolated is shown in the Minimum Spanning Tree in Figure 2.4. The genotypes common to both the stream and the cattle are clearly visible and group together.

The AMOVA analysis showed that, while most of the variation (74-75%) resides between animals within farms, there is significant clustering of genotypes (>25%) between farms. Some of this can be attributed to the adjacency of farms in the catchment (10.8%), but not their adjacency to the main catchment stream (<1%) (Table 2.3 and 2.4). This suggests that transmission is more likely to occur between farms that share a boundary, than through the stream network.

2.5 Discussion

This study provides important information about the genetic diversity of STEC and *C. jejuni* between farms in a defined catchment environment. It was limited to a single catchment and aimed to recover a representative set of isolates for molecular genotyping from as many farms as possible, rather than conduct a large-scale analysis of the prevalence of carriage of *E. coli* and *C. jejuni* in different livestock groups on each farm. Therefore, the strategy of compositing faecal material from four animals for each sample was adopted in an attempt to maximise recovery rate. The recovery rate for O26 isolates was higher as compared to O103 and O145 and may reflect the availability of selective media (CT-RMAC) for O26, whereas no well-established selective media is available for isolation of O103 and O145.

The prevalence of *E. coli* O26 (41.7%) was higher compared to other serotypes included in this study. This finding is in agreement with other studies (Bonardi et al., 2007; Jeon et al., 2006; Pearce et al., 2006) which, in contrast to this study, were not conducted in a single catchment. For

example, in a Scottish national survey which focused exclusively on non-O157 STEC, 6,086 faecal samples were collected from calves of age more than one year and from 338 farms to determine the prevalence of *E. coli* O26, O103, O111 and O145 using IMS. The weighted mean prevalence of *E. coli* O26 (4.6%) was higher than O103 (2.7%), O145 (0.7%) and O111 which was absent. The prevalence of serogroup O157 was not recorded (Pearce et al., 2006). An Italian study which analysed 182 faecal samples collected from cattle from slaughter plants also reported the higher prevalence of *E. coli* O26 (3%) compared to O157 (0.5%) using IMS, however, no O103, O111 or O145 isolates were obtained (Bonardi et al., 2007). Similarly, a Korean study also reported the higher prevalence of *E. coli* O26 (6.7%) compared to O111 (4.6%) in 809 faecal samples collected from beef and dairy cattle aged between eight months and five years, but O103, O145 or O157 numbers were not investigated (Jeon et al., 2006). Whether O26 is better-adapted to colonise cattle as compared to other clinically-important STEC serogroups, such as O103, O111, O145 and O157, is unknown (O'Reilly et al., 2010). The outer membrane protein, intimin, encoded by *eae*, plays an important role in STEC attachment and colonisation in cattle (Wales et al., 2005). There are at least 20 intimin variants (Blanco et al., 2004a, 2006b; Garrido et al., 2006; Lacher et al., 2006; Ramachandran et al., 2003) and several studies have indicated that different *E. coli* serogroups posses a specific intimin type. For example, serogroup O26 often possesses intimin type β, serogroup O103 intimin type θ or ϵ and serogroups O145 and O157 intimin type γ (Blanco et al., 2004a; Cookson et al., 2007a; Kozub-Witkowski et al., 2008; Posse et al., 2007). Host specificity and tissue tropism displayed by different STEC may be influenced by the various *eae* types (Fitzhenry et al., 2002, 2003; Phillips and Frankel, 2000; Phillips et al., 2000).

Most of the *E. coli* O26 isolates (13/18) were positive for *stx1, eae, ehxA*. Although *E. coli* O26 has rarely been isolated from human diarrhoeal disease cases in New Zealand, it is recognised as an important and emerging pathogen in other countries (Brooks et al., 2005; Lathrop et al., 2009). Infection with STEC O26 often does not cause complications associated with HUS and is often associated with mild diarrhoeal disease or asymptomatic cases (Ethelberg et al., 2009). Therefore, there is a potential of underestimating the rate of infection due to *E. coli* O26 as many laboratories in New Zealand (and internationally) only test for STEC in young children with bloody diarrhoea, and faecal samples may not be assessed for the presence of *E. coli* O26 or other non-O157 STEC (Baker et al., 1999). The presence of *stx1, eae, ehxA* virulence genes in *E. coli* O26 isolates from calves has great public health significance as this is the most common virulence profile observed in *E. coli* O26 isolates from human cases (Schmidt et al., 1999b; Zhang et al., 2000b). Several other studies have also reported the presence of *stx1* and *eae* virulence genes in *E. coli* O26 isolates from cattle (Blanco et al., 1997; Burnens et al., 1995; Orden et al., 1998; Pearce et al., 2004). Although, carriage of the *stx2* gene in *E. coli* O26 isolates has also been reported in German cattle (Geue et al., 2002), no *stx2*-positive STEC O26 isolates were identified in this study. Five O26, two O103 and one O145 isolates were *eae*, *ehxA*-positive which is typical of atypical enteropathogenic *E. coli* (aEPEC). The aEPEC pathotype has been associated with prolonged diarrhoeal disease and several studies have demonstrated the close genetic relationship and common virulence determinants between aEPEC and STEC strains suggesting a dynamic relationship between aEPEC and STEC through the loss and acquisition of *stx*-encoding bacteriophage (Bielaszewska et al., 2008;

Hernandes et al., 2009; Mellmann et al., 2005; Whittam et al., 1993).

The prevalence of O26 was higher in younger calves (60%) than adults (8%) in this study, and this is consistent with other international studies. A case-control study conducted in the United States of America in dairy calves indicated that calves were three times more likely to shed STEC after weaning than before weaning (Garber et al., 1995). A study in Denmark also showed that calves between 2-6 months of age were more likely to be infected with STEC than calves with the age of <2 months and >6 (Nielsen et al., 2002). Similarly, Paiba et al. (2003) also reported the higher prevalence of STEC in calves between 2-12 months of age than calves <2 months and >12 months of age. Data reveal that most (28 of 30) of the samples positive for O26 by RT-PCR were from Y0 calves and could be associated with the well-developed rumen of adult calves where there is high concentration of volatile fatty acids and low pH which would inhibit the growth of STEC (Rasmussen et al., 1993). This age-associated prevalence data could also be due to differences in nutrition, immunity and management practices. For example, higher shedding of STEC has been reported in calves fed straw as compared to those that were not (Ellis-Iversen et al., 2007; Rugbjerg et al., 2003).

PFGE was used in this study to explore the genetic diversity of isolates and their spatial separation or relatedness from separate farms. Water is likely to represent an important transmission source for a number of zoonotic pathogens including STEC which have previously been isolated from various water sources in New Zealand (Donnison and Ross, 2009). Therefore, we investigated the role that the location of farms in the catchment may have in the population structure of STEC between farms. Data of this study indicate that there is a trend of within farm relatedness of *E. coli* O26 isolates within the animals of the same farm, but limited between animals of different farms. Similar results were found previously where molecular epidemiological investigation of 163 STEC O157 isolates indicated that those from the same farms had similar molecular profiles in contrast to isolates from different farms indicating limited transmission of STEC O157 between farms (Faith et al., 1996). Similarly, Cobbold and Desmarchelier (2001) also reported that STEC isolates seem to be specific to individual farms. A study conducted in Argentina to establish the relatedness between cattle and water STEC isolates using PFGE reported more diversity in water isolates (5 *XbaI* profiles) compared to cattle isolates (3 *XbaI* profiles). Moreover, the virulence profiles of isolates from both sources were also different (Tanaro et al., 2010). However, this study was of a small scale and due to limited number of isolates further analysis of population structure and differentiation was not performed.

In this study the prevalence of *C. jejuni* in cattle faeces was high (73.6%). Other studies have also reported the high prevalence of *C. jejuni* in cattle faeces (Gilpin et al., 2008; Stanley et al., 1998a). Cattle are considered an important reservoir of *C. jejuni* and potential source of infection for humans (Stanley and Jones, 2003). A New Zealand study reported bovine (11-18% of human cases attributed to bovine) as second important source of transmission of *C. jejuni* to humans after poultry (58-76% of human cases attributed to poultry) (Mullner et al., 2009a). Six of the 13 STs (ST 42, ST 50, ST 53, ST 61, ST 474, ST 2026) isolated from cattle in this study are recognised as important human pathogens in New Zealand (Mullner et al., 2009a). More samples were positive

from Y0 calves as compared to Y1 calves. This finding is in agreement with other studies (Bae et al., 2005; Nielsen, 2002). Susceptibility to colonisation with *Campylobacter* may be higher for the intestine of young calves than adult cattle and may be the reason for the higher prevalence of *C. jejuni* in Y0 calves (Giacoboni et al., 1993). Moreover, age dependent reduction in infection by enteric pathogens is associated with development of intestinal micro-flora (Nakagawa et al., 1969; Nurmi and Rantala, 1973).

Water is considered an important source of transmission of *Campylobacter* and many water-borne *Campylobacter* outbreaks have been reported (Clark et al., 2003; Kuusi et al., 2005; Richardson et al., 2007). *Campylobacter* have been isolated from rivers and water ways (Bolton et al., 1987; Carter et al., 2009; Jones, 2001) and transmission of *Campylobacter* from water to livestock has been suggested due to isolation of identical biotypes of campylobacters from water and cattle (Stanley et al., 1998a). In this study diversity in *C. jejuni* isolates from catchment streams was much higher as compared to cattle isolates. Other studies from New Zealand also reported similar findings (Carter et al., 2009; Devane et al., 2005). Devane et al. (2005) identified 32 heat stable (HS) serotypes among the 616 isolates tested. Of the 32 HS serotypes 28 were present in isolates from water samples whereas only 12 were present in the isolates from dairy cattle faeces. The difference in diversity of cattle and catchment stream isolates also suggested that most of the water isolates were most likely to have come from other sources including wildlife inhabiting the area (ducks, starlings and pukeko) (French et al., 2011b). A study conducted in Cheshire, UK to investigate the molecular epidemiology of *C. jejuni* in dairy cattle, wild life and the environment also reported that most of the water related *C. jejuni* were similar to those present in wild birds (Kwan et al., 2008). The most prevalent ST in water, ST 2381, is also the most prevalent isolate in many other river systems in New Zealand (Carter et al., 2009). To date this genotype has only been isolated from pukeko (*Porphyrio porphyrio*) (French et al., 2011b) and takahe (*Porphyrio hochstetteri*) and is not recognised as a human pathogen. However, it is also important to note that a significant proportion of the isolates from the water samples are likely to be of cattle origin but not isolated from cattle faecal samples in this study such as ST 45, and these are the ones most strongly associated with human disease in New Zealand, particularly in rural areas and in children (Mullner et al., 2009a, 2010). Kwan et al. (2008) also reported water as an important source of infection for humans as six of the eight clonal complexes isolated from environmental water were similar to those isolated from human patients. Therefore, surface water could be an important source of human infection via untreated drinking water and recreational activities (Mullner et al., 2009a). However, a study conducted in the Taieri river catchment, New Zealand showed that there was no overlap between genotypes from environmental water and human cases when PFGE profiles and Penner serotyping were used together (Eyles et al., 2006). The relationship between environmental water isolates and human infection is very complex and further studies are required to understand the role of *C. jejuni* strains common to animal and environmental water in epidemiology of human campylobacteriosis (Skelly and Weinstein, 2003).

AMOVA provides evidence of clustering of *C. jejuni* population within farms (74-75%) and between farms (>25%) grouped by geographical boundaries. Grove-White et al. (2011) also reported that most of the genetic variation (80-84%) in *C. jejuni* population was in the animals within farm

($P<0.001$) and some variation (16-20%) between farms ($P<0.001$). This finding could have very important implications for transmission and control of *C. jejuni*. Direct contact between cattle in neighbouring farms, sharing of equipment and personnel, and surface water run-off between farms could be important mechanisms for local transmission, underlining the importance of maintaining good between-farm biosecurity.

In summary, STEC and *C. jejuni* were prevalent in cattle in the catchment, and individual farms were associated with a subset of genotypes circulating among the young stock located on that farm. The pattern of genotypes of STEC O26 and *C. jejuni* was suggestive of a high level of within farm transmission, and limited between-farm transmission. Although this study was conducted on a limited number of farms in a single catchment, it has revealed some significant associations that provide insight into the nature and scale of dissemination of potential human pathogens within and between farms.

Table 2.1: Virulence factors associated with O157 and non-O157 serogroups obtained from 72 composite faecal samples. No samples were positive for serogroup O111 by RT-PCR.

Serogroups	Pooled faecal samples RT-PCR positive			Isolates obtained	Virulence profiles *(stx1, stx2, eae, ehxA)*	
	Total	Y0[a]	Y1[b]		*stx1, eae, ehxA*	*eae, ehxA*
O157	2	2	0	0	0	0
O26	30	28	2	18	13	5
O103	7	4	3	2	0	2
O145	3	3	0	1	0	1

[a] Calves born in 2009.
[b] Calves born in 2008.

Table 2.2: The distribution of *C. jejuni* sequence types (STs) isolated from cattle and water from catchment stream. The STs indicated by "W" are associated with wildlife in New Zealand.

ST	Cattle	Water	Shared	Wildlife ST
21	6	0	0	
42	1	10	1	
43	0	1	0	
45	0	7	0	W
50	11	11	1	
53	14	2	1	
61	10	4	1	
257	1	0	0	
474	1	7	1	
520	1	0	0	
677	0	1	0	W
1324	0	1	0	W
2026	2	2	1	
2233	0	1	0	
2378	0	2	0	W
2381	0	22	0	W
2538	0	1	0	W
3222	3	0	0	
3675	0	2	0	W
3845	0	7	0	W
4336	0	18	0	W
4342	0	1	0	
U2381	0	2	0	
4493	1	0	0	
4491	0	1	0	
4495	0	1	0	
3846	0	1	0	
4492	1	0	0	
U563	0	4	0	
4494	1	0	0	

Table 2.3: Global Analysis of Molecular Variance considering the farm structure outlined in Figure 2.1a.

Source of variation	Sum of squares	Variance components	% of variation	P (random value> or=observed value)
Among groups (A-E)	18.7	0.22	10.8	<0.00001
Among population within groups	34.9	0.32	15.7	0.007
Within population	43.7	1.5	73.5	<0.00001
Total	97.4	2		

Table 2.4: Global Analysis of Molecular Variance considering the farm structure outlined in Figure 2.1b.

Source of variation	Sum of squares	Variance components	% of variation	P (random value> or=observed value)
Among groups (X and Y)	3.3	0.01	0.6	0.30
Among population within groups	50.3	0.5	24.8	<0.0001
Within population	43.7	1.5	74.6	<0.0001
Total	97.4	2.0		

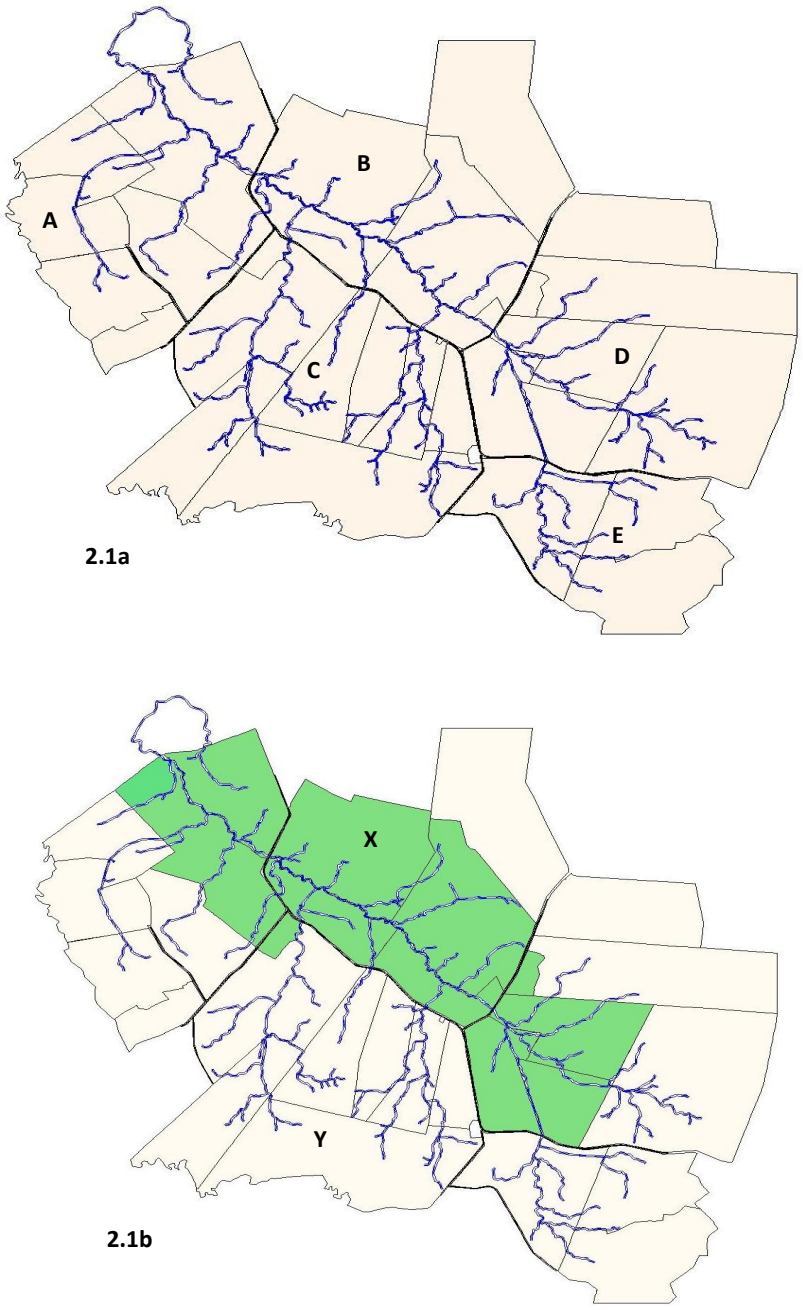


Figure 2.1: Five distinct groups marked A-E were evident (Fig 2.1a). The road network is shown in bold black and the stream network in blue lines. Figure 2.1b shows an alternative grouping of farms according to whether they were adjacent to the main stream (X=green) or away from the main stream (Y=orange)

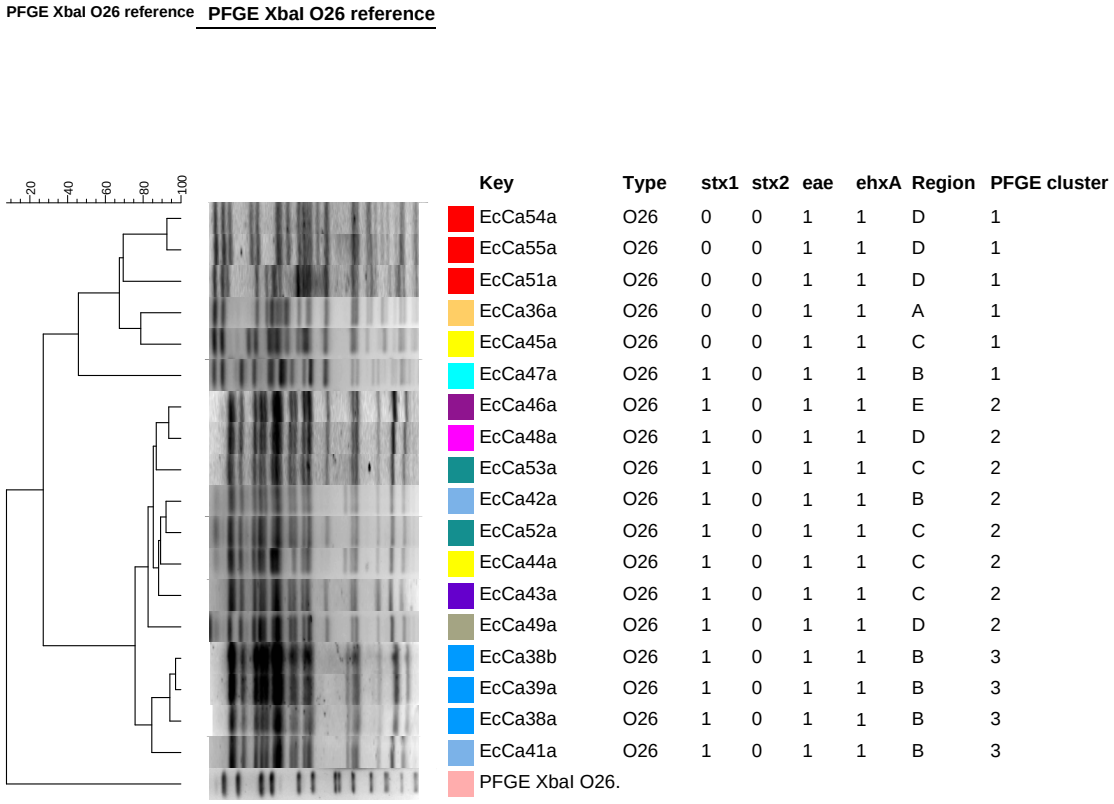


Figure 2.2: Clustering (UPGMA dendrogram) of the PFGE profiles of 18 O26 isolates from 17 composite faecal samples taken from 11 farms in catchment. Each farm is colour coded and the catchment region is indicated. Note two isolates from sample EcCa38 are included in the analysis. The last four columns indicate the presence of the *stx, eae* and *ehxA* virulence genes. The last lane is the *Salmonella* serotype Braenderup reference standard (H9812).

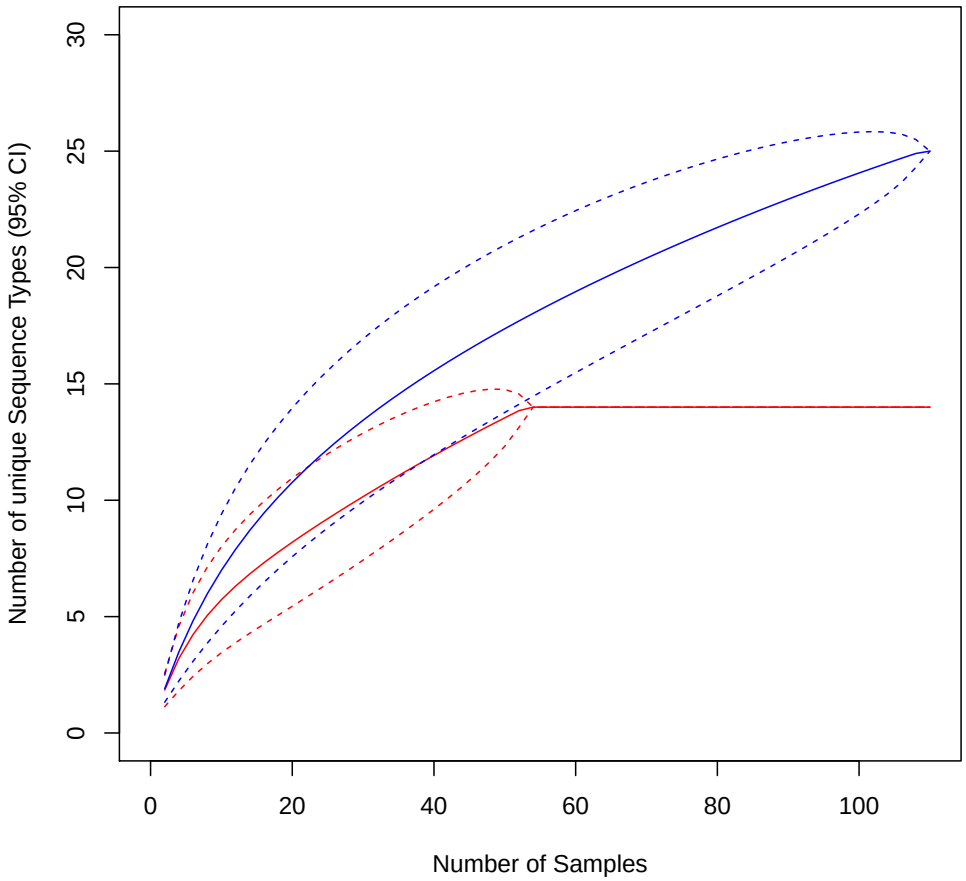


Figure 2.3: Genotype (sequence types = STs) richness in cattle and water samples. Rarefaction curves indicating the estimated number of unique STs in cattle (red) and water (blue) for varying sample sizes, with 95% confidence intervals.

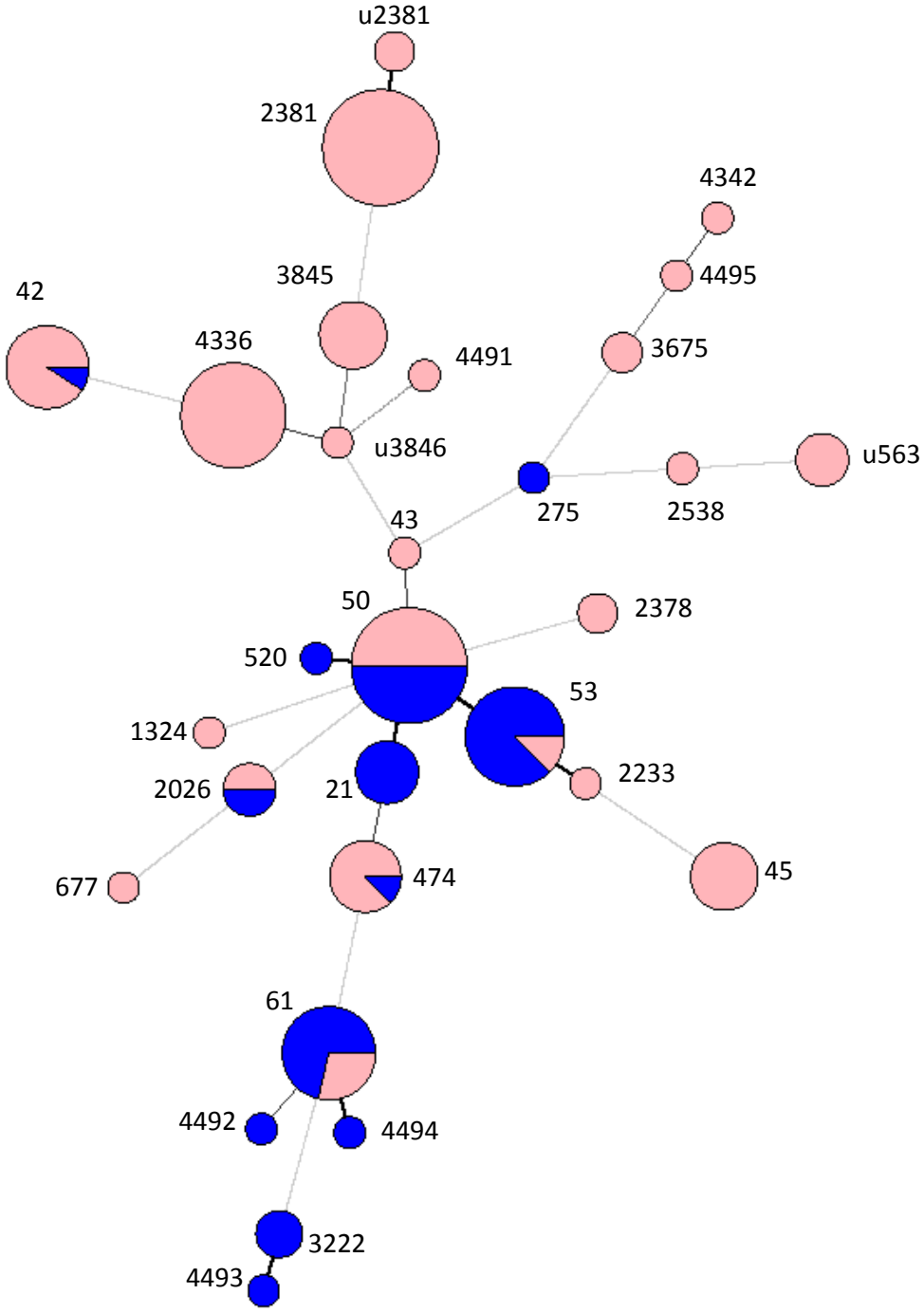


Figure 2.4: A Minimum Spanning Tree showing the multilocus sequence types (STs) isolated from cattle (pink) and water (blue) in the catchment stream. Each circle represents a ST; the size of the circle is proportional to the number of isolates and the area of each circle represents the relative frequency of each source. Isolates are grouped according to their genetic relatedness; short solid lines between STs indicate they vary at just one of the seven MLST loci (i.e. they are Single Locus Variants or SLVs).

3 Chapter 3

Distribution of *Escherichia coli* strains harbouring STEC-associated virulence factors (*stx1, stx2, eae, ehxA*) from bobby calves in the North Island of New Zealand

3.1 Abstract

The objective of this study was to determine the distribution of Shiga toxin-producing *Escherichia coli* (STEC) virulence markers (*stx1, stx2, eae* and *ehxA*) in *E. coli* strains isolated from young calves aged fewer than seven days (bobby calves). In total, 299 recto-anal mucosal swabs (RAMS) were collected from animals at two slaughter plants in the North Island of New Zealand. RAMS were enriched in buffered peptone water for 24 hours at 37 °C and inoculated onto tryptone bile X-glucuronide (TBX) and sorbitol MacConkey agar (CT-SMAC). Each plate was then incubated at 37 °C for 18-24 hours. Blue and white colonies (1 each) were selected at random from TBX plates. Similarly, purple and grey colonies (1 each) were selected at random from CT-SMAC plates. Isolates were analysed using multiplex PCR to detect *stx1, stx2, eae* and *ehxA* genes and those that were positive for any of these STEC virulence genes were further analysed using real time PCR (RT-PCR) for the detection of DNA sequences specific for serogroups O26, O103, O111, O145 and O157. STEC isolates negative for *E. coli* O26, O103, O111, O145 and O157 were serotyped using O and H antisera. Of the 975 *E. coli* isolates analysed, 512 isolates were from CT-SMAC plates; of which 299 were sorbitol fermenting (58.4%) and 213 non-sorbitol fermenting (41.6%), and 463 isolates were from TBX plates; of which 227 were β-glucuronidase positive (49%) and 236 were β-glucuronidase negative (51%). In total, 60 of 975 (6.2%) isolates were positive for one or more virulence markers. Most of the isolates with one or more toxin genes were obtained from CT-SMAC (47/512; 9.1%) as compared to TBX (13/463; 2.8%) (Chi square test, P=0.00006). The most common combination of virulence markers were those previously noted in atypical enteropathogenic *E. coli* (aEPEC), e.g. *eae, ehxA* (n=35) followed by *eae* (n=9). Only eight STEC were identified of which four were *stx2, eae, ehxA*-positive (3 O157:H7 and 1 O68:H24), three were *stx1, eae, ehxA* (2 O26 and 1 O71:HR) and a single *stx2*-only isolate (ONT:HNM). Of the 35 *eae, ehxA* isolates three were identified as O26, seven were O103 and eight were O145 using serogroup specific RT-PCR. Similarly, three of the nine *eae* positive isolates were O26 using serogroup specific RT-PCR. The remaining isolates were negative using serogroup specific RT-PCR for O26, O103, O111, O145 and O157 and their serogroups were not determined. 8/299 (2.6%) calves were positive for STEC and 38/299 (12.7%) for aEPEC. All the isolates can be divided into 15 clusters with >70% similarity cut off using *XbaI* on the basis of PFGE. Healthy calves from the dairy industry are asymptomatic carriers of both STEC and aEPEC

3.2 Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are considered to be important food-borne pathogens causing outbreaks and sporadic cases of diarrhoea, haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) in humans globally (Bettelheim, 2007; Karmali et al., 2010). Cattle and sheep are well established reservoirs of STEC (Armstrong et al., 1996; Caprioli et al., 2005; Paiba et al., 2003) where direct contact with infected animals and consumption of contaminated food and water are important pathways for transmission of STEC to humans (Armstrong et al., 1996; Renwick et al., 1993; Swerdlow et al., 1992). Internationally the majority of the human cases are associated with STEC O157 (Caprioli et al., 2005) but other STEC serogroups are also associated with human disease with six non-O157 STEC (O26, O45, O103, O111, O121 and O145) responsible for 70% of non-O157 human cases in the United States of America (Brooks et al., 2005). As a consequence of the threat to human health through the contamination of the environment or food products, the US Department of Agriculture (USDA) and European Food Safety Authority (EFSA) recommended the routine testing of important non-O157 STEC in meat of the ruminants (Eblen, 2006; European Food Safety Authority, 2007). Most recently the Food Safety and Inspection Service (FSIS) of the United States of America extended the list of meat adulterants by including non-O157 STEC from serogroups O26, O45, O103, O111, O121 and O145 (Food Safety and Inspection Service, 2011a,b).

STEC elaborate a variety of virulence factors including Shiga toxin (*stx*) which inhibit protein synthesis, especially in renal endothelial cells (Louise and Obrig, 1995; Sandvig, 2001), *E. coli* attaching and effacing (*eae*) and enterohaemolysin (*ehxA*) (Gyles, 2007; Paton et al., 1997). The *eae* gene encodes the outer membrane protein intimin (Jerse and Kaper, 1991; Jerse et al., 1990) which mediates the attachment of *E. coli* to epithelial lining of intestine and effacement of microvilli (Moon et al., 1983). The plasmid encoded enterohaemolysin, encoded by *ehxA*, is an important virulence marker which can cause haemolysis of washed sheep erythrocytes (Beutin et al., 1988, 1989; Schmidt et al., 1996a; Taneike et al., 2002). The exact role of the enterohaemolysin in mechanism of disease is not clearly understood. However, in-vitro studies have shown increased level of proinflammatory cytokine interleukin-1β from human monocytes in response to enterohaemolysin from STEC O128:H12 (Taneike et al., 2002).

In addition to STEC, cattle and sheep may also be a reservoir of enteropathogenic *E. coli* (EPEC) (Aidar-Ugrinovich et al., 2007; Cookson et al., 2002). EPEC contain *eae* and are able to form attaching and effacing (AE) lesions on cultured epithelial cells in-vivo but lack *stx* (Kaper, 1996). The formation of micro-colonies on cultured epithelial cells by many typical EPEC (tEPEC) isolated from human diarrhoeal cases is mediated by bundle forming pili (*bfp*). However, atypical EPEC (aEPEC) which lack *bfp* are commonly recovered from ruminants and may also be associated with human diarrhoeal disease (Trabulsi et al., 2002).

After the first recorded isolation of STEC in New Zealand in 1980 (Wilson and Bettelheim, 1980) the rate of infection in New Zealand increased to 1.3 cases per 100,000 population in 1998 and 3.5 cases per 100,000 population in 2011 (Environmental Science and Research, 2011). In New

Zealand the rate of STEC cases has been noted as being three times higher in rural areas compared with urban areas, however, no large outbreaks have been reported to date with most cases being sporadic (Thorbun, 2010). Shiga toxin bacteriophage insertion typing studies in New Zealand have provided further support that environmental and direct contact pathways are likely to be more important in transmission of STEC to humans than food pathways (French et al., 2011a) in line with the strong association observed between cattle density and the incidence of human STEC cases (Thorbun, 2010).

The dairy and meat industries make a significant contribution to the New Zealand economy (Cavanagh, 2003) and processing methods, especially in the meat industry, are influenced by meat inspection guidelines imposed by some countries such as the United States of America, which has a zero tolerance policy for STEC O157 and some non-O157 (O26, O45, O103, O111, O121, and O145) in meat (Food Safety and Inspection Service, 2011a). Relatively few data are available to assist in the epidemiological analysis of STEC and EPEC in New Zealand cattle. Therefore, this study was conducted with the aim of isolating *E. coli* from bobby calves RAMS to determine the distribution of STEC virulence factors (*stx1, stx2, eae, ehxA*) in randomly-chosen isolates. This will also assist in determining whether bobby calves may represent a source of STEC or EPEC infection for humans in New Zealand.

3.3 Materials and methods

Two abattoirs (A and B) selected in the North Island of New Zealand were each visited six times between July and October 2008 (Irshad et al., 2012). Recto-anal mucosal swab samples (RAMS) were collected systematically (every 10^{th} calf on the chain) from calves using sterile cotton swabs (Copan, Brescia, Italy) immediately after slaughter. Twenty five calves were sampled on each visit and in total, 299 calves were sampled.

RAMS were then placed in the transport media provided by the manufacturer and transported to the laboratory on ice where they were enriched in buffered peptone water (BPW) for 24 hours at $37\,^{\circ}\mathrm{C}$. Each enrichment broth was serially diluted and inoculated onto tryptone bile X-glucuronide (TBX; Fort Richard, Auckland, New Zealand) and sorbitol MacConkey agar, supplemented with 50 μg/ml cefixime and 2.5 mg/ml potassium tellurite (CT-SMAC). Each plate was then incubated at $37\,^{\circ}\mathrm{C}$ for 18-24 hours. One blue colony indicative of β-glucuronidase positive and one white colony indicative of β-glucuronidase negative activity were selected at random from TBX plates. Similarly one purple colony indicative of sorbitol fermenting activity and one grey or colourless colony indicative of non-sorbitol fermenting activity were selected at random from CT-SMAC plates. Isolates were sub-cultured onto the same culture media (CT-SMAC and TBX) to ensure purity, and a single colony was stored at $-80\,^{\circ}\mathrm{C}$ in nutrient broth containing 15% (v/v) glycerol.

Isolates were analysed using multiplex PCR to detect *stx1, stx2, eae* and *ehxA* genes. Isolates were re-grown on TBX or CT-SMAC plates at $37\,^{\circ}\mathrm{C}$ for 18-24 hours. A loop from a single colony was suspended in 500μl of 2% Chelex (Bio-Rad, Auckland, New Zealand) solution and heated at $95\,^{\circ}\mathrm{C}$ for 10 minutes for isolation of DNA. The lysed bacterial cell suspension was then cooled in

the refrigerator for two minutes and re-centrifuged at 12000 g for two minutes. The supernatant containing the DNA was transferred to another tube. Each PCR reaction contained 1 x reaction buffer (Invitrogen, Auckland, New Zealand), 0.2μM of each primer (Paton and Paton, 1998a; Sharma and Dean-Nystrom, 2003), 0.1mM of each dNTP (Fermentas, Auckland, New Zealand), 0.15mM $MgCl_2$ (Invitrogen, Auckland, New Zealand), 1 unit of *Taq* DNA Polymerase (Invitrogen, Auckland, New Zealand), 2μl of DNA, and made to a final volume of 25μl with sterile water. The amplification was carried out in a Gene Amp PCR system 9700 (Applied Biosystems, Melbourne, Australia) which was programmed for 5 minutes at 96 °C, 40 cycles of 30 seconds at 96 °C, 30 seconds at 60 °C, 30 seconds at 72 °C, with final extension of 5 minutes at 72 °C, after which the PCR products were electrophoresed through an agarose (2% w/v) gel (Invitrogen, Auckland, New Zealand) and visualised using ethidium bromide under ultra violet illumination. The positive and negative controls for *stx1*, *stx2*, *eae* and *ehxA* were included in each run.

Isolates positive for virulence genes were confirmed as being *E. coli* by using PCR described by (Yokoigawa et al., 1999). Briefly, each PCR reaction contained 1 x reaction buffer (Invitrogen, Auckland, New Zealand), 0.2μM of each primer (Forward 5′-CTGGAAGAGGCTAGCCTGGAC GAG-3′ and reverse 5′-AAAATCGGCACCGGTGGAGCGATC-3′), 0.1mM of each dNTP (Fermentas, Auckland, New Zealand), 0.15mM $MgCl_2$ (Invitrogen, Auckland, New Zealand), 1 unit of *Taq* DNA Polymerase (Invitrogen, Auckland, New Zealand), 2μl of DNA and made to a final volume of 20μl with sterile water. The amplification was carried out in a Rotor Gene 6000 series thermal cycler. It was programmed for initial denaturation step for two minutes at 94 °C, 25 cycles of 20 seconds at 94 °C, 20 seconds at 62 °C, and 20 seconds at 72 °C and final extension at 72 °C for 90 seconds. The PCR products were electrophoresed through an agarose gel (1% w/v) and visualised as described previously.

Isolates positive for *stx, eae* or *ehxA* gene were analysed by real time PCR (RT-PCR) to determine whether they were positive for O26, O103, O111, O145 and O157 (described in section 2.3.1). STEC isolates negative for *E. coli* O26, O103, O111, O145 and O157 by RT-PCR were serotyped (Enteric Reference Laboratory, Environmental Science and Research Ltd, New Zealand) using O and H antisera (Statens Serum Institute, Copenhagen, Denmark).

All isolates containing virulence genes obtained in this study (60 isolates) were genotyped using pulse field gel electrophoresis (PFGE) to determine the genetic relatedness of the isolates. PFGE was performed following the standard protocol described by PulseNet USA (Centres for Disease Control and Prevention, 2009). Each *E. coli* isolate was grown overnight on Trypticase Soy agar with 5% defibrinated sheep blood (Fort Richard, Auckland, New Zealand) and suspended in cell suspension buffer (100mM Tris: 100mM EDTA, pH 8.0) in a Falcon tube. The concentration of cell suspension was adjusted to between 0.40-0.45 using a Dade Microscan Turbidity meter. Each bacterial suspension was transferred to a 1.5ml centrifuge tube. Proteinase K (20μl of 20mg/ml) (Total Lab System, Auckland, New Zealand) and 400μl of SeaKem Gold agarose (1% w/v) gel (Invitrogen, Auckland, New Zealand) in Tris/EDTA buffer (10mM Tris: 1mM EDTA, pH 8.0) were added to each tube, mixed and dispensed into a block former to make plugs. After solidification each plug was transferred to a 5ml plastic tube with a lid and 5ml of cell lysis buffer (50mM Tris:

50mM EDTA, pH 8.0 + 1% Sarcosyl) and 25μl of proteinase K (20mg/ml) (Total Lab System, Auckland, New Zealand) were added to each tube. The plugs were incubated at 56 °C for 2 hours in a shaking water bath. After lysis, plugs were washed with ultrapure distilled water (2 washes, 15 minutes at 56 °C) and Tris/EDTA buffer (3 washes, 15 minutes at 56 °C) (10mM Tris: 1mM EDTA, pH 8.0). Plugs were digested with 5μl *XbaI* (10 units/μl) (Roche Diagnostics, Auckland, New Zealand) for 2 hours at 37 °C in a heating block. The plugs were loaded into SeaKem Gold agarose (1% w/v) gel (Invitrogen, Auckland, New Zealand) in Tris borate EDTA buffer (TBE) and PFGE was performed using CHEF DR II system (Bio-Rad Laboratories, Auckland, New Zealand) at 14 °C in 0.5 x TBE, with a plus ramp of initially 6.8 seconds and finally 35.4 seconds, for 20 hours at constant voltage of 6 V/cm. The gel was stained in ethidium bromide (0.5μg/ml) for 20 minutes and visualised using short wave length ultraviolet transillumination. Digital images of the gel were captured using a Gel Doc imaging system (Bio-Rad, Milan, Italy) and saved as TIFF files. The digital images were analysed using Bionumeric version 6.6 (www.applied-maths.com) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Dice coefficient with $>$70% similarity cut off were used to construct the clusters of *E. coli* isolates with one or more virulence genes.

The distribution of positive and negative farms from which the calves that were sampled originated from (n=193) were mapped using R package maptools (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austraia). The coordinates (latitude and longitude) of the farms (180) were obtained using Agribase data (Sanson and Pearson, 1997) and Google earth (www.google.com/earth). The latitude and longitude of each farm was then converted to XY coordinates. The coordinates of 13 farms were missing. The sampled farms were aggregated to 5x5 km grid squares to maintain the anonymity of the farms. The prevalence of calves positive for *E. coli* isolates with one or more virulence genes in three regions (Manawatu, Taranai and Waikato) of the North Island of New Zealand was estimated and adjusted for lack of independence of calves within farms using R package "survey" and function "svydesign".

3.4 Results

In total 299 RAMS were collected from bobby calves originating from 193 farms in the North Island of New Zealand. *E. coli* isolates (975) obtained from RAMS were analysed by multiplex PCR and of these 512 isolates were obtained from CT-SMAC (299 isolates were sorbitol fermenting and 213 were sorbitol non-fermenting) and 463 from TBX agar plates (227 isolates were β-glucuronidase positive and 236 isolates were β-glucuronidase negative). Sixty isolates were positive for at least one of the virulence factors of which 47/512 (9.1%) were isolated on CT-SMAC plates; 42/299 (8.4%) were sorbitol fermenting and 5/213 (2.3%) were non sorbitol fermenting (Chi square test. P=0.00001). Similarly, 13/463 (2.8%) were isolated on TBX plates; 9/227 (3.9%) were β-glucuronidase positive and 4/236 (1.6%) were β-glucuronidase negative (Chi square test, p=0.23). The rate of isolation of *E. coli* isolates with one or more virulence genes was significantly higher on CT-SMAC plates (47/512; 9.1%) as compared to TBX plates (13/463; 2.8%) (Chi square test, P=0.00006). The most common combination of virulence genes in the

isolates was *eae, ehxA* (35) followed by *eae* (9); *ehxA* (8); *stx2, eae, ehxA* (4); *stx1, eae, ehxA* (3) and *stx2* (1) (Table 3.1). Two of the three *stx1, eae, ehxA* isolates were identified as serogroup O26 using RT-PCR and the remaining strain was O71:HR. Three of the four *stx2, eae, ehxA* isolates were identified as O157 using RT-PCR and the remaining strain was O68:H24. The serotype of the *stx2*-positive isolate could not be readily determined using currently available sera, and was designated ONT:HNM. Six of the eight *stx* positive isolates were detected on CT-SMAC plates and the remaining two on TBX plates (Chi square test, P=0.35). In total, 53/299 (17.7%) calves were positive for one or more virulence genes (*stx1, stx2, eae and ehxA*). Of these 2.6% (8/299) calves were positive for STEC, 12.3% (37/299) for aEPEC and 2.6% (8/299) for *ehxA* positive isolates. There was no significant difference in the proportion of calves positive for *E. coli* isolates with one or more virulence genes from Waikato (23.1%; CI=11.9%-40%), Manawatu (21.1%; CI=13.1%-32%) and Taranaki regions (15.7%; CI=10.3%-23%) (Table 3.2). Using RT-PCR, three of the 35 *eae, ehxA* positive-isolates were identified as O26, seven were O103 and eight were O145. Similarly, using RT-PCR three of the nine *eae* positive isolates were identified as O26. None of the remaining isolates gave a positive reaction with O26, O103, O111, O145 and O157-specific RT-PCR primers and their respective serogroups were not determined.

The distribution of those farms from which calves harbouring *E. coli* isolates analysed in this study is shown in the Figure 3.1. Isolates positive for virulence genes were found in all important dairy producing areas of the North Island of New Zealand from which calves were sampled.

Using *XbaI*-PFGE, the 60 isolates from which at least one STEC-associated virulence factor was identified, could be divided into 15 clusters (A-O) with >70% similarity cut off using *XbaI* (Figure 3.2). Five of eight O26 isolates were present in cluster L and the remaining isolate being in cluster B which is *eae*-positive only. Two *eae*-positive O26 isolates could not be genotyped using *XbaI*-PFGE. All the O103 isolates (7) were present in cluster I. Of eight O145 isolates seven were present in cluster I and the remaining one isolate was present in cluster O. All O157 isolates (3) grouped together in cluster M. All O103 isolates present in cluster I were obtained from Taranaki (6) and Manawatu regions (1). Of six O103 isolates from Taranaki four were recovered from one farm and the remaining two from two different farms. All O145 isolates in cluster I were obtained from Taranaki from four different farms. Similarly, four of five O26 isolates in cluster L were obtained from four different farms of Taranaki and and the remaining one isolate from Manawatu region. All O157 isolates (3) in cluster M were recovered from three different farms of Taranaki region.

3.5 Discussion

Cattle have been identified as reservoir for both STEC O157 and non-O157 STEC and may provide a source of human infection through direct animal contact and faecally contaminated material, or through general environmental contamination. This study has provided evidence that young calves are potentially important carriers of STEC and aEPEC in the North Island of New Zealand. The results of this study should be extrapolated with care as samples were collected from only two abattoirs and the prevalence of STEC and/or aEPEC in other areas of North Island of New

Zealand may be influenced by variables such as the concentration of dairy farms and climatic conditions. It is highly likely that the prevalence of both STEC and aEPEC are underestimated as no preliminary screening method such as an initial screen for *stx* or *eae* was used. However, the use of two culturing media, selection of four randomly chosen colonies with an absence of screening of enriched samples for *stx* and/or *eae* could help in reducing the bias towards subculture of *stx*- and/or *eae*-positive isolates.

A low proportion of STEC (2.6%) isolates were recovered in this study with seven *stx*- and *eae*-positive strains and a single *stx* only isolate obtained. These data are in contrast to that previously described study conducted in the North Island of New Zealand where STEC (139) were isolated from 53 of 187 (28.3%) RAMS samples taken from cattle (calves, heifers and dairy cattle) (Cookson et al., 2006b). In that study, the prevalence of *stx* only isolates (48/139; 34.5%) was higher than the prevalence of both *stx*- and *eae*-positive (21/139; 15.1%) isolates and the remaining 70 isolates (50.3%) were *eae*-positive only (Cookson et al., 2006b). However, in the Cookson et al. (2006b) study RAMS were taken from animals of different age groups (calves, heifers and dairy cattle) from just four farms in two regions of the lower North Island. A further study also reported a higher prevalence (8.7%; 36/412) of STEC in Spanish cattle where faecal samples were collected from calves, heifers and adult cattle over a period of one year. These samples were inoculated onto MacConkey agar and four *E. coli* colonies from each plate were analysed for presence of *stx, eae* and *ehxA* genes (Orden et al., 2002).

STEC strains of zoonotic importance (Bettelheim, 2007; Beutin et al., 2004) belonging to serogroups O26, O68, O71 and O157 isolated in this study have previously been isolated from healthy cattle and sheep in New Zealand, Australia and Spain (Bettelheim, 2007; Buncic and Avery, 1997; Cookson et al., 2006c; Hornitzky et al., 2002; Orden et al., 2003). Further STEC strains from these serogroups have been reported from human cases (Bettelheim, 2007; Beutin et al., 2004) with STEC strains O157 and O26 frequently isolated from ruminants and human cases but STEC O68 and O71 only been rarely isolated from these hosts (Bettelheim, 2007). Bettelheim (2007) reported isolation of O68 from healthy cattle and human cases on three and seven occasions respectively. Similarly, O71 have also been isolated from healthy sheep and human cases on two and three occasions respectively. STEC O71 (*stx1* and *stx2*-positive) has also been isolated from a diarrhoeic lamb (Orden et al., 2003).

The zoonotic potential of these serogroups should not be underestimated for two reasons. Firstly, the low isolation of these serogroups may be due to unavailability of standardised isolation methods and secondly, the recent emergence of enteroaggregative enterohaemorrhagic *E. coli* (EAEHEC) O104:H4 in Europe affecting 3222 individuals including 810 HUS cases have shown that less virulent STEC can become highly virulent by acquiring other virulence factors (Brzuszkiewicz et al., 2011; Frank et al., 2011).

In this study all STEC O157 isolates were positive for the *stx2* gene whereas all STEC O26 isolates were positive for the *stx1* gene. Previous studies have also reported higher prevalence of *stx2* in STEC O157 (Irshad et al., 2012) isolates and *stx1* in STEC O26 isolates (Cookson et al., 2006c; Pearce et al., 2006). For example, all STEC O157 isolates (10) obtained from New Zealand calves

were *stx2*-positive (Irshad et al., 2012). Similarly, Cookson et al. (2006c) reported that all O26 isolates (2) obtained from cattle were positive for *stx1* gene. The analysis of *E. coli* O26 isolates from Scottish cattle also reported a higher prevalence of *stx1*-positive O26 isolates (122/249; 49%) (Pearce et al., 2006).

Previous studies have shown an association between *stx* genotype and STEC virulence (Friedrich et al., 2002; Kawano et al., 2012). STEC isolates with *stx2* gene are considered to have high virulence due to their association with HC and HUS cases (Beutin et al., 2004; Boerlin et al., 1999; Friedrich et al., 2002). Boerlin et al. (1999) reported that the rate of isolation of *stx2*-positive isolates was higher (39/60; 72.2%) from HUS and HC cases than *stx1*-positive isolates (29/75; 53.7%). Another study also found that 12 of the 15 (80%) STEC isolates obtained from HUS and HC cases were positive for *stx2*, one for *stx1* and *stx2* (6.6%) and the remaining two for *stx1* (13.3%) (Pradel et al., 2008). STEC isolates with *stx2c* gene have also been isolated from HUS and HC cases but they are considered less virulent than STEC isolates positive for *stx2* genes. For example, Kawano et al. (2012) analysed 74 *stx2c*-positive STEC isolates from human infection cases in Japan and found that 96% (71/74) of the isolates were associated with asymptomatic or mild human infection and only 4% (3/71) were recovered from patients with serious disease such as HC.

STEC O26 is also an important human food-borne pathogen and has caused large outbreaks of HUS where the vehicle of infection was food (Hiruta et al., 2001; Werber et al., 2002). In comparison to much of the rest of the world, in central and southern Europe the numbers of human cases of diarrhoeal disease due to STEC O26 are higher (Tozzi et al., 2003; Zhang et al., 2000a). However, in New Zealand very few human cases due to STEC O26 have been reported and this may be due to under reporting of STEC cases where O26 is not isolated or identified on selective media (Baker et al., 1999). Most of the STEC O26 that are associated with human diarrhoeal disease are *stx1, eae* and *ehxA* although some isolates from Europe have been identified that are *stx2, eae* and *ehxA* positive (Beutin et al., 2004). In this New Zealand study a typical virulence profile of *stx1, eae* and *ehxA* was noted for all the STEC O26 isolates. This virulence profile has great public health significance as this is the most common virulence profile observed in STEC O26 isolates from human cases internationally (Schmidt et al., 1999a; Zhang et al., 2000b).

The proportion of calves positive for aEPEC (12.7%) in this study was estimated to be higher than that of STEC (2.6%). However, there was no difference in prevalence of STEC and aEPEC in cattle in other studies (Cookson et al., 2006c; Orden et al., 2002). This difference may be due to differences in sampling and analysis of samples. In the present study RAMS were collected from calves of fewer than seven days of age from two North Island abattoirs and these samples were plated onto CT-SMAC and TBX plates. *E. coli* isolates obtained from these plates were analysed using multiplex PCR for *stx1, stx2, eae* and *ehxA*. In contrast Cookson et al. (2006b) sampled calves, heifers and adult cattle from four farms of lower North Island of New Zealand. A Spanish study collected faecal samples directly from rectum of calves, heifers and adult cattle. These samples were plated onto MacConkey agar plates which were incubated overnight and four suspected *E. coli* colonies were collected and analysed for presence of *stx1, stx2* and *eae* genes by

PCR (Orden et al., 2002).

Most of the EPEC isolated from ruminants in previous studies lack *bfp* and are therefore considered as aEPEC (Hornitzky et al., 2005; Trabulsi et al., 2002). The provenance of aEPEC is uncertain as several studies have shown that aEPEC may eventuate from STEC strains that have lost the *stx*-encoding bacteriophage, or where the complete *E. coli* adherence factor (EAF) plasmid, or the operon encoding *bfp* on EAF plasmid is lost (Bielaszewska et al., 2008; Bortolini et al., 1999; Hernandes et al., 2009; Mellmann et al., 2005). In contrast, the acquisition of the *stx*-encoding bacteriophage by EPEC has also been reported (Whittam et al., 1993) suggesting that transition to and from aEPEC and STEC is a dynamic process in complex microbial environments such as ruminant or human gut. To our knowledge there are no instances of isolates being *bfp* and *stx*-positive. As isolates that are *bfp* and *stx*-positive have not been recognised, the classification of *eae*-positive isolates remain unclear as some may be tEPEC (*bfp*-positive), others may be aEPEC (EAF plasmid lost) or STEC-like (*stx* genes lost). The number of calves positive for aEPEC (37/299; 12.3%) in this study was lower than previous study (44% in calves) conducted in lower North Island of New Zealand (Cookson et al., 2006c) but this difference may be due to differences in study design of two studies. Some of the aEPEC serogroups (O26, O103 and O145) isolated in this study may be of zoonotic importance due to their strong association with HC and HUS cases (Bielaszewska et al., 2008).

Although *ehxA* is considered an important virulence marker of STEC due to its association with disease in humans (Beutin et al., 1988; Schmidt et al., 1995), the role of enterohaemolysin in human disease is not clearly understood. The vast majority of human clinical cases of disease caused by STEC are associated with strains that are *eae* and *ehxA* positive (Gyles et al., 1998), however some serotypes such as O113:H21 (*eae*-negative, *ehxA*-positive), are notable exceptions (dos Santos et al., 2010). In this study all the STEC isolates except one were positive for *eae* and *ehxA* indicating their potential for causing disease in humans. However, STEC isolates without *eae* and *ehxA* from diarrhoea and HUS patients have also been recovered, but their recovery from human cases is rare (Beutin and Martin, 2012; Morabito et al., 1998; Mossoro et al., 2002). The virulence of *eae* and *ehxA*-negative isolates should not be underestimated. The recent European outbreak of EAEHEC O104, involving isolates negative for *eae* and *ehxA* further illustrates the potential public health significance of STEC isolates negative for *eae* and *ehxA*. Most of the aEPEC (35/44; 79.5%) in the present study were also positive for *ehxA*. Another study also reported higher detection of plasmid related genes such as *ehxA*, *etpD*, *espP* and *katP* in aEPEC (40/80; 50%) as compared to tEPEC (6.9%) (Bugarel et al., 2011).

In the study by Cookson et al. (2006c) most *eae* negative STEC were isolated from TBX plates (38/48, 79.16%) compared to CT-SMAC (10/48, 20.84%). However in this study, where very young calves were sampled, only a single *eae*-negative STEC (ONT:HNM) was isolated from TBX plates. Similarly, in this study aEPEC were isolated more frequently on CT-SMAC (34) as compared to TBX (10). Cookson et al. (2006b) also reported higher isolation of aEPEC on CT-SMAC (67/70: of which 41 were sorbitol fermenting and 26 non-sorbitol fermenting) as compared to TBX (3/70). Although it is difficult to compare those studies due to differences in study design,

these findings suggest the possibility of underestimation of STEC or EPEC prevalence if a single selective medium is used for isolation of *E. coli*.

PFGE is a molecular subtyping tool and has been used successfully for surveillance (Bender et al., 1997; Swaminathan et al., 2001), outbreak investigation (Gouveia et al., 1998; Johnson et al., 1995; Keene et al., 1997; Roberts et al., 1995) and to assess genetic relatedness of *E. coli* O157 isolates from different cattle herds (Lee et al., 1996; Shere et al., 1998). In this study isolates having the same serogroup generally clustered together with few exceptions. For example, a single *E. coli* O145 isolate grouped separately from the remaining seven O145 isolates and a single O26 from the remaining five O26 isolates. The basis of this differential clustering is unknown. It may be due to the presence of some additional virulence genes which are absent from other isolates. The presence of similar isolates obtained from different farms in the same region in similar PFGE clusters provided evidence of localised geographical clustering of farms positive for various *E. coli* serotypes. For example, O103 isolates obtained from different farms in the same region grouped together by PFGE. Similar results were observed for O26, O145 and O157 isolates. This geographical clustering may be due to localised transmission of various *E. coli* serogroups between farms in the same region.

This study showed that STEC and aEPEC of public health significance are present in bobby calves in New Zealand and, therefore, they may represent an important source of environmental contamination and possible human infection. However, further studies using molecular characterisation and comparison of these isolates with isolates from human cases are required to estimate the nature and scale of risk to the human population from these *E. coli*. Additional non-O157 serogroups (O26, O45, O103, O111, O121 and O145) have recently been legislated as adulterants by FSIS (Food Safety and Inspection Service, 2011a). Some of these serogroups were isolated in this study where both *stx*-positive and *stx*-negative strains were characterised. Other non-O157 serogroups may have been missed by this 'snapshot' cross-sectional study and more targeted methods such as RT-PCR or immuno-magnetic separation will be required to unequivocally determine their presence.

Table 3.1: Distribution of virulence genes *(stx1, stx2, eae, ehxA)* in *E. coli* isolates obtained from recto-anal mucosal swabs (n=299) of bobby calves on sorbitol MacConkey agar (CT-SMAC) and tryptone bile X-glucuronide plates. Sorbitol fermenting (SF) and non-sorbitol fermenting (NSF) colonies were obtained on CT-SMAC plates while β-glucuronidase positive (βGP) and β-glucuronidase negative (βGN) colonies were obtained on TBX plates.

Virulence genes	CT-SMAC		TBX		Total
	SF	NSF	βGP	βGN	
eae, ehxA	27	2	6	0	35
eae	5	0	1	3	9
ehxA	7	0	1	0	8
stx1, eae, ehxA	3	0	0	0	3
stx2, eae, ehxA	0	3	0	1	4
stx2	0	0	1	0	1
Total	42	5	9	4	60

Table 3.2: The proportion of samples positive for *E. coli* isolates containing virulence genes *(stx1, stx2, eae, ehxA)* from 299 recto-anal mucosal swabs of bobby calves from three regions of the North Island of New Zealand.

Region	No. of Samples	Positive for virulence genes	Prevalence (%) adjusted for clustering 95%CI
Taranaki	197	31	15.7 (10.3-23)
Manawatu	76	16	21.1 (13.1-32)
Waikato	26	6	23.1 (11.9-40)
Total	299	53	17.7 (13.2-23)

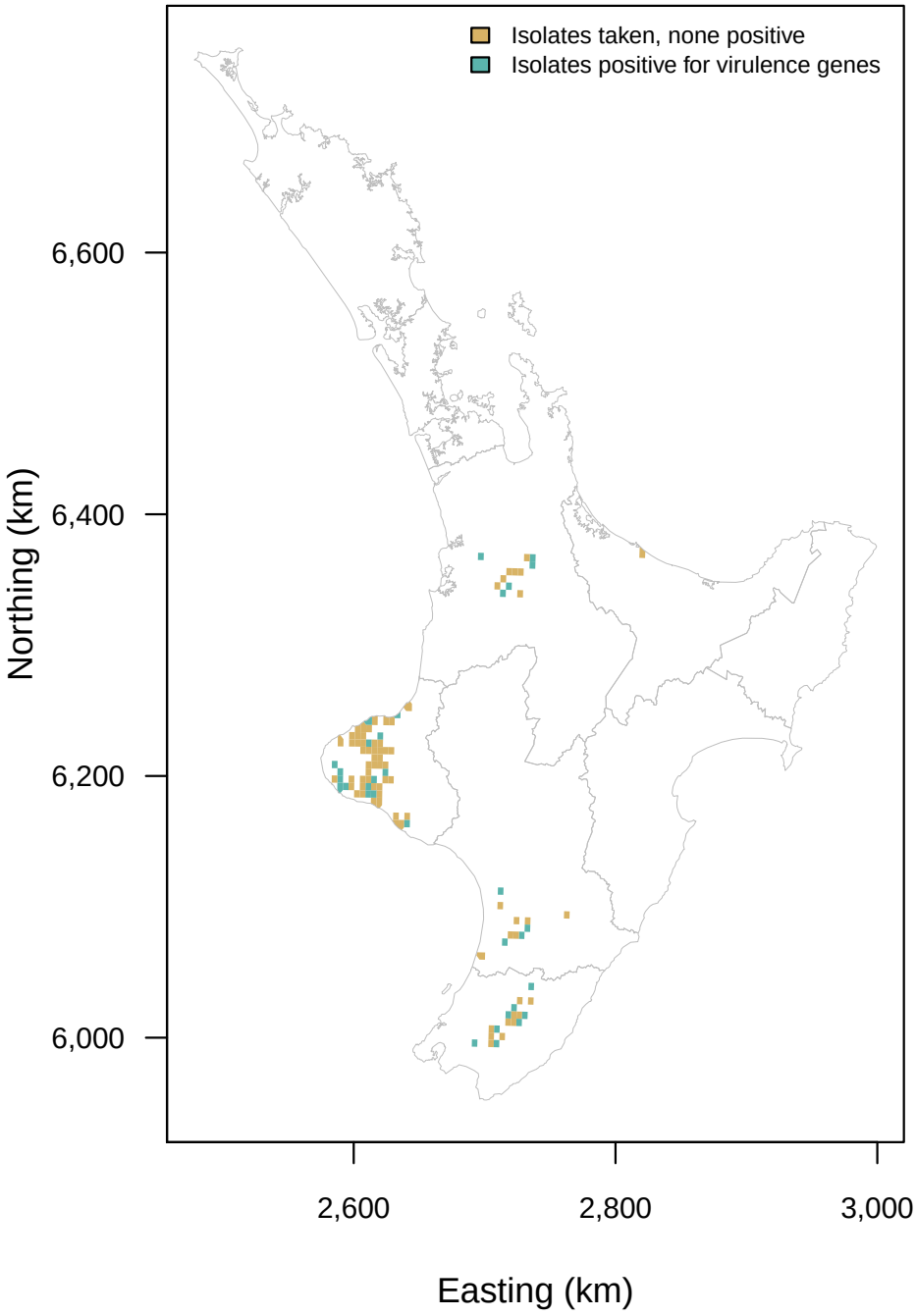
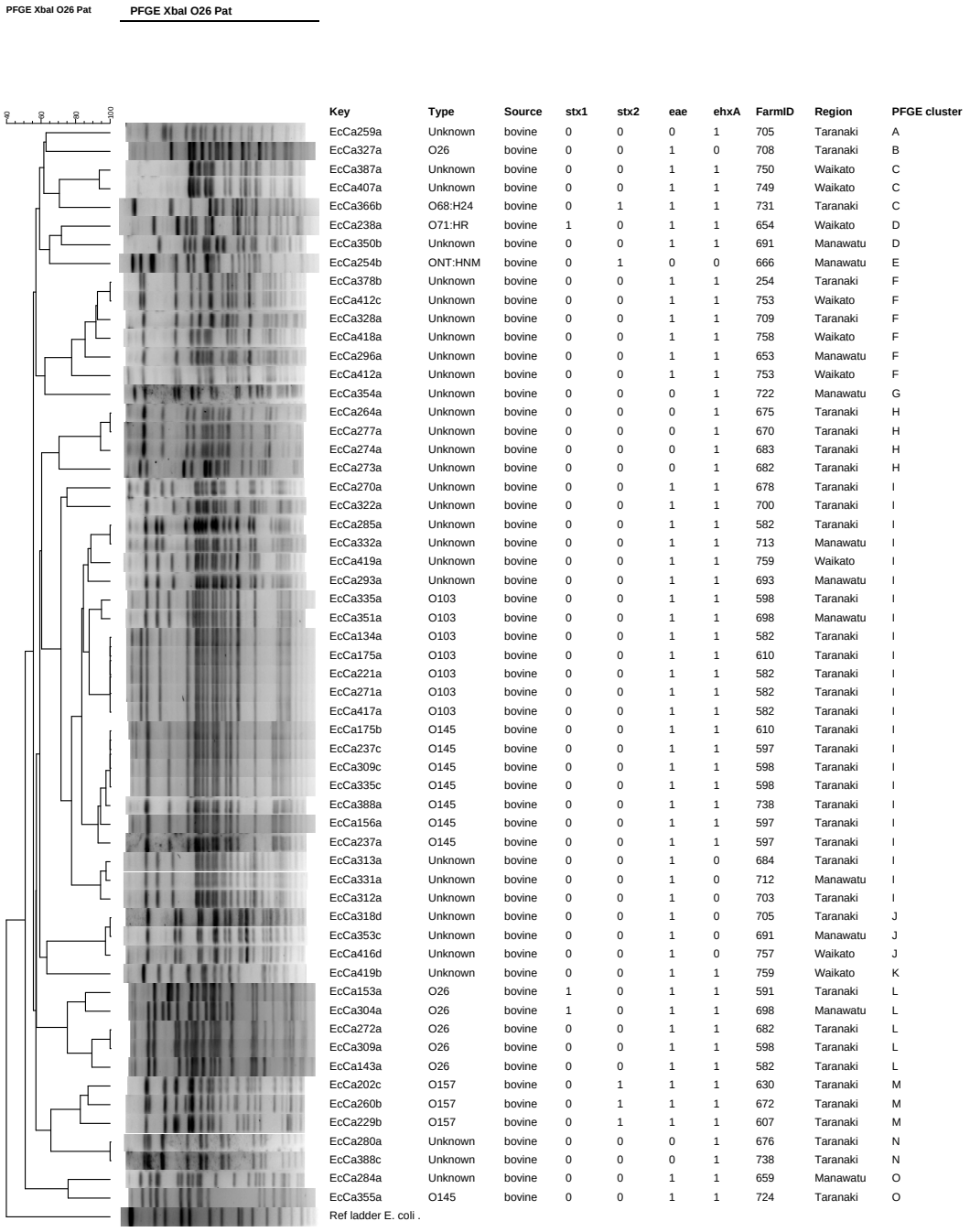


Figure 3.1: Map showing the distribution of *E. coli* isolates positive for virulence genes (*stx1, stx2, eae, ehxA*) in the North Island of New Zealand. The data are aggregated to 5x5km grid cells. If a single animal was positive the grid cell was coloured green. If no animals sampled were positive the cell was coloured beige.



Key	Type	Source	stx1	stx2	eae	ehxA	FarmID	Region	PFGE cluster
EcCa259a	Unknown	bovine	0	0	0	1	705	Taranaki	A
EcCa327a	O26	bovine	0	0	1	0	708	Taranaki	B
EcCa387a	Unknown	bovine	0	0	1	1	750	Waikato	C
EcCa407a	Unknown	bovine	0	0	1	1	749	Waikato	C
EcCa366b	O68:H24	bovine	0	1	1	1	731	Taranaki	C
EcCa238a	O71:HR	bovine	1	0	1	1	654	Waikato	D
EcCa350b	Unknown	bovine	0	0	1	1	691	Manawatu	D
EcCa254b	ONT:HNM	bovine	0	1	0	0	666	Manawatu	E
EcCa378b	Unknown	bovine	0	0	1	1	254	Taranaki	F
EcCa412c	Unknown	bovine	0	0	1	1	753	Waikato	F
EcCa328a	Unknown	bovine	0	0	1	1	709	Taranaki	F
EcCa418a	Unknown	bovine	0	0	1	1	758	Waikato	F
EcCa296a	Unknown	bovine	0	0	1	1	653	Manawatu	F
EcCa412a	Unknown	bovine	0	0	1	1	753	Waikato	F
EcCa354a	Unknown	bovine	0	0	0	1	722	Manawatu	G
EcCa264a	Unknown	bovine	0	0	0	1	675	Taranaki	H
EcCa277a	Unknown	bovine	0	0	0	1	670	Taranaki	H
EcCa274a	Unknown	bovine	0	0	0	1	683	Taranaki	H
EcCa273a	Unknown	bovine	0	0	0	1	682	Taranaki	H
EcCa270a	Unknown	bovine	0	0	1	1	678	Taranaki	I
EcCa322a	Unknown	bovine	0	0	1	1	700	Taranaki	I
EcCa285a	Unknown	bovine	0	0	1	1	582	Taranaki	I
EcCa332a	Unknown	bovine	0	0	1	1	713	Manawatu	I
EcCa419a	Unknown	bovine	0	0	1	1	759	Waikato	I
EcCa293a	Unknown	bovine	0	0	1	1	693	Manawatu	I
EcCa335a	O103	bovine	0	0	1	1	598	Taranaki	I
EcCa351a	O103	bovine	0	0	1	1	698	Manawatu	I
EcCa134a	O103	bovine	0	0	1	1	582	Taranaki	I
EcCa175a	O103	bovine	0	0	1	1	610	Taranaki	I
EcCa221a	O103	bovine	0	0	1	1	582	Taranaki	I
EcCa271a	O103	bovine	0	0	1	1	582	Taranaki	I
EcCa417a	O103	bovine	0	0	1	1	582	Taranaki	I
EcCa175b	O145	bovine	0	0	1	1	610	Taranaki	I
EcCa237c	O145	bovine	0	0	1	1	597	Taranaki	I
EcCa309c	O145	bovine	0	0	1	1	598	Taranaki	I
EcCa335c	O145	bovine	0	0	1	1	598	Taranaki	I
EcCa388a	O145	bovine	0	0	1	1	738	Taranaki	I
EcCa156a	O145	bovine	0	0	1	1	597	Taranaki	I
EcCa237a	O145	bovine	0	0	1	1	597	Taranaki	I
EcCa313a	Unknown	bovine	0	0	1	0	684	Taranaki	I
EcCa331a	Unknown	bovine	0	0	1	0	712	Manawatu	I
EcCa312a	Unknown	bovine	0	0	1	0	703	Taranaki	I
EcCa318d	Unknown	bovine	0	0	1	0	705	Taranaki	J
EcCa353c	Unknown	bovine	0	0	1	0	691	Manawatu	J
EcCa416d	Unknown	bovine	0	0	1	0	757	Waikato	J
EcCa419b	Unknown	bovine	0	0	1	1	759	Waikato	K
EcCa153a	O26	bovine	1	0	1	1	591	Taranaki	L
EcCa304a	O26	bovine	1	0	1	1	698	Manawatu	L
EcCa272a	O26	bovine	0	0	1	1	682	Taranaki	L
EcCa309a	O26	bovine	0	0	1	1	598	Taranaki	L
EcCa143a	O26	bovine	0	0	1	1	582	Taranaki	L
EcCa202c	O157	bovine	0	1	1	1	630	Taranaki	M
EcCa260b	O157	bovine	0	1	1	1	672	Taranaki	M
EcCa229b	O157	bovine	0	1	1	1	607	Taranaki	M
EcCa280a	Unknown	bovine	0	0	0	1	676	Taranaki	N
EcCa388c	Unknown	bovine	0	0	0	1	738	Taranaki	N
EcCa284a	Unknown	bovine	0	0	1	1	659	Manawatu	O
EcCa355a	O145	bovine	0	0	1	1	724	Taranaki	O
Ref ladder E. coli .									

Figure 3.2: Clustering using UPGMA and Dice co-efficient of the PFGE profiles of *E. coli* isolates positive for *stx1*, *stx2*, *eae* and *ehxA* from calves with >70% similarity cut off using *XbaI*. The last lane is the *Salmonella* serotype Braenderup reference standard (H9812).

4 Chapter 4

Epidemiology of non-O157 Shiga toxin-producing *Escherichia coli* in very young ("bobby") calves in the North Island, New Zealand

4.1 Abstract

The prevalence and spatial distribution of *E. coli* O26, O103, O111 and O145 in bobby calves in New Zealand and the relationship between serum IgG, weight, sex and breed and serotype prevalence was determined by collecting recto-anal mucosal swabs (RAMS) (n=299) and blood samples (n=299) from two slaughter plants in the North Island. The analysis of RAMS by real time PCR (RT-PCR) revealed that 134/299 (44.8%) samples were positive for O26, 68/299 (22.7%) for O103 and 47/299 (15.7%) for O145 while not a single sample was positive for O111. Processing of positive samples for isolation using immuno-magnetic separation (IMS) and specific media resulted in 49 O26, four O103 and five O145 isolates. More than 50% of O26 isolates (25/49; 51%) were positive for *stx1*, *eae*, *ehxA* followed by *eae*, *ehxA* (17/49; 34.6%) and *eae* (7/49; 14.2%) by multiplex PCR. All O103 (4/4; 100%) and O145 (5/5; 100%) isolates were positive for *eae*, *ehxA*. O26 isolates were grouped into four clusters (A-D) of >70% similarity using pulse field gel electrophoresis (PFGE), using the *XbaI* restriction enzyme. K function analysis did not indicate spatial clustering of the farms positive for O26, O103 and O145. Mapping showed the presence of farms positive for O26, O103 and O145 in the three important dairy producing regions of the North Island (Manawatu, Taranaki and Waikato) supplying the slaughter plants. There was no association between PCR-prevalence and the variables IgG, weight, sex and breed however, calves positive for O103 were more likely to be positive for O26 and vice versa (P=0.01). Similar association was observed between calves positive for O145 and O103 (P=0.01). It was also observed that lighter calves were more likely to be positive for O26 as compared to heavier calves (P=0.05).

4.2 Introduction

Shiga toxin-producing *Escherichia coli* (STEC), also known as verocytotoxin-producing *Escherichia coli* (VTEC) have emerged as an important public health concern globally. They have been associated with outbreaks and sporadic cases of diarrhoea, haemorrhagic colitis (HC), haemolytic uraemic syndrome (HUS) and thrombocytopenic purpura in humans (Bettelheim, 2007; Karmali et al., 2010).

More than 100 serotypes of STEC have been reported to be associated with disease in humans (World Health Organization, 1998). STEC O157 is considered most important due to its association with large outbreaks, and many HUS and HC cases in most parts of the world (Mainil and Daube, 2005). However, recently non-O157 STEC especially STEC O26, O103, O111 and O145 have emerged as important pathogens in some parts of the world (Johnson et al., 2006). The numbers of human cases due to non-O157 STEC are on the rise worldwide (European Food Safety Authority, 2010; Johnson et al., 2006). In New Zealand the first human case associated with non-O157 STEC was reported in 1980, when *E. coli* O26 and O39 were isolated from human diarrhoeal cases (Wilson and Bettelheim, 1980). Few human cases of non-O157 STEC are reported in New Zealand annually, for example, there were only 14 cases in 2011 (Environmental Science and Research, 2011). However, it is likely that infection is under reported as many faecal samples from asymptomatic or mild cases may not be assessed for the presence of non-O157 STEC and most of the laboratories do not look for non-O157 STEC (Baker et al., 1999). Despite under reporting of non-O157 in New Zealand, 10% of the isolates recovered from human cases are non-O157 (O26, O84, O91, O113, O117, O128, O130, O145 and O153) (Hudson et al., 2000).

Ruminants, especially cattle are considered the most important reservoir of STEC (Bettelheim, 2007; Hancock et al., 1998). Humans can acquire STEC infection by direct contact with animals or indirectly through consumption of food or water contaminated by their faeces (Armstrong et al., 1996; Renwick et al., 1993). Therefore, cattle play a significant role in the epidemiology of STEC.

In New Zealand both cattle and sheep are considered to be important reservoirs of STEC (Buncic and Avery, 1997; Cookson et al., 2006b,c). However, limited information is available about the epidemiology of STEC in cattle especially "bobby calves" which are defined as dairy calves culled from herds at an age of fewer than seven days. In New Zealand 1.4 million calves are slaughtered annually and their meat is exported to other countries (Anonymous, 2009). The USA has a zero tolerance policy for STEC O157 in the meat and six non-O157 STEC (O26, O45, O103, O111, O121 and O145) have recently been added to the list of meat adulterants (Food Safety and Inspection Service, 2011a). The objectives of this study were to enhance understanding about the epidemiology of carriage of non-O157 STEC (O26, O103, O111 and O145) in bobby calves in the North Island of New Zealand and to examine the hypothesis that carriage of non-O157 STEC is associated with the concentration of maternally derived immunity. It is likely that the information provided by the project would help in reducing the rising number of human cases in New Zealand and also in devising appropriate control strategies to avoid possible losses to economically important red meat and dairy export industries of New Zealand (exports equivalent to NZ$10.8 billion)

(Cavanagh, 2003).

4.3 Materials and methods

Recto-anal mucosal swab (RAMS) and blood samples were collected from bobby calves from two different slaughter plants (A and B) in the North Island of New Zealand (Irshad et al., 2012). Each slaughter plant was visited six times during July and October 2008. Every 10^{th} calf on the chain was sampled and in total twenty five calves were sampled on each visit for RAMS and blood. Blood samples were collected in vaccutainers (Becton Dickinson, Auckland, New Zealand) from free draining blood. RAMS were collected using sterile swabs (Copan, Brescia, Italy) at the slaughter plant after the slaughter of the animal. RAMS were then placed in the transport media provided by the manufacturer. Blood and RAMS were transported to the laboratory on ice. The details of the sampled animals; address of the farm, tag number, weight, sex and breed of the animal were recorded.

RAMS were enriched in buffered peptone water (BPW) at $37\,^{\circ}\mathrm{C}$ for 24 hours. After enrichment, 820μl aliquot of each enriched broth was mixed with 180μl of glycerol and preserved at $-80\,^{\circ}\mathrm{C}$ in cryovials. After thawing at room temperature for 5 minutes, 100μl of each sample was re-enriched in BPW at $37\,^{\circ}\mathrm{C}$ for six hours. DNA was extracted from a 1ml aliquot of enriched broth. This aliquot was centrifuged at 12000 g for two minutes, the supernatant discarded; the pellet re-suspended in 500μl of 2% Chelex (Bio-Rad, Auckland, New Zealand) solution and heated at $95\,^{\circ}\mathrm{C}$ for 10 minutes. The lysed bacterial cell suspension was refrigerated for two minutes and re-centrifuged at 12000 g for two minutes. The supernatant containing the DNA was transferred to another tube and tested using real time PCR (RT-PCR) for the presence of *wzx* (O26) (Perelle et al., 2004), *wzx* (O103) (Fratamico et al., 2005) *wbdI* (O111) (Perelle et al., 2004) and *wzx1* (O145) genes (Fratamico et al., 2009).

Each PCR reaction contained 1x reaction buffer (Invitrogen, Auckland, New Zealand), 0.1mM of each dNTP (Fermentas, Auckland, New Zealand), 0.15mM $MgCl_2$ (Invitrogen, Auckland, New Zealand), 0.03mM SYTO-9 (Invitrogen, Auckland, New Zealand), 1 unit of *Taq* DNA Polymerase (Invitrogen, Auckland, New Zealand), 2μl of template DNA and made to final volume of 20μl with sterile water. The concentration of primer in each reaction for O26, O111 and O145 was 500nM and 100nM for O103 respectively.

The amplification was carried out in a Rotor Gene 6000 series thermal cycler (Bio Strategy, Auckland, New Zealand). For detection of O26, O103 and O111 an initial denaturing step of $96\,^{\circ}\mathrm{C}$ for 10 minutes was followed, by 40 cycles of $95\,^{\circ}\mathrm{C}$ for 10 seconds, $60\,^{\circ}\mathrm{C}$ for 30 seconds and $72\,^{\circ}\mathrm{C}$ for 10 seconds, after which the PCR product was detected by thermal melt from $75\,^{\circ}\mathrm{C}$ to $95\,^{\circ}\mathrm{C}$ at a rate of $0.05\,^{\circ}\mathrm{C}$ per second. Similarly, for O145 an initial denaturing step of $94\,^{\circ}\mathrm{C}$ for two minutes was followed, by 35 cycles of $94\,^{\circ}\mathrm{C}$ for 20 seconds, $60\,^{\circ}\mathrm{C}$ for 60 seconds and $72\,^{\circ}\mathrm{C}$ for 60 seconds, after which the PCR product was detected by thermal melt from $75\,^{\circ}\mathrm{C}$ to $95\,^{\circ}\mathrm{C}$ at a rate of $0.05\,^{\circ}\mathrm{C}$ per second. The efficiency of primers used in RT-PCR for detection of O26, O103, O111 and O145 was assessed in three different ways. Firstly, faecal samples negative for O26,

O103, O111, O145 and O157 were spiked with *E. coli* isolate from each serogroup. DNA was isolated from each spiked sample after enrichment at 37 °C for 24 hours. Each DNA sample was analysed with RT-PCR primers for O26, O103, O111, O145 and O157. Samples spiked with *E. coli* O26 gave positive amplicons with O26 primers only and did not give any product with other primers. The same was observed for O103, O111, O145 and O157 primers when tested against DNA isolated from samples spiked with O26, O103, O111, O145 and O157. Thirdly, PCR products from five samples positive by RT-PCR for the serogroups O26, O103 and O145 which did not yield isolates were sequenced. These products had sequence identical to *wzx* (O26), *wzx* (O103), and *wzx1* (O145) genes respectively.

RAMS samples positive for O26, O103, O111 or O145 by RT-PCR were subjected to isolation of the respective serogroup using immuno-magnetic separation (IMS). Immuno-magnetic beads, used for IMS are coated with polyclonal antibodies raised against *E. coli* O26, O103, O111 and O145 lipopolysaccharide. The magnetic beads (20μl) (Invitrogen, Auckland, New Zealand) were mixed with a 1ml of enriched sample and IMS was performed according to the manufacturer's instructions. The bead suspension (100μl) was inoculated onto rhamnose MacConkey agar supplemented with cefixime (50μg/ml) and potassium tellurite (2.5mg/ml) (CT-RMAC) (Fort Richard, Auckland, New Zealand) for isolation of O26 and sorbitol MacConkey agar (SMAC) (Fort Richard, Auckland, New Zealand) for isolation of O103, O111 and O145. The plates were incubated at 37 °C for 24 hours and observed for the presence of *E. coli* O26 colonies (grey colour/colourless colonies on CT-RMAC) and O103, O111 and O145 colonies (pink/purple colonies on SMAC). Suspect colonies were sub-cultured to MacConkey agar (Fort Richard, Auckland, New Zealand). Potentially positive O26, O103, O111 and O145 colonies were identified using RT-PCR. Multiplex PCR was performed to detect the presence of Shiga toxin 1 (*stx1*), Shiga toxin 2 (*stx2*), *E. coli* attaching and effacing (*eae*) and enterohaemolysin (*ehxA*) genes on colonies positive for non-O157 (O26, O103, O111 and O145).

Each PCR reaction contained 1x reaction buffer (Invitrogen, Auckland, New Zealand), 0.2μM of each primer (Paton and Paton, 1998a; Sharma and Dean-Nystrom, 2003), 0.1mM of each dNTP (Fermentas, Auckland, New Zealand), 0.15mM $MgCl_2$ (Invitrogen, Auckland, New Zealand), 1 unit of *Taq* DNA Polymerase (Invitrogen, Auckland, New Zealand), 2μl of DNA and was made to a final volume of 25μl with sterile water.

The amplification was carried out in a Gene Amp PCR system 9700 (Applied Biosystems, Melbourne, Australia). An initial denaturing cycle of 5 minutes at 96 °C was followed by 40 cycles of, 30 seconds at 96 °C, 30 seconds at 60 °C, 30 seconds at 72 °C, followed by a final cycle of 5 minutes at 72 °C, after which the PCR products were electrophoresed through an agarose (2% w/v) gel (Invitrogen, Auckland, New Zealand) and visualised using ethidium bromide under ultraviolet illumination.

O26 isolates were genotyped using pulse field gel electrophoresis (PFGE) following the standard procedure described by PulseNet USA (Centres for Disease Control and Prevention, 2009). Briefly, each *E. coli* isolate grown on Trypticase Soy agar with 5% defibrinated sheep blood (Fort Richard, Auckland, New Zealand) was mixed with cell suspension buffer (100mM Tris: 100mM

EDTA, pH 8.0) to make a bacterial suspension. The plugs were made by mixing 400μl of bacterial suspension with 20μl of proteinase K (20mg/ml) (Total Lab System, Auckland, New Zealand) and 400μl of Seakam Gold agarose (1 %w/v) (Invitrogen, Auckland, New Zealand) in Tris/EDTA buffer (10mM Tris: 1mM EDTA, pH 8.0) and dispensing this mixture to block former. The lysis of the plugs was carried out in the mixture of cell lysis buffer (50mM Tris: 50mM EDTA, pH 8.0 + 1% Sarcosyl) (5ml) and proteinase K (25μl of 20mg/ml) (Total Lab System, Auckland, New Zealand) at 56 °C for 2 hours in a shaking water bath. After lysis, plugs were washed with ultrapure distilled water (2 washes) and Tris/EDTA buffer (3 washes) (10mM Tris: 1mM EDTA, pH 8.0). Each washing was for 15 minutes at 56 °C in a shaking water bath. Digestion of agarose embedded DNA was performed with 5μl *XbaI* (10units/μl) (Roche Diagnostics, Auckland, New Zealand) for 2 hours at 37 °C in a heating block. The plugs were cut to appropriate size with sharp blade and loaded into SeaKem Gold agarose (1% w/v) gel (Invitrogen, Auckland, New Zealand) in Tris borate EDTA buffer (TBE). Electrophoresis was carried out with CHEF DR II system (Bio-Rad Laboratories, Auckland, New Zealand) at 14 °C in 0.5 x TBE, with plus ramp of initially 6.8 seconds and finally 35.4 seconds, for 20 hours at constant voltage of 6 V/cm. After electrophoresis gel was stained in ethidium bromide (0.5μg/ml) for 20 minutes and visualised using short wave length ultraviolet light and an image of the gel was saved. Bionumeric version 6.6 (www.applied-maths.com) was used to create dendrogram of the PFGE profiles. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Dice coefficient with >70% similarity cut off were used to construct the clusters of *E. coli* isolates with one or more virulence genes.

All O26 isolates were analysed using *stx*-encoding bacteriophage insertion (SBI) typing. Briefly multiplex PCR was used to determine SBI genotypes of *E. coli* O26 isolates using primers shown in Table 4.1. The amplicon obtained by PCR was applied to capillary electrophoresis using DNA analyser 3730 (Applied Biosystems, Foster City, CA) and GeneMarker software was used for analysis of data (Soft Genetics, LLC, State College, PA).

Calf serum samples were sent to New Zealand Veterinary Pathology Laboratory, Palmerston North for quantification of IgG antibodies. IgG antibodies were measured using a commercially available turbidimetric immuno-assay (TIA) kit (Midland Products Corporation, Mt. Maunganui, New Zealand) and Hitachi P800 analyser (Diamond Diagnostics, Holliston, United States of America).

4.3.1 Statistical analysis

The farms PCR positive and negative for O26, O103 and O145 were mapped using R package maptools (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria). The addresses of these farms (193) were recorded from the slaughter plant records and coordinates (Latitude and Longitude) of 180 farms were obtained using Agribase data (Sanson and Pearson, 1997) and Google earth (www.google.com/earth). The latitude and longitude of each farm was converted to XY coordinates and farms were then assigned to 5x5 km grid cells for mapping and to maintain the anonymity of the farms. The coordinates of 13 farms were missing.

K function analysis was used to determine the localised (also known as second-order spatial clus-

tering), of positive (case) and negative (control) farms (Ripley, 1976) by comparing the spatial properties of various spatial point patterns with each other. In this case, location of each farm is a spatial point pattern. For this technique assumption of underlying process to be stationary is not required i.e. intensity of the process does not depend upon the location in space. In this case the distribution of positive and negative farms for each serogroup were compared at a range of distance from 1-10 km by calculating the difference between two K functions (Bailey and Gatrell, 1995). The difference between two K functions was plotted against distance with 95% upper and lower simulation envelopes. If the difference between the two K functions fell above the upper envelope then it means that the positive farms were significantly more clustered than negative farms.

The prevalence of *E. coli* O26, O103 and O145 in calves was estimated and adjusted for clustering of calves within farms using R package "survey" and function "svydesign".

The association between potential risk factors (concentration of IgG, weight, sex, breed and RT-PCR status of the calves for O26, O103, O145 and O157) and *E. coli* O26, O103, O145 and O157 RT-PCR status of the calves was assessed using logistic regression model. The statistical analysis was conducted using software R 2.8.1 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria).

Seven generalised linear mixed-effects models were fitted for each variable (concentration of IgG, weight, sex, breed, RT-PCR status of the calves for *E. coli* O26, O103, O145 and O157), being the *j-th* PCR result from farm *i*, and being the *j-th* IgG result from farm *i*. The following logistic regression model was fitted with a random effect at the farm level:

$$\text{logit}(P_{ij})=\beta_0 + \beta_1 IgG_{ij} + \mu_i + \varepsilon_{ij} \ldots (1)$$

Where

$$\mu_i \sim N(0, \nu^2)$$
$$\varepsilon_{ij} \sim N(0, \sigma^2)$$

μ_i = Farm random effect

N= Normal distribution

β= Regression coefficient

σ^2 = Variance of residuals

ν^2= Variance for random effect for farm

The model shown in equation (1) was fitted again by replacing variable IgG with weight, sex and breed, respectively.

The generalised mixed-effects model was also fitted with all the variables (concentration of IgG, weight, sex, breed and RT-PCR status of the calves for *E. coli* O26, O103, O145 and O157) to observe the combined effect on RT-PCR results, where:

$$\text{logit}(P_{ij})=\beta_0 + \beta_1 IgG_{ij} + \beta_2 Weight_{ij} + \beta_3 sex_{ij} + \beta_4 breed_{ij} + \beta_5 RT-PCRO26_{ij} + \beta_6 RT-PCRO103_{ij} + \beta_7 RT-PCRO145_{ij} + \beta_8 RT-PCRO157_{ij} + \mu_i + \varepsilon_{ij}$$

Multiple correspondence analysis was used to simultaneously assess the correspondence between explanatory variables (IgG, weight, sex, breed and RT-PCR results) and an outcome variable (RT-PCR results). Each column in the data represent category of variable and each row represent the results from each sample. The variable RT-PCR results were coded as indicator matrices. The analysis was performed using R package ca and using each variable as supplementary variable (one at a time). Maps were generated to visualise the results of the analysis.

4.4 Results

In total 299 RAMS and serum samples were collected from bobby calves originating from two abattoirs, A and B in the North Island of New Zealand. There were 134/299 (44.8%; 95% CI= 39.5%-50%) samples positive for *E. coli* O26, 68/299 (22.7%; 95% CI= 17.8%-29%) samples positive for *E. coli* O103, 47/299 (15.7%; 95% CI= 11.6%-21%) positive for *E. coli* O145 but not a single sample was positive for *E. coli* O111 by RT-PCR. There was no significant difference in prevalence of *E. coli* O26 in male (97/211; 46%; 95% CI= 39.5%-53%) and female calves (37/88; 42%; 95% CI= 31.8%-53%). The prevalence of *E. coli* O103 was also similar in male (46/211; 21.8%; 95% CI= 16.6%-28%) and female calves (22/88; 25%; 16.5%-36%), and likewise there was no significant difference in the prevalence of O145 in male (33/211; 15.6%; 95% CI= 11.2%-21%) and female calves (14/88; 15.9%; 95% CI= 9.1%-26%). The mean level of IgG was similar in calves positive for *E. coli* O26 (2280.4 mg/dL), O103 (2167.7 mg/dL) and O145 (2306.1 mg/dL) by RT-PCR and calves negative for *E. coli* O26 (2018.1 mg/dL), O103 (2129.6 mg/dL) and O145 (2083.1 mg/dL) (Figure 4.1). There was no significant difference in average weight of the calves positive for *E. coli* O26 (15.9 Kg) O103 (16.3 Kg) and O145 (16.9 Kg) and the calves negative for *E. coli* O26 (16.9 Kg), O103 (16.5 Kg) and O145 (16.3 Kg) by RT-PCR (Figure 4.2).

Results of linear mixed effects-models fitted consecutively for each variable (IgG, weight, sex, breed and RT-PCR results for O26, O103, O145 and O157) and models in which all the variables were fitted together for O26, O103 and O145 are shown in Tables 4.2, 4.3 and 4.4. There was no significant relationship between any of the *E. coli* serotypes and the variables: concentration of IgG, sex and breed of the calves. However, heavier calves tended to be less positive for O26 as compared to lighter calves (P=0.05) (Table 4.2). There was also a relationship between PCR prevalence of O26 and O103 and between O103 and O145; log odds of being positive for O26 by RT-PCR were likely to increase 0.67 times with each sample positive for O103 and vice versa (P=0.01) (Table 4.2 and 4.3). Similarly, the log odds of being positive for O145 by RT-PCR were likely to increase 0.80 times (P=0.01) with each sample positive for O103 and vice versa (Table 4.3 and 4.4).

The results of multiple correspondence analysis are shown in appendix-1 (Figures 7.1-7.6). The results of the analysis showed no correspondence between RT-PCR results and explanatory variables. For example Figure 7.1 showed that PCR results for O26, O103 and O145 were present in the centre of the map. The centre of the map depicts the average profile therefore variables present in the central position are homogeneous. The inertia represents the variance of a MCA model. In

Figure 7.1 first and second principal axis corresponded to 23.4% and 16.3% of total inertia which means that 39.7% of the variance in the model is explained by first two dimensions.

The spatial distribution of the farms positive or negative for *E. coli* O26, O103 and O145 is shown in the Figure 4.3. The farms positive for O26, O103 and O145 are present in all three of the important dairy producing areas of the North Island supplying the abattoirs sampled in the study. K function analysis was used to observe the spatial clustering of farms positive for *E. coli* O26, O103 and O145. There was no evidence of localised clustering (second order) in farms positive for *E. coli* O26, O103 and O145 that were more than 10 km apart as the difference between two K functions did not cross the upper 95% CI envelope (Figures 4.4).

Analysis of RT-PCR positive samples for *E. coli* O26 (134), O103 (68) and O145 (47) by IMS gave 49 *E. coli* O26, four *E. coli* O103 and five *E. coli* O145 isolates. Of 49 *E. coli* O26 isolates 25 isolates were positive for *stx1*, *eae*, *ehxA*, and 17 for *eae*, *ehxA* and seven for *eae*. However, all *E. coli* O103 and O145 isolates were positive for *eae*, *ehxA* virulence genes.

Using SBI all O26 isolates can be divided into two genotypes; genotype 9 and 20. All *stx1*, *eae*, *ehxA* isolates had genotype 20 whereas all *eae*, *ehxA* and *eae* isolates had genotype 9. PFGE of 49 *E. coli* O26 isolates revealed various PFGE profiles as shown in Figure 4.5. These can be broadly divided into four clusters (A, B, C and D). All the isolates in cluster A were positive for *stx1*, *eae*, *ehxA* except one isolate which was positive for *eae*, *ehxA*. The remaining *eae*, *ehxA* isolates were present in cluster B. Isolates positive for *eae* only were present in cluster C (two isolates) and D (one isolate).

4.5 Discussion

The beef and dairy industries are key contributors to the New Zealand economy (Cavanagh, 2003). However, limited information is available about the prevalence of non-O157 STEC in cattle. Although non-O157 STEC serogroups O5, O26, O84 and O128 have been isolated from New Zealand cattle (n=187) and sheep (n=132) previously (Cookson et al., 2006b) but no specific methods such as RT-PCR or IMS were included for specific selection of O26, O103, O111 and O145.

RT-PCR has been reported to be a fast and sensitive method for detection of non-O157 STEC, including O26, O103, O111 and O145 (Perelle et al., 2004) and has been used successfully for detection of non-O157 STEC in food and faecal samples (Perelle et al., 2004; Sharma, 2002). In this study the prevalence of O26 (44.8%) was higher compared to O103 (22.7%) and O145 (15.7%). Similar to this study, a Scottish study also reported a higher prevalence of O26 (4.6%) followed by O103 (2.7%) and O145 (0.7%) (Pearce et al., 2006). An Italian study also reported a higher prevalence of *E. coli* O26 (3%) compared to O157 (0.5%) in 182 faecal samples collected from cattle using IMS. However, no O103, O111 or O145 isolates were obtained (Bonardi et al., 2007). A Korean study analysed 809 faecal samples collected from beef and dairy cattle aged between eight months and five years using IMS. They reported a higher prevalence of *E. coli* O26 (6.7%) compared to O111 (4.6%) but *E. coli* O103, O145 and O157 numbers were not investigated (Jeon

et al., 2006). The higher sensitivity of RT-PCR may be the reason for the higher prevalence of O26, O103 and O145 in this study compared to the Scottish, Italian and Korean studies where culturing was used as the diagnostic test (Bonardi et al., 2007; Jeon et al., 2006; Pearce et al., 2006). However, this difference in prevalence may also be due to difference in sample size, management practices and environmental factors.

The differences in prevalence of O26, O103 and O145 may also be due to differences in transmission dynamics of O26, O103 and O145. O'Reilly et al. (2010) used dynamic models to quantify the transmission dynamics of O26 and O103 in terms of within group basic reproduction number (R_0). After fitting different dynamic models they reported that O26 appeared to be well adapted to cattle host with an R_0 value greater than 1. This means that there is higher cattle to cattle transmission of O26 compared to transmission through alternative non-cattle reservoir. On the other hand, O103 appeared to be well adapted to the non-cattle environment with an R_0 value of less than 1. This difference may also be due to differences in intimin subtypes. Intimin plays an important role in STECs attachment and colonisation of enterocytes of cattle. There are 20 variants of intimin and several studies have shown the relationship between intimin type and serogroup of *E. coli*. For example, serogroup O26 often possesses intimin type β, serogroup O103 intimin type θ or ϵ and serogroups O145 and O157 intimin type γ (Blanco et al., 2004c; Cookson et al., 2007a; Kozub-Witkowski et al., 2008; Posse et al., 2007). Different intimin types may be responsible for differences in patterns of colonisation, tissue tropism and host specificity (Fitzhenry et al., 2002, 2003; Phillips and Frankel, 2000; Phillips et al., 2000).

Higher rates of isolation for O26 compared to O103 and O145 may be due to the availability of specific media for isolation of O26 whereas no specific media is available for isolation of O103 and O145. Presence of higher levels of competing microflora or inhibitors present in the samples may be another explanation of low recovery of isolates from positive samples (Fach et al., 2001; Vernozy-Rozand et al., 2005). STEC may also be present in the stressed condition which may inhibit its isolation.

The most common virulence profile of *E. coli* O26 isolates was *stx1*, *eae*, *ehxA*. This is in agreement with another study where more than 90% of O26 isolates displayed this profile (Pearce et al., 2004). This virulence profile of O26 isolates has great public health significance because this is the most common profile present in O26 isolates from human diarrhoeal cases (Schmidt et al., 1999a; Zhang et al., 2000b). All the O103 and O145 isolates were negative for the *stx* gene. Similar to this study Pearce et al. (2006) also reported the rare carriage of *stx* gene in O103 and O145 isolates. However, the absence of an *stx* gene in O103 and O145 isolates may also be related to lower recovery of these isolates from positive samples. Twenty four O26, four O103 and five O145 isolates were positive for the *eae* gene. Isolates positive for the *eae* gene from cattle are termed "atypical entero-pathogenic *E. coli*" (aEPEC). They are also of public health importance because they are genetically closely related to STEC and can easily become STEC by acquiring the *stx* gene from bacteriophages (Bielaszewska et al., 2008; Hernandes et al., 2009; Mellmann et al., 2005; Trabulsi et al., 2002; Whittam et al., 1993).

K function analysis has been used successfully to find the second order clustering of disease cases

for various diseases and can be helpful in understanding the process of transmission of disease (Benschop et al., 2009; Fenton et al., 2009). Fenton et al. (2009) applied K function analysis to investigate the presence of spatial clustering of dairy farms positive for *S. enterica* serovar Agama and *S. enterica* serovar Dublin in England and Wales and observed spatial clustering for farms positive with these serotypes at a distance up to 30 km. Another study reported the second order clustering of O157 positive farms between 3 and 4 km in the North Island of New Zealand (Irshad et al., 2012). However, no spatial clustering of farms positive for O26, O103 and O145 was observed in this study (Figure 4.4).

PFGE has been used successfully for molecular typing of STEC isolates. It is considered a highly discriminatory technique for molecular typing of STEC isolates (Davis et al., 2003; Olive and Bean, 1999; Terajima et al., 2006). PFGE results indicate that O26 isolates can be grouped on the basis of the presence or absence of a particular virulence gene. The presence of a single *eae*, *ehxA* positive isolate in cluster A (*stx1*, *eae*, *ehxA* positive isolates) may indicate the close relationship between aEPEC and STEC isolates.

SBI typing has been previously used for genotyping of *E. coli* O157 isolates (Besser et al., 2007; Irshad et al., 2012). Besser et al. (2007) analysed 282 *E. coli* O157 isolates from humans and 80 isolates from bovine by SBI typing and found more diversity in bovine isolates (13 genotypes) as compared to human isolates (9 genotypes). However, genotype 1 and 3 were most commonly found genotypes among human (261/282; 89%) and bovine isolates (41/80; 51%). In contrast to that a New Zealand study reported more diversity among human *E. coli* O157 isolates (5 genotypes) as compared to bovine isolates (3 genotypes) (Irshad et al., 2012). To our knowledge this is the first study to report the SBI typing of *E. coli* O26 isolates. *E. coli* O26 isolates could be divided into two genotypes (9 and 20). All the isolates positive for *stx1*, *eae*, *ehxA* belonged to genotype 20 whereas all *eae*, *ehxA* isolates belonged to genotype 9 which showed limited diversity among *E. coli* O26 isolates by SBI typing.

A higher concentration of antibodies in the serum has been reported from the calves that were fed colostrum or vaccinated against STEC (Bretschneider et al., 2007; Dziva et al., 2007; Potter et al., 2004; van Diemen et al., 2007; Widiasih et al., 2004b). Potter et al. (2004) reported reduction in the shedding of *E. coli* O157 after vaccination. Similarly, Rugbjerg et al. (2003) reported that calves that were fed colostrum were less likely to shed *E. coli* O157 than the calves that were not fed colostrum. However, this study could not find any difference between the concentration of IgG in calves that were RT-PCR positive or negative for *E. coli* O26, O103 and O145. Several other studies have reported conflicting findings about the efficacy of STEC vaccination. These studies reported a higher level of IgG in the serum after vaccination, however it did not provide protection against intestinal colonisation by STEC after challenge (Dziva et al., 2007; van Diemen et al., 2007).

Studies have shown that diarrhoea or respiratory disease has significant impact on growth of the calves (Donovan et al., 1998; Wittum and Perino, 1995). For example reduction in weight gain has been observed in feedlot cattle suffering from respiratory disease (Karren et al., 1987; Wittum and Perino, 1995). The poor health and body condition of the calves may be indirectly related

to passive transfer of immunity (Donovan et al., 1998). In this study heavier calves were less likely to be positive for *E. coli* O26 as compared to lighter calves but no association between weight of the calves and RT-PCR results for O103 and O145 was observed. The exact reason for this difference is not known. Low concentration of IgG in lighter calves may be one possible explanation but there was no difference in concentration of IgG in lighter and heavier calves in this study. Therefore further studies are required to understand association between weight of the calves and their susceptibility to *E. coli* O26.

Hereford, Jersey and Friesian calves were equally likely to be positive for O26, O103 and O145 in this study. However, Widiasih et al. (2004a) reported a difference in the shedding of *E. coli* O157 and O26 in calves of different breeds. The shedding of *E. coli* O157 (10.7%) was higher in Japanese Black calves whereas the shedding of *E. coli* O26 (8.9%) was higher in Holstein calves. The likelihood of shedding of *E. coli* O157 was lesser ($P<0.01$) in Romosinuano cows compared with Angus or Brahmas cows (Riley et al., 2003). In contrast some other studies could not find differences in shedding of STEC in different breeds. For example, there was no difference in shedding of STEC in calves of age 0-8 months of calves of different breeds (Holstein, Japanese Black and cross of Holstein and Japanese Black) (Shinagawa et al., 2000). Similarly, Hancock et al. (1994) reported that there was no difference in shedding of *E. coli* O157 and different breeds.

This study provided useful information about the prevalence of *E. coli* serogroups (O26, O103, O111 and O145) of economic and public health significance. This study showed that *E. coli* O26 was most prevalent in RAMS samples collected from calves followed by O103 and O145 and not a single sample was positive for O111. Maternally derived immunity and calves positive with *E. coli* O26, O103 and O145 by RT-PCR were not associated with each other. Therefore, colostrum feeding may not be effective in reducing colonisation and shedding of *E. coli* O26, O103 and O145. Although this study provided valuable information about the epidemiology of non-O157 STEC of economic and public health concern but it was a small scale study therefore further investigations are required to assess the risk factors and to devise appropriate on farm and pre-slaughter control strategies for *E. coli* O26, O103 and O145 in bobby calves.

Table 4.1: Primers used for PCR in *stx*-encoding bacteriophage insertion (SBI) typing.

Sequence (5′-3′)	Target
CACAGACTGCTGCAGTGAGG	*stx1*
CAGTTAATGTGGTGGCGAAG	
AGGATGACACATTTACAGTGAAGGTT	*stx2*
CACAGGTACTGGATTTGATTGTGAC	
CGACAGGCCCGTTATAAAAA	*stx2c*
GGCCACTTTTACTGTGAATGTATC	
CACCGGAAGGACAATTCATC	Bacteriophage-*yehV* left junction
AACAGATGTGTGGTGAGTGTCTG	
AAGTGGCGTTGCTTTGTGAT	Variant bacteriophage-*yehV* right junction
AGCGATACAGATCTCAACAC	
AAGTGGCGTTGCTTTGTGAT	Bacteriophage-*yehV* right junction
GATGCACAATAGGCACTACGC	
AAGTGGCGTTGCTTTGTGAT	*yehV* insertion site region
AACAGATGTGTGGTGAGTGTCTG	
CCGACCTTTGTACGGATGTAA	Bacteriophage-*wrbA* left junction
CGAATCGCTACGGAATAGAGA	
AGGAAGGTACGCATTTGACC	Bacteriophage-*wrbA* right junction
ATCGTTCGCAAGAATCACAA	
AGGAAGGTACGCATTTGACC	*wrbA* insertion site region
CGAATCGCTACGGAATAGAGA	
CCGTAACGACATGAGCAACAAG	Bacteriophage-*argW* left junction
GCAGTATCACGCAGAGCTGAAG	
GCATCTCACCGACGATAACA	Bacteriophage-*argW* right junction
AATTAGCCCTTAGGAGGGGC	
CCGTAACGACATGAGCAACAAG	*argW* insertion site region
AATTAGCCCTTAGGAGGGGC	
GACAGCAGAAACAACGGATTTAAC	Bacteriophage-*sbcB* left junction
CCAGCGTGGGATAAAAGAGAATC	
GTGCTTGGGTCTTTTCTCTG	Bacteriophage-*sbcB* right junction
TCCAGGCGTAAGGATCGTAG	
GACAGCAGAAACAACGGATTTAAC	*sbcB* insertion site region
TCCAGGCGTAAGGATCGTAG	

Table 4.2: The univariable and multivariable relationships between the likelihood a calf is positive by RT-PCR for *E. coli* O26 and five explanatory variables. The reference category for breed was Friesian

Variables	Coefficient	Standard Error	P-value
Univariable analysis			
IgG	$1x10^{-4}$	$7\ x10^{-5}$	0.14
Weight	-0.05	0.02	0.05
Sex (Male)	0.21	0.025	0.39
Breed (Hereford)	-0.12	0.41	0.77
Breed (Jersey)	0.41	0.26	0.11
PCRO103	0.67	0.28	0.01
PCRO145	0.45	0.31	0.15
PCRO157	0.43	0.30	0.15
Multivariable model			
IgG	$1x10^{-4}$	$7\ x10^{-5}$	0.17
Weight	-0.05	0.028	0.05
Sex (Male)	0.21	0.26	0.42
Breed (Hereford)	-0.22	0.43	0.60
Breed (Jersey)	0.3	0.28	0.28
PCRO103	0.67	0.29	0.01
PCRO145	0.35	0.33	0.28
PCRO157	0.52	0.32	0.10

Table 4.3: The univariable and multivariable relationships between the likelihood a calf is positive by RT-PCR for *E. coli* O103 and five explanatory variables. The reference category for breed was Friesian

Variables	Coefficient	Standard Error	P-value
Univariable analysis			
IgG	$1.6x10^{-5}$	$8.9\ x10^{-5}$	0.85
Weight	-0.01	0.03	0.71
Sex (Male)	-0.17	0.29	0.54
Breed (Hereford)	0.66	0.44	0.13
Breed (Jersey)	0.003	0.32	0.99
PCRO26	0.67	0.28	0.01
PCRO145	0.80	0.34	0.01
PCRO157	0.18	0.35	0.60
Multivariable model			
IgG	$1.3x10^{-5}$	$9.5\ x10^{-5}$	0.88
Weight	-0.005	0.03	0.87
Sex (Male)	-0.12	0.31	0.69
Breed (Hereford)	0.62	0.46	0.17
Breed (Jersey)	-0.11	0.33	0.72
PCRO26	0.67	0.29	0.01
PCRO145	0.74	0.35	0.03
PCRO157	0.11	0.36	0.75

Table 4.4: The univariable and multivariable relationships between the likelihood a calf is positive by RT-PCR for *E. coli* O145 and five explanatory variables. The reference category for breed was Friesian

Variables	Coefficient	Standard Error	P-value
Univariable analysis			
IgG	$8x10^{-5}$	$1x10^{-4}$	0.41
Weight	0.03	0.03	0.92
Sex (Male)	-0.02	0.34	0.95
Breed (Hereford)	0.33	0.55	0.55
Breed (Jersey)	0.30	0.38	0.42
PCRO26	0.45	0.31	0.15
PCRO103	0.80	0.34	0.01
PCRO157	-0.18	0.44	0.66
Multivariable model			
IgG	$6x10^{-5}$	$1x10^{-4}$	0.52
Weight	0.005	0.03	0.87
Sex (Male)	-0.04	0.36	0.90
Breed (Hereford)	0.26	0.57	0.64
Breed (Jersey)	0.26	0.39	0.90
PCRO26	0.34	0.33	0.29
PCRO103	0.74	0.35	0.03
PCRO157	-0.23	0.45	0.59

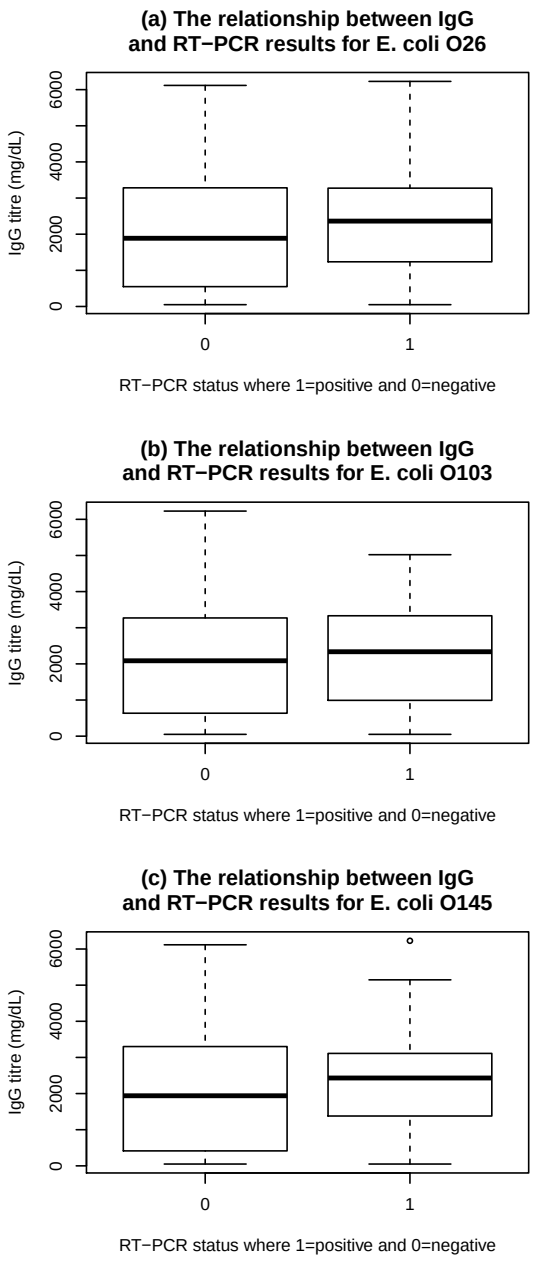


Figure 4.1: Box plot showing the relationship between IgG and RT-PCR results for O26, O103 and O145

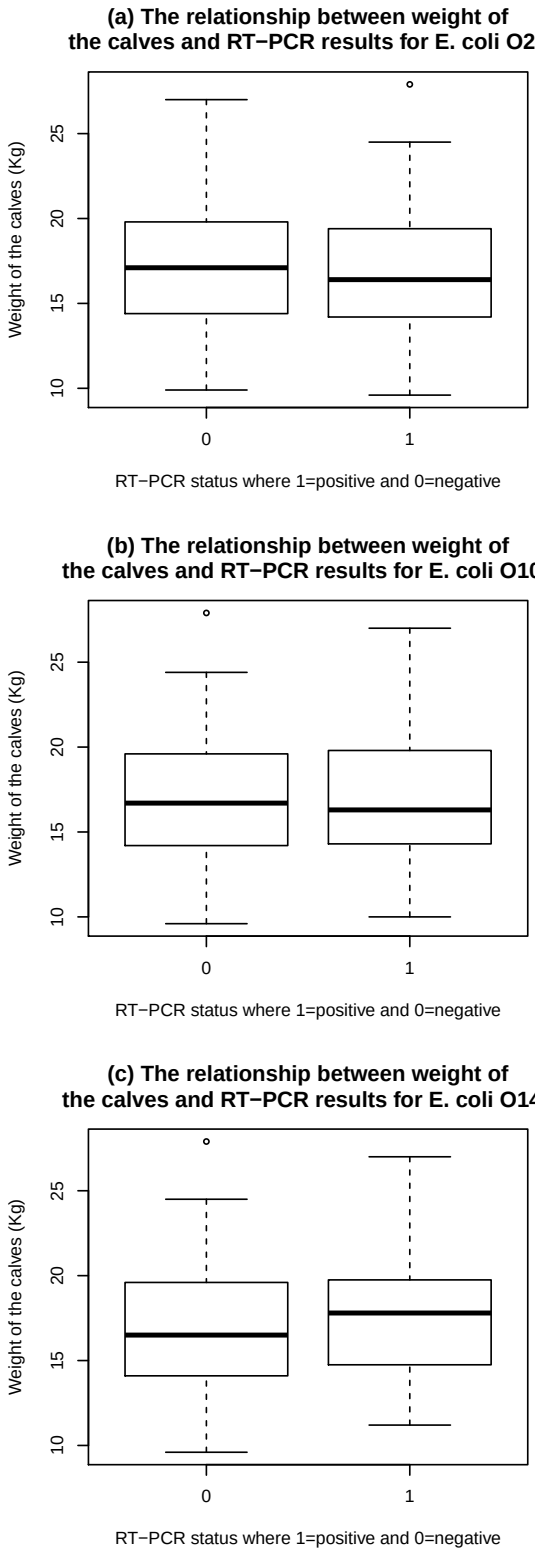


Figure 4.2: Box plot showing the relationship between weight and RT-PCR results for O26, O103 and O145

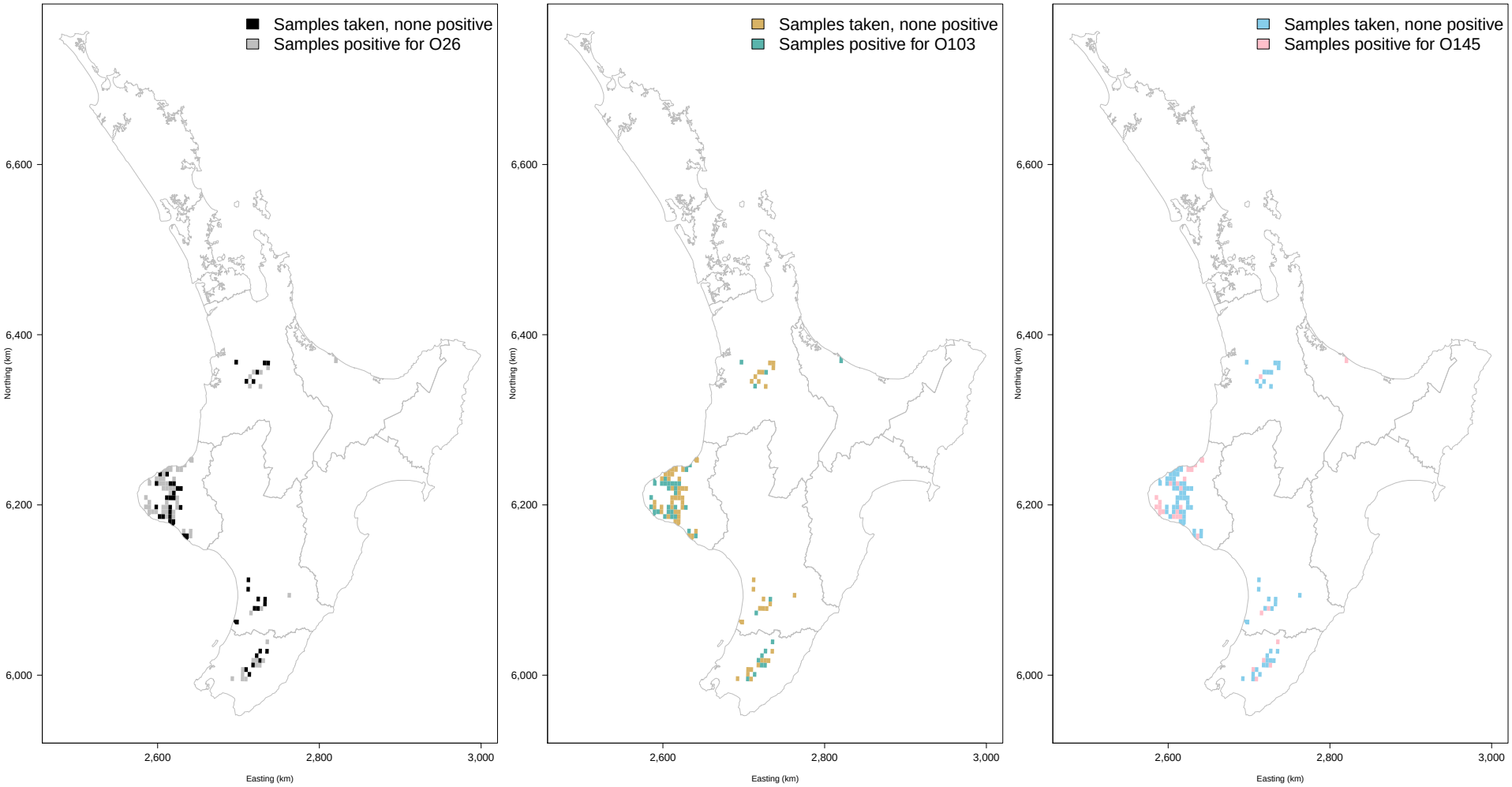


Figure 4.3: Maps showing the distribution of the farm of origin of *E. coli* O26, O103 and O145-positive calves in North Island, New Zealand. The data are aggregated to 5x5 km grid cells. Only cells containing farms that submitted calves to the abattoir that were subsequently sampled are displayed.

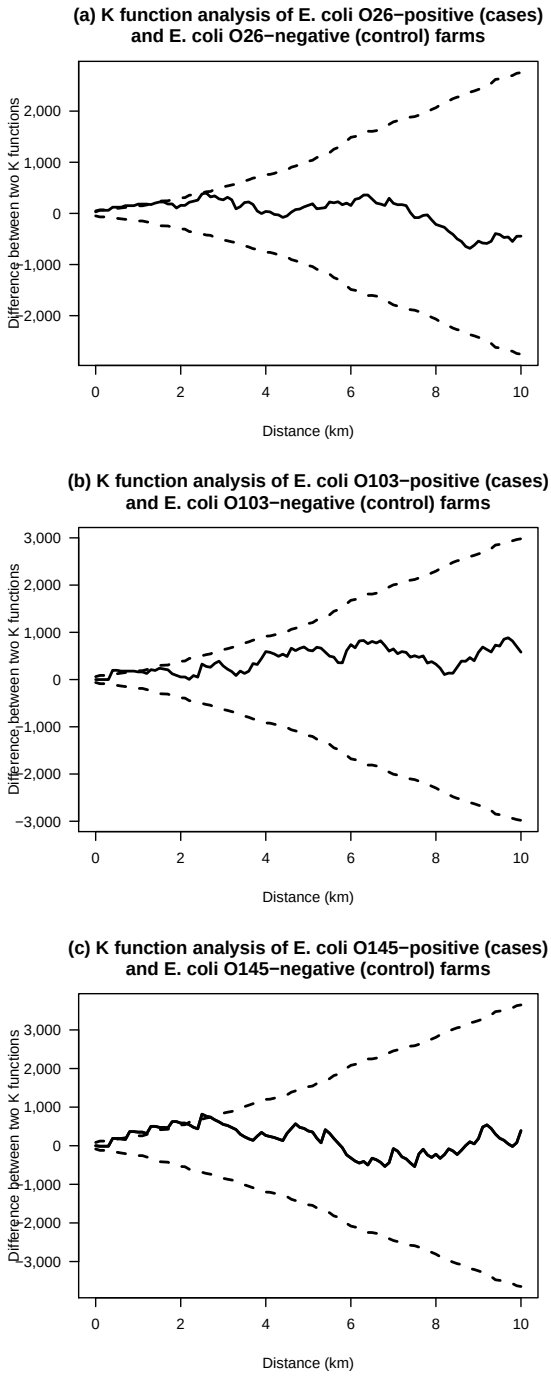
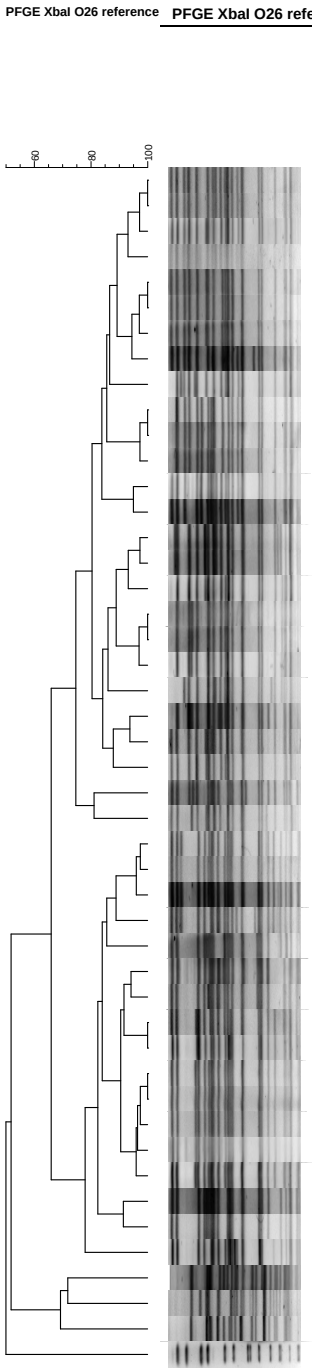


Figure 4.4: K function analysis of *E. coli* O26, O103 and O145-positive (cases) and *E. coli* O26, O103 and O145-negative (control) farms. The black line represents the difference between the two K functions and dashed lines represent the upper and lower simulation envelopes.



Key	Type	Source	stx1	genotype	eae	ehxA	FarmID	Region	PFGE cluster
EcCa76a	O26	bovine	1	20	1	1	645	Taranaki	A
EcCa77a	O26	bovine	1	20	1	1	635	Taranaki	A
EcCa87a	O26	bovine	1	20	1	1	698	Manawatu	A
EcCa74a	O26	bovine	1	20	1	1	644	Taranaki	A
EcCa106a	O26	bovine	1	20	1	1	739	Taranaki	A
EcCa107a	O26	bovine	1	20	1	1	739	Taranaki	A
EcCa108a	O26	bovine	1	20	1	1	740	Taranaki	A
EcCa69a	O26	bovine	1	20	1	1	622	Taranaki	A
EcCa92b	O26	bovine	1	20	1	1	703	Taranaki	A
EcCa101a	O26	bovine	1	20	1	1	734	Taranaki	A
EcCa102a	O26	bovine	1	20	1	1	732	Taranaki	A
EcCa105a	O26	bovine	1	20	1	1	638	Taranaki	A
EcCa64a	O26	bovine	1	20	1	1	580	Taranaki	A
EcCa71a	O26	bovine	1	20	1	1	625	Taranaki	A
EcCa67a	O26	bovine	1	20	1	1	246	Taranaki	A
EcCa68a	O26	bovine	1	20	1	1	614	Taranaki	A
EcCa85a	O26	bovine	1	20	1	1	650	Manawatu	A
EcCa103a	O26	bovine	1	20	1	1	735	Taranaki	A
EcCa104a	O26	bovine	1	20	1	1	254	Taranaki	A
EcCa100b	O26	bovine	1	20	1	1	733	Taranaki	A
EcCa97a	O26	bovine	1	20	1	1	709	Taranaki	A
EcCa66a	O26	bovine	1	20	1	1	591	Taranaki	A
EcCa80a	O26	bovine	1	20	1	1	655	Manawatu	A
EcCa94a	O26	bovine	1	20	1	1	593	Taranaki	A
EcCa110a	O26	bovine	0	9	1	1	760	Waikato	A
EcCa98a	O26	bovine	1	20	1	1	710	Taranaki	A
EcCa100a	O26	bovine	0	9	1	1	733	Taranaki	B
EcCa75a	O26	bovine	0	9	1	1	582	Taranaki	B
EcCa70a	O26	bovine	0	9	1	1	618	Taranaki	B
EcCa83a	O26	bovine	0	9	1	0	678	Taranaki	B
EcCa109a	O26	bovine	0	9	1	1	757	Waikato	B
EcCa65a	O26	bovine	0	9	1	1	582	Manawatu	B
EcCa79a	O26	bovine	0	9	1	1	653	Manawatu	B
EcCa82a	O26	bovine	0	9	1	1	678	Taranaki	B
EcCa89a	O26	bovine	0	9	1	1	700	Taranaki	B
EcCa92a	O26	bovine	0	9	1	1	703	Taranaki	B
EcCa93a	O26	bovine	0	9	1	1	684	Taranaki	B
EcCa95a	O26	bovine	0	9	1	1	704	Taranaki	B
EcCa84a	O26	bovine	0	9	1	1	682	Taranaki	B
EcCa90a	O26	bovine	0	9	1	1	598	Taranaki	B
EcCa72a	O26	bovine	0	9	1	1	638	Taranaki	B
EcCa99a	O26	bovine	0	9	1	1	607	Taranaki	B
EcCa86a	O26	bovine	0	9	1	1	690	Waikato	B
EcCa73a	O26	bovine	0	9	1	0	643	Taranaki	C
EcCa78a	O26	bovine	0	9	1	0	651	Manawatu	C
EcCa96a	O26	bovine	0	9	1	0	708	Taranaki	D
PFGE XbaI O2.									

Figure 4.5: Clustering (UPGMA dendrogram) of the PFGE profiles of *E. coli* O26 isolates recovered from bobby calves from the North Island of New Zealand with >70% similarity cut off using *XbaI*. The last lane is the *Salmonella* serotype Braenderup reference standard (H9812).

5 Chapter 5

Genotyping of *Escherichia coli* isolates using pulse field gel electrophoresis and allelic profiling (based on 8 genes) from young calves in the North Island of New Zealand

5.1 Abstract

Diarrhoeagenic *Escherichia coli* (*E. coli*) are a diverse group of enteric pathogens often differentiated by the presence or absence of specific virulence factors. *E. coli* isolates (n=137) obtained from recto-anal mucosal swab (chapters 3 and 4) and faecal samples (chapter 2) of apparently healthy calves in the North Island of New Zealand were genotyped using pulse field gel electrophoresis (PFGE) and allelic profiling (based on 8 genes). The subtypes of *eae* and *ehxA* genes of *E. coli* isolates were determined using PCR-restriction fragment length polymorphism (RFLP) analysis. Endonuclease digestion of *ehxA* PCR products with *TaqI* resulted in six *ehxA* subtypes (A-F) distinguished by different RFLP profiles upon electrophoresis. The most common subtype was C (79) followed by F (27), B (4) and A (4), and one isolate each was positive for subtypes D and E. The subtypes of five *ehxA* positive isolates could not be identified. Upon inoculation of the 137 isolates onto washed sheep blood agar plates, 82 showed a narrow zone of haemolysis characteristic of enterohaemolytic activity, 28 showed a broad zone of haemolysis characteristic of α-haemolysin (α-hly) and 27 isolates were non-haemolytic. Endonuclease digestion with *HhaI* or *HaeIII* enzymes permitted four *eae* subtypes to be identified that corresponded to subtypes δ/κ, β, ϵ and γ. Of the 129 *eae* positive isolates 82 were β followed by ϵ (34), γ (11) and δ/κ (2). All 74 O26 isolates were subtype β, all O103 (13 isolates) were subtype ϵ and all O157 (3 isolates) were subtype γ. However, for O145 isolates two different *eae* subtypes γ (7 isolates) and ϵ (7 isolates) were identified. *E. coli* isolates were also analysed for plasmid-associated alleles *espP*, *etpD*, *katP* and α-*hly*. Of 137 isolates 93 (67.8%) were positive for *espP*, 32 for *etpD* (23.3%), 76 for *katP* (55.4%) and nine for α-*hly* (6.5%). PERMANOVA and multidimensional scaling were used to observe the multivariate relationship between virulence gene allelic profiles (based on 8 genes) and PFGE restriction patterns of the *E. coli* isolates. RFLP *eae* and RFLP *ehxA* were the most important contributors to the variance in PFGE profiles and contributed to approximately 37% of the observed variation. The genotyping ability of allelic profiling was also compared with PFGE profiling using multidimensional scaling. This analysis indicated that isolates having similar allelic profiles had similar PFGE profiles and tended to cluster together. *E. coli* serogroups and *eae* and *ehxA* subtypes observed in these serogroups have also been observed from human *E. coli* isolates indicating the potential public health significance of these isolates. The results also showed that allelic profiling (based on 8 genes) may be used for genotyping of *E. coli* isolates and provide a similar pattern of genotype clustering to PFGE.

5.2 Introduction

Enteropathogenic *E. coli* (EPEC) and Shiga toxin-producing *E. coli* (STEC) are two important pathotypes of diarrhoeagenic *E. coli* (Nataro and Kaper, 1998). EPEC and STEC are commonly recovered from cattle, sheep, pigs and humans and are considered important causes of diarrhoea in humans (Aidar-Ugrinovich et al., 2007; Armstrong et al., 1996; Caprioli et al., 2005; Cookson et al., 2002; Janke et al., 1989; Trabulsi et al., 2002).

More than 400 serotypes of STEC have been isolated from cattle (Blanco et al., 2004c). However, *E. coli* O157, O26, O103, O111 and O145 are considered of clinical importance due to their association with haemorrhagic colitis (HC) and haemorrhagic uraemic syndrome (HUS) cases in humans (Johnson et al., 2006; Mainil and Daube, 2005). In addition to Shiga toxin (*stx*), STEC may also possess a number of other virulence factors including intimin (*eae*) and enterohaemolysin (*ehxA*) (Gyles, 2007; Paton et al., 1997). STEC carrying *eae* have been reported to be involved more frequently in severe diarrhoea and HUS cases (Karmali, 1989; Paton and Paton, 1998b).

Although large EPEC associated infantile diarrhoeal outbreaks of disease have almost disappeared from developed countries, these outbreaks are frequently reported from developing countries (Trabulsi et al., 2002). In EPEC pathogenesis, attachment and effacement (AE) lesion formation on the epithelial lining of gastro-intestinal tracts of ruminants (Dean-Nystrom et al., 1998; Wales et al., 2001) is mediated by a protein known as intimin encoded by *eae* gene. The *eae* gene is located on a pathogenecity island known as the locus of enterocyte effacement (LEE) (McDaniel and Kaper, 1997; McDaniel et al., 1995). LEE also produces Tir (translocated intimin receptor) which is inserted into the epithelial cell membrane and acts as a receptor for intimin (Kenny et al., 1997). Host cell cytoskeletal rearrangement is associated with AE lesions and cup like pedestal formation due to the accumulation of filamentous actin (Knutton et al., 1989; Moon et al., 1983).

The C-terminal end of intimin has a variable amino acid sequence and mediates attachment to the Tir. Based on DNA and amino acid sequence variation there are at least 20 intimin types and subtypes (Blanco et al., 2004c, 2006a,b; Garrido et al., 2006; Lacher et al., 2006). Some association between intimin types and *E. coli* serogroups has been reported. For example, serogroup O26 often possesses intimin type β, serogroup O103 intimin type θ or ϵ and serogroups O145 and O157 intimin type γ (Blanco et al., 2004c; Cookson et al., 2007a; Kozub-Witkowski et al., 2008; Posse et al., 2007). Various *eae* types may influence the host specificity and tissue tropism displayed by different STEC (Fitzhenry et al., 2002, 2003; Phillips and Frankel, 2000; Phillips et al., 2000). Therefore, knowledge of different types of intimin may be helpful in pathological, epidemiological and immunological studies (Blanco et al., 2006a).

Plasmid-associated enterohaemolysin, encoded by *ehxA* is another important STEC virulence marker (Beutin et al., 1988; Cookson et al., 2006a; Lehmacher et al., 1998; Schmidt et al., 1995) and in *E. coli* O157:H7, the *ehx* operon is located on a 94kb large plasmid termed as pO157 (Beutin et al., 1988) with a gene order *ehx*CABD. The *ehxA* locus has >60% similarity to the α-haemolysin (α-*hly*) gene of *E. coli* (Schmidt et al., 1996b). Enterohaemolysis of washed sheep erythrocytes by various STEC serotypes including O157:H7 and O111:H- has also been reported

(Beutin et al., 1989; Schmidt and Karch, 1996; Schmidt et al., 1995) however, some *ehxA*-positive serotypes including O157:H- and O111:H- do not show haemolytic activity on washed sheep blood agar (Eklund et al., 2006; Schmidt and Karch, 1996) which may indicate the differing culture conditions required for enterohaemolytic activity or contrasting regulation of this operon in these serotypes. Although the exact role of *ehxA* in STEC-associated disease is not known, the presence of *ehxA* in many STEC isolates from clinical cases (Beutin et al., 1989; Schmidt et al., 1995), the haemolysis of erythrocytes (Beutin et al., 1989; Schmidt and Karch, 1996; Schmidt et al., 1995), the detection of antibodies against enterohaemolysin from sera of HUS cases (Schmidt et al., 1995) and the isolation of non-STEC O157:H7- having *ehxA* from HUS cases (Schmidt et al., 1999b) all indicate the potential importance of *ehxA* in pathogenesis. Other important loci and/or potential virulence factors present on the large plasmid commonly found in STEC are a catalase peroxidase (*katP*) (Brunder et al., 1996), serine protease (*espP*) (Brunder et al., 1997) and a type II secretion pathway (*etp*) (Schmidt et al., 1997). These loci/virulence factors have been reported to be associated with STEC from human and bovine sources. Interestingly, many STEC-associated loci/virulence factors have also been identified in *stx*-negative *E. coli* isolated from humans and ruminant faeces (Brunder et al., 1999; Cookson et al., 2010). Many of these isolates are *eae*-positive and have been described therefore as EPEC. In contrast to EPEC associated with infantile diarrhoea, where initial attachment to cultured epithelial cells is mediated by bundle forming pili (*bfp*), these *stx*-negative *E. coli* from ruminants are *bfp*-negative. Therefore EPEC that possess *bfp* have been described as typical EPEC (tEPEC), and those *bfp*-negative isolates with STEC-associated characteristics more commonly isolated from ruminants, have been described as atypical EPEC (aEPEC) (Trabulsi et al., 2002).

This study was conducted to determine the occurance of *eae* and *ehxA* subtypes in *E. coli* isolates from calves, and to determine the association between *eae* and *ehxA* subtypes with various STEC and EPEC serogroups. Further the virulence characteristics (virulotyping) were compared with the pulse field gel electrophoresis (PFGE) patterns to examine the multivariate relationship between virulence gene allelic profiles (based on 8 genes) and the restriction pattern of the whole bacterial genome. The information provided by this study would be helpful in understanding the relationship of intimin and enterohaemolysin subtypes with EPEC and STEC serogroups and would provide valuable information on the characterisation of STEC and aEPEC from calves.

5.3 Materials and methods

E. coli isolates obtained from previous studies were used in this study. In total, 137 isolates were analysed of which 129 were *eae* positive and 121 were *ehxA* positive. Of these, 117 isolates were obtained from recto-anal mucosal swabs of slaughtered bobby calves from two slaughter plants of North Island of New Zealand and the remaining 20 isolates analysed in this study were obtained from the faecal samples of calves in a river catchment area of New Zealand. Most of these isolates were positive for *eae*, *ehxA* (69 isolates) followed by *stx1*, *eae*, *ehxA* (40 isolates), *eae* (16 isolates), *stx2*, *eae*, *ehxA* (4 isolates) and *ehxA* (8 isolates). All the *stx* positive isolates (44) were subjected to serogrouping/serotyping and the remaining isolates (93) were tested for O26, O103, O111, O145

and O157 using real time PCR (RT-PCR) (described in section 2.3.1).

All 121 isolates positive for *ehxA* gene were subtyped using *ehxA* PCR-RFLP. The *ehxA* gene (2,997-bp) was amplified using PCR with primers *ehxA*-RFLP-F (5′-ATGACAGTAAATAAAATAA AGAAC-3′) and *ehxA*-RFLP-R (5′-TCAGACAGTTGTCGTTAAAGTTG-3′) (Cookson et al., 2007b). The DNA was isolated by mixing a single colony in 2% Chelax solution and heating the suspension at 95 °C for 10 minutes. The lysed suspension was refrigerated for two minutes and centrifuged at 12000 g for two minutes. The supernatant containing the DNA was dispensed to another tube and amplified using Rotor Gene 6000 series thermal cycler (Bio Strategy, Auckland, New Zealand). Each PCR reaction contained 1 x reaction buffer (Invitrogen, Auckland, New Zealand), 0.1mM of each dNTP (Fermentas, Auckland, New Zealand), 0.15mM $MgCl_2$ (Invitrogen, Auckland, New Zealand), 1 unit of *Taq* DNA Polymerase (Invitrogen, Auckland, New Zealand), 2μl of template DNA and made to final volume of 20μl with sterile water. The concentration of primer in each reaction was 0.1μM. Rotor Gene 6000 series thermal cycler (Bio Strategy, Auckland, New Zealand) was programmed for initial denaturation step at 95 °C for 5 minutes, 40 cycles of 45 seconds at 95 °C, 45 seconds at 52 °C, 120 seconds at 72 °C and final extension step at 72 °C for 5 minutes. The presence of *ehxA* 2,997-bp product was confirmed by agarose (1% w/v) gel (Invitrogen, Auckland, New Zealand) electrophoresis and visualising using ethidium bromide (0.5μg/ml) under ultraviolet illumination. Each amplified *ehxA* positive product (4.5 μl) was digested with mixture of 10 x restriction enzyme buffer (0.55μl) and 5 units of *TaqI* restriction endonuclease (Invitrogen, Auckland, New Zealand). Restriction mixtures were incubated at 65 °C for 90 minutes. After incubation each digest mix was electrophoresed through agarose (2% w/v) gel (Invitrogen, Auckland, New Zealand) and visualised using ethidium bromide and ultraviolet illumination to confirm the presence of different banding patterns. The *ehxA* RFLP gel images were photographed using a Gel Doc imaging system (Bio-Rad, Milan, Italy) and saved as TIFF files. These images were then compared with the images published in the paper (Cookson et al., 2007b) corresponding to the subtypes A, B, C, D, E and F.

Sheep blood agar plates (Fort Richard, Auckland, New Zealand) were used to detect the presence of haemolytic activity of isolates. Isolates were inoculated onto sheep blood agar plates which were then incubated at 37 °C for 24 hours. After 24 hours plates were observed for the presence of zones of haemolysis around the colonies. The results were recorded as no haemolysis, narrow zone of haemolysis and broad zone of haemolysis depending upon the size of haemolytic zone.

The *eae* gene of *E. coli* isolates (129) was subtyped using *eae* PCR-RFLP. The *eae* gene amplicon of size 1,848-bp was amplified using primers RFLP-F (5′-TCRGATTCWAAACTRTTAACTCA-3′) and RFLP-R (5′-TAAWYTCRGTAATRGCYTKRSTTT-3′). The DNA was isolated from each isolate using the method described previously. The PCR mixture contained 1x reaction buffer (Invitrogen, Auckland, New Zealand), 0.1mM of each dNTP (Fermentas, Auckland, New Zealand), 0.15mM $MgCl_2$ (Invitrogen, Auckland, New Zealand), 1 unit of *Taq* DNA Polymerase (Invitrogen, Auckland, New Zealand), 2μl (0.2μM) of each primer, 2μl of template DNA and made to final volume of 20μl with sterile water. This PCR mixture was subjected to amplification of *eae* gene using Rotor Gene 6000 series thermal cycler (Bio Strategy, Auckland, New Zealand). It was

programmed for initial denaturation at 94 °C for 5 minutes, 40 cycles of 60 seconds at 94 °C, 60 seconds at 54 °C, 120 seconds at 72 °C and final extension step at 72 °C for 5 minutes. The presence of 1,848-bp *eae* gene amplicon was confirmed using agarose (1% w/v) gel (Invitrogen, Auckland, New Zealand) electrophoresis described previously. Each *eae* amplicon (17 μl) was divided into two parts and digested separately using two enzymes *HaeIII* and *HhaI* to give two distinct RFLP profiles. For digestion with *HaeIII*, each amplicon (8.5 μl) was mixed with *HaeIII* restriction buffer (1μl) and 5 units of *HaeIII* enzyme (0.5μl). Digestion mixtures were incubated at 37 °C for 2 hours. Similarly, with *HhaI* each amplicon (8.5 μl) was mixed with *HhaI* restriction buffer (1μl) and 5 units of *HhaI* enzyme (0.25μl). Digestion mixtures were incubated at 37 °C for 2 hours. After incubation each digestion mixture was electrophoresed, visualised, photographed and saved as described previously. The *eae* gene sequences were downloaded from GenBank, trimmed to match with the 1,848-bp *eae* PCR-RFLP amplicon and virtual gels were constructed using Vector NTI advance (v. 11, Invitrogen Corporation). Each DNA fragment was digested separately with *HaeIII* and *HhaI* and ran *in silico*.

The 1,848-bp *eae* amplicon identified as subtype α by PCR-RFLP was re-amplified; the PCR product was purified using ethanol precipitation and sent to Massey Genome Service, (Massey University, Palmerston North, New Zealand) for sequencing. Sequencing was performed using the BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, Auckland, NZ) and the primers (RFLP F and RFLP R) used originally to amplify the product and capillary separation of sequencing reactions was performed on an ABI3730 (Applied Biosystems). ABI trace files were trimmed using Contig Express and homologous sequences were identified using the Basic Local Alignment Search Tool (BLAST).

PCR was performed to detect the presence of *katP*, *espP*, *etpD* and α-*hly* genes in *E. coli* isolates. The primers, *katP*-F (5′-CTTCCTGTTCTGATTCTTCTGG-3′) and *katP*-R (5′-ACTTATTTCTCG CATCATCC-3′) were used to amplify 2,125-bp fragment of *katP* gene (Brunder et al., 1996). For amplification of *espP* gene (amplicon size=1,830-bp) *espP*-F (5′-AAACAGCAGGCACTTGAACG-3′) and *espP*-R (5′-GGAGTCGTCAGTCAGTAGAT-3′) primers were used (Brunder et al., 1999). Each amplified *espP* product was digested with *AluI* enzyme and digested product was electrophoresed through agarose (2% w/v) gel (Invitrogen, Auckland, New Zealand) and visualised using ethidium bromide staining and ultraviolet illumination to confirm the presence of different banding patterns. The primers *etpD*-F (5′-CGTCAGGAGGATGTTCAG-3′) and *etpD*-R (5′-CGACTGCACCTGTTCCTGATTA-3′) were used to amplify the 1,061-bp fragment of *etpD* gene (Cookson et al., 2002). The α-*hly* gene amplicon of size 542-bp was amplified using α-*hly*-F (5′-GTCTGCAAAGCAATCCGCTGCAAATAAA-3′) and α-*hly*-R (5′-CTGTGTCCACGAGTTGGT TGATTAG-3′) (Mueller et al., 2009). The amplification was carried out using a Rotor Gene 6000 series thermal cycler (Bio Strategy, Auckland, New Zealand). It was programmed for initial denaturation step at 96 °C for 2 minutes, 30 cycles of 45 seconds at 96 °C, 45 seconds at 56 °C, 120 seconds at 72 °C and final extension step at 72 °C for 10 minutes. The presence of α-*hly* 542-bp product was confirmed by agarose (1% w/v) gel (Invitrogen, Auckland, New Zealand) electrophoresis and visualising using ethidium bromide staining (0.5μg/ml) under ultraviolet illumination.

All isolates containing *eae* and/or *ehxA* genes were genotyped using PFGE to determine the genetic relatedness of isolates. PFGE was performed following standard protocol described by PulseNet USA (Centres for Disease Control and Prevention, 2009). Briefly, a bacterial suspension was made by mixing few colonies of an isolate with cell suspension buffer (100mM tris: 100mM EDTA, pH 8.0) and the concentration of the bacterial suspension was adjusted between 0.40-0.45 using a Dade Microscan Turbidity meter. The plugs were prepared by mixing each bacterial suspension with proteinase K (25μl of 20mg/ml) (Total Lab System, Auckland, New Zealand) and 400μl of SeaKem Gold agarose (1% w/v) gel (Invitrogen, Auckland, New Zealand) in Tris/EDTA buffer (10mM tris: 1mM EDTA, pH 8.0) and dispensing this mixture into block formers. These plugs were lysed in mixture of cell lysis buffer (50mM tris: 50mM EDTA, pH 8.0 + 1% Sarcosyl) (5ml) and proteinase K (25μl of 20mg/ml) (Total Lab System, Auckland, New Zealand) at 56 °C for 2 hours in a shaking water bath. These plugs were washed twice with ultrapure distilled water and three times with Tris/EDTA buffer (10mM tris: 1mM EDTA, pH 8.0). Each washing was for 15 minutes at 56 °C in a shaking water bath. After washing, digestion of the plugs was carried out with 5μl of *XbaI* (10 units/μl) (Roche Diagnostics, Auckland, New Zealand) for 2 hours at 37 °C in a heating block. The plugs were then loaded into SeaKem Gold agarose (1% w/v) gel (Invitrogen, Auckland, New Zealand) in tris borate EDTA buffer (TBE). Electrophoresis was carried out with CHEF DR II system (Bio-Rad Laboratories, Auckland, New Zealand) at 14 °C in 0.5 x TBE, with plus ramp of initially 6.8 seconds and finally 35.4 seconds, for 20 hours at constant voltage of 6 V/cm. After electrophoresis gel was stained in ethidium bromide (0.5μg/ml) for 20 minutes and visualised under short wave length ultraviolet light. The PFGE gel images were photographed using a Gel Doc imaging system (Bio-Rad, Milan, Italy) and saved as TIFF files. These images were added to the database and dendrogram of the PFGE profiles was created using Bionumric version 6.6 (www.applied-maths.com). Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Dice coefficient with >70% similarity cut off were used to construct the clusters of *E. coli* isolates with one or more virulence genes.

Hierarchical clustering was performed using software R (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria) with the function "hclust" and the distance matrix calculated by custom R script to group isolates into various clusters on the basis of results obtained from eight PCR (-RFLP) analysis: *stx1*, *stx2*, *eae*, *ehxA*, *espP*, *etpD*, *katP* and α-*hly*. The similarity between isolates was calculated by using the method described by Xu and Wunsch II (2009).

$$a\ (a_i, a_j) = N\ (\text{matches})/[N\ (\text{matches}) + w\ .\ N\ (\text{mismatches})]$$

Where,

N (matches) = Number of similarities between isolate *i* and isolate *j*

w = weight applied to the number of dissimilarities (Dice co-efficient weighting factor=0.5) (Dice, 1945)

The distance metric was then calculated using the equation

d = 1-a (a_i, a_j)

For example the similarity score of two isolates having N (matches) = 6 and N (mismatches) = 2 would be 0.85 and the distance score would be 0.15. Similarly, two exactly similar isolates would have a similarity score of 1.

We also examined the relationship between PFGE profile and virulence gene allelic profile (virulotype) using multivariate methods including multidimensional scaling (MDS) and permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001; Anderson et al., 2008). PERMANOVA is an analogue to ANOVA and is used in this study to associate variation in PFGE profiles and virulotype. We tested the hypothesis that the clustering of isolates observed with PFGE could be explained by virulotype, and estimated the proportion of variance attributable to each virulence gene assay.

$$\text{PFGE} \sim \beta_1 stx1 + \beta_2 stx2 + \beta_3 \text{RFLP}\, ehxA + \beta_4 \text{RFLP}\, eae + \beta_5 etpD + \beta_6 espP + \beta_7 katP + \beta_8 \text{serogroup}.. \quad (1)$$

This hypothesis was tested using software PRIMER 6 and PERMANOVA+ (PRIMER-E Ltd, Plymouth, UK) and package PERMANOVA (Anderson, 2001; Anderson et al., 2008). PRIMER 6 and PERMANOVA+ (PRIMER-E Ltd, Plymouth, UK) was also used to create MDS plot (Clarke and Warwick, 2001) of PFGE profiles and allelic profiles of isolates. MDS is a mathematical procedure of representing the information contained in a data set as points in space. It detects useful underlying dimensions that could explain observed similarities and dissimilarities between the variables under investigation and gives visual representation of the similarities or distances among set of variables on the plot based on a criterion function known as stress. Stress gives the magnitude of the lack of correspondence between the distance among points indicated by MDS plot and the input matrix. MDS plots the variables in such a way that the variables similar to each other are plotted close to each other and variables different from each other are plotted away from each other on the plot.

5.4 Results

The isolates positive for *stx*, *eae*, *ehxA* (44); *eae*, *ehxA* (69) and *ehxA* (8) were analysed by *ehxA* PCR-RFLP to identify various subtypes of *ehxA*. Restriction of *ehxA* PCR products with *TaqI* resulted in six *ehxA* PCR-RFLP subtypes A, B, C, D, E and F. The most common subtype was C (79) followed by F (27), B (4) and A (4) whereas one isolate each was positive for subtype D and E. The subtypes of five *ehxA* positive isolates initially identified using internal PCR primers (Paton and Paton, 1998a) could not be identified as the PCR primers for the amplification of the full length *ehxA* were not specific for amplification under these conditions. This may be due to sequence variation of the *ehxA* gene of these strains at the primer binding site or the absence of a full length gene. The most common serotype was O26 (64 isolates) followed by O145 (14 isolates), O103 (13 isolates each) and O157 (3 isolates). An association between *ehxA* subtype and *E. coli* serogroup was also observed. All the O26 isolates contained *ehxA* subtype C, all O103

isolates contained *ehxA* subtype F and all O157 isolates contained *ehxA* subtype B. However, O145 isolates contained *ehxA* subtype C (7 siolates) and F (7 isolates) (Table 5.1).

Of 121 *ehxA* positive isolates 82 showed a narrow zone of haemolysis, 19 showed broad zone of haemolysis and 20 isolates were non-haemolytic. The most common *ehxA* subtype associated with haemolysis was subtype F (26/27; 96.2%) followed by subtype C (72/79; 91.1%). However, whilst 19 of 27 (70.3%) of *ehxA* subtype F isolates showed a broad zone of haemolysis, 7 (25.9%) showed a narrow zone of haemolysis and one *ehxA* F subtype isolate was non-haemolytic (Table 5.2). The O26 isolates also had three characteristic phenotypes, i.e. non-haemolytic, broad/narrow zones of haemolysis (Table 5.3). All O103 isolates (13) showed broad zone of haemolysis, while 6/14 (42.8%) O145 isolates showed broad zone of haemolysis, 7/14 (50%) produces a narrow zone of haemolysis and one isolate was non-haemolytic on sheep blood agar plates. Similarly, the three O157 isolates were non-haemolytic on sheep blood agar plates (Table 5.3). All nine *E. coli* O26 isolates showing broad zone of haemolysis were positive for α-*hly* gene, lacked the *ehxA* gene and were rhamnose fermenting. In contrast the other isolates (O103 and O145) showing a broad zone of haemolysis lacked the α-*hly* gene but always contained *ehxA* subtype F and *eae* subtype ϵ.

A total of 129 isolates positive for *eae* gene obtained from calves were analysed by *eae* PCR-RFLP to identify various *eae* subtypes. A total of four profiles of *eae* subtypes (δ/κ, β, ϵ and γ) were observed from *E. coli* isolates. All *eae* restriction profiles exactly matched with *eae* PCR-RFLP profiles produced *in silico* from GenBank *eae* sequences except five isolates which gave slightly different profile with *HaeIII* enzyme but exactly same profile with *HhaI* enzyme (profile similar to *eae* subtype ϵ). Of the 129 *eae* positive isolates, 82 were the β subtype, 34 ϵ, 11 γ and two δ/κ. Most of the *stx*, *eae*, *ehxA* isolates were β (41/44) and γ (3/44) whereas most of the aEPEC (*eae, ehxA*, positive or *eae* positive isolates) were β subtype (41/85) and ϵ subtype (34/85) (Table 5.4). An association between *eae* subtype and *E. coli* serogroup was also observed; for example all the 74 O26 isolates including 39 STEC were *eae* subtype β. Similarly all O103 (13 isolates) were *eae* subtype ϵ and all O157 (3 isolates) were *eae* subtype γ. However, two different *eae* subtypes, γ (7 isolates) and ϵ (7 isolates) were associated with the O145 isolates identified in this study (Table 5.5). Two *eae* subtypes were obtained from the same RAMS from 11 different calves. For example, one isolate positive for *eae* subtype ϵ and another positive for *eae* subtype β were obtained from the same animal on five different occasions. Similarly, isolates positive for *eae* subtype δ/κ and for *eae* subtype ϵ, and subtypes β and γ were obtained from the same animal on one and three occasions respectively. On two occasions, two different *E. coli* isolates belonging to two different serogroups, O103 and O145 but with the same *eae* subtype (ϵ) were obtained from a single calf.

E. coli isolates (137) were also analysed for *espP*, *etpD*, *katP* and α-*hly* genes by PCR. In total, 93 (67.8%) isolates were positive for *espP*, 32 (23.3%) for *etpD*, 76 (55.4%) for *katP* and nine (6.5%) for α-*hly* gene. Further analysis of *espP* amplicons by RFLP resulted in four *espP* subtypes (A, B, D and E). The most commonly found plasmid-associated gene in STEC isolates was *ehxA* (44/44; 100%) followed by *espP* (43/44; 97.7%), *katP* (42/44; 95.4%) and *etpD* (4/44; 9%). Similar findings were observed for non-STEC isolates where the most commonly

found plasmid-associated gene was *ehxA* (77/93; 82.7%) followed by *espP* (50/93; 53.7%), *katP* (33/93; 35.4%) and *etpD* (28/93; 30.1%). Various combinations (9) of plasmid-associated genes (*ehxA*, *espP*, *etpD* and *katP*) were observed in *E. coli* isolates. *E. coli* O26 isolates showed four combinations with the most common combination being *ehxA*+, *espP*+, *etpD*- and *katP*+ (62/74; 83.7%), for O103 isolates the most common combination was *ehxA*+, *espP*-, *etpD*+ and *katP*- (13/13; 100%) whereas 50% of O145 isolates had *ehxA*+, *espP*+, *etpD*-, *katP*+ and the other 50 % had *ehxA*+, *espP*-, *etpD*+, *katP*- combination of plasmid-associated genes. The combination of plasmid-associated genes in all three O157 isolates was *ehxA*+, *espP*+, *etpD*+, *katP*+. Isolates from unknown serogroups showed various combinations of plasmid-associated genes (Table 5.6).

All isolates could be divided into 23 clusters (A-W) on the basis of PFGE using a cut off of $>$70% similarity (Figure 5.1). All STEC O26 isolates grouped together in cluster A (28) and C (11) whereas all non-STEC O26 isolates grouped in cluster A (1), B (25), H (3) and I (1). It is important to note that all STEC O26 isolates except one from the river catchment study (chapter 2) grouped in cluster C whereas all STEC O26 isolates from bobby calves study (chapters 3 and 4) grouped in cluster A. The single STEC isolate (O68:H24) was present in cluster I with a single non-STEC O26 isolate. O103 isolates were present in three different clusters L (1), P (2) and T (10 of 24). All O145 isolates were present in cluster D (7 of 8), K (1) and T (6 of 24). O157 isolates (3) grouped differently from all other isolates in cluster M. The remaining isolates of unknown serogroup grouped in cluster D (1 isolate), E (6 isolates), F (1 isolate), J (2 isolates), N (2 isolates), O (3 isolates), Q (3 isolates), R (1 isolate), S (1 isolate), T (8 isolates), U (1 isolate), V (1 isolate) and W (1 isolate).

On the basis of hierarchical clustering *E. coli* isolates could be divided into 22 groups and seven clusters as shown in the Figure 5.2. All the isolates (63 isolates) in cluster 1 were *E. coli* O26. The isolates in cluster 2 (15) belonged to serogroups O145 (7), O26 (1) and the serogroup of seven isolates was not known. Eight isolates of unknown serogroup clustered in cluster 3. The serogroup of two of three isolates in cluster 4 was unknown and the remaining one isolate was an O71:HR serotype. Most of the isolates (10 of 16 isolates) in cluster 5 were O26 positive with the serogroup of the remaining six isolates unknown. There were 27 isolates in cluster 6 of various serogroups, for example, 13 isolates were O103 positive, seven isolates were O145 positive and the serogroup of the remaining seven isolates was unknown. The five isolates in cluster 7 belonged to serogroup O68 (1 isolate), O157 (3 isolates) and the serogroup of the one isolate was unknown.

The model mentioned in equation (1) was run using all the variables (*stx1*, *stx2*, RFLP *ehxA*, RFLP *eae*, *etpD*, *espP*, *katP* and serogroup). The results of the model indicated that *etpD* and *stx2* contributed no information over and above that explained by the other variables. The model was also run by first removing the variable serogroup and then removing *etpD* and *stx2* and adding variable serogroup. The results of these models are shown in Tables 5.7 and 5.8. These data indicate that the variable serogroup provided all the information about differences in PFGE profile that could be attributed in the univariate analysis to *stx2* and *etpD*. Therefore, either serogroup or *stx2*/*etpD* were included in subsequent model. In addition Table 5.8 also showed that with serogroup in the model *katP* is no longer a significant contributor to the variation seen in PFGE

profiles. However, with *stx2*/and *etpD* in the model *katP* is still useful. The most important contributors to the variance in PFGE profiles were RFLP *eae* and RFLP *ehxA* and their total contribution towards the variance in PFGE profiles was approximately 37%. Similar results were observed when the previously mentioned models were run with *E. coli* isolates from bobby calves study (chapters 3 and 4) and excluding isolates obtained from calves in the river catchment (chapter 2). The results of these models are shown in appendix-2 (Tables 7.1, 7.2 and 7.3). The MDS plot of distance matrix of PFGE and allelic profile also showed that isolates having similar allelic profiles had similar PFGE profiles and they tend to cluster together (Figure 5.3). For example all STEC O26 isolates clustered together in MDS plot as did the PFGE profiles of STEC O26. Similarly, most of the O103 and O145 isolates clustered together in MDS plot. Similar clustering was observed for O103 and O145 isolates with PFGE analysis.

5.5 Discussion

EPEC and STEC are considered important food-borne pathogens causing gastro-enteritis in humans (Bettelheim, 2007; Karmali et al., 2010; Trabulsi et al., 2002). EPEC contains *eae* and can cause attachment and effacement lesions but lack *stx* (Hornitzky et al., 2005). EPEC, isolated from ruminants mostly lack bundle forming pilli (*bfp*) and are considered as aEPEC (Kaper, 1996; Trabulsi et al., 2002). Isolation of aEPEC from diarrhoeal cases is relatively higher than that of tEPEC in industrialised countries (Nguyen et al., 2005; Ratchtrachenchai et al., 2004; Rodrigues et al., 2004) but still the role of aEPEC in causation of diarrhoea is not well defined as it has been isolated with similar frequency from healthy and diarrhoeal infants. For example a Swiss study reported no difference ($P>0.05$) in the rate of isolation of aEPEC from children under age of 16 years having diarrhoea (30/187; 16%) and those having no diarrhoea (15/137; 11%) (Pabst et al., 2003). STEC without *eae* have been isolated from HUS cases (Bielaszewska et al., 2011) but strong associations between STEC carrying *eae* and HUS have been reported (Beutin et al., 1994; Eklund et al., 2001). This variation in disease sequelae (diarrhoea or severe illness) of aEPEC and STEC may be associated with specific serogroups, the presence of various *eae* subtypes or other virulence factors. Therefore, knowledge of various *E. coli* serogroups and *eae* subtypes in addition to other virulence genes is essential.

The analysis of 137 *eae* positive isolates by PCR-RFLP resulted in four *eae* subtypes in this study. These results are in contrast to another study conducted in the lower North Island of New Zealand (Cookson et al., 2007a) in which 139 *E. coli* isolates obtained from cattle and sheep were analysed and 11 different *eae* subtypes were observed. However, most of the isolates (78) analysed in this study are from two previous studies which were specifically designed for isolation of O26, O103, O111, O145 and O157 supplemented with 59 isolates obtained by randomly selecting *E. coli* colonies using two different media CT-SMAC (47) and TBX (12). Furthermore, isolates analysed in this study were obtained exclusively from calves, whereas isolates analysed by Cookson et al. (2007a) were from cattle and sheep of different ages. The poorly developed gastro-intestinal tract (GIT) of calves fed a milk-replacer diet may not provide suitable conditions for colonisation of some *eae* types. In this study the most common *eae* subtype was β and is in agreement with

another study from the lower North Island of New Zealand where the most common *eae* subtype observed from cattle and sheep *E. coli* isolates was also β (33/139; 23.7%) (Cookson et al., 2007a). Previous studies investigating virulence determinants of STEC O26 isolates from human cases have shown association between the severity of human disease and the virulence profile *stx1*, *eae*, *ehxA* (Schmidt et al., 1999a; Zhang et al., 2000b) found in all the STEC O26 isolates of this study.

Initially the *eae* subtype of two isolates was identified as α by PCR-RFLP. This subtype has rarely been isolated from ruminants (Ramachandran et al., 2003) therefore the *eae* gene of these two isolates was sequenced to confirm the presence of this rare *eae* subtype. Upon analysis however, the sequence matched with *eae* subtype δ/κ. This is an example of the limitations of PCR-RFLP despite being a cheap and quick method of subtyping, sequencing of PCR product provides the best method to differentiate between various *eae* subtypes.

An association between *eae* subtype and *E. coli* serogroup was observed in this study. All isolates of *eae* subtype β were associated with serogroup O26 whereas *eae* subtypes ϵ and γ were associated with serogroup O103 and O157 respectively. However, two different *eae* subtypes (ϵ and γ) were associated with serogroup O145. Other studies have also reported the association between *eae* subtype and *E. coli* serogroup. For example *eae* subtype β is commonly associated with serogroup O26, *eae* subtype ϵ or θ with serogroup O103 and *eae* subtype γ with serogroups O145 and O157 (Blanco et al., 2004a; Cookson et al., 2007a; Kozub-Witkowski et al., 2008; Posse et al., 2007). These serogroups (O26, O103, O145 and O157) are of great public health significance due to their association with disease in humans (Johnson et al., 2006; Mainil and Daube, 2005). The virulence of *E. coli* isolates from these serogroups, in addition to *stx*, may also be due to AE lesion formation associated with specific *eae* subtypes. Previous studies have reported that various *eae* subtypes may influence host specificity and tissue tropism shown by different *E. coli* serogroups (Fitzhenry et al., 2002, 2003; Phillips and Frankel, 2000; Phillips et al., 2000). For example, *eae* subtype α is specifically expressed by tEPEC and it forms AE lesions on small intestine and follicle associated epithelium (FAE) whereas *eae* subtype γ attaches to FAE only (Phillips et al., 2000) and preferred site for AE lesion formation is recto-anal mucosal junction (Naylor et al., 2003). Similar to *eae* subtype γ, *eae* subtype ϵ attaches to FAE (Fitzhenry et al., 2003).

Enterohaemolysin is considered an important virulence marker due to its association with HUS and HC cases in humans. However, the exact role of *ehxA* in human disease mechanism and bacterial colonisation of ruminants remains to be fully elucidated (Beutin et al., 1988; Dopfer et al., 2012; Lehmacher et al., 1998). aEPEC are considered genetically closely related to STEC and can become STEC by acquiring *stx*-encoding bacteriophage (Bielaszewska et al., 2008; Hernandes et al., 2009; Mellmann et al., 2005; Whittam et al., 1993). Therefore, further information is required to understand the role of *ehxA* in disease mechanism in humans and also to understand the role of *ehxA* in the colonisation of cattle by different serotypes of *E. coli*. In this study six *ehxA* subtypes were identified using PCR-RFLP and the most common *ehxA* subtypes were C and F (106/121; 87.6%). Cookson et al. (2007b) also reported six *ehxA* subtypes by analysing 251 *E. coli* isolates from cattle, sheep and humans using PCR-RFLP. However, in contrast to this study the most common *ehxA* subtypes were A and C (230/251; 91.6%). This difference may be due

to the reason that isolates analysed by Cookson et al. (2007b) were from three different sources (cattle and sheep of different ages and humans) whereas isolates analysed in this study were from calves only. More scarcely identified subtypes in this study were D and E (1 isolate each). This finding is in agreement with a previous study where 5/251 (1.9%) isolates were subtyped as D and E (Cookson et al., 2007b). Further *ehxA* subtyping studies are required to confirm these findings. An association between *ehxA* subtype and *E. coli* serotype was observed in this study where for example, all *E. coli* O26 isolates whether STEC or aEPEC were identified as subtype C. Similarly, all O103 and O157 isolates were subtyped as F and B respectively. However, O145 isolates were subtyped as C and F. Cookson et al. (2007b) also reported the similar association between *E. coli* serotypes (O26, O103 and O157) and *ehxA* subtypes (C, F and B) but in contrast to this study all *E. coli* O145 isolates belong to subtype C.

STEC serotypes positive for *ehxA* have been isolated from diarrhoeic and healthy calves (Aidar-Ugrinovich et al., 2007). Therefore, the role of *ehxA* positive STEC isolates in the causation of diarrhoea in calves is not known. In this study samples were taken from apparently healthy calves after slaughter therefore, no conclusion can be made about the association of isolation of *ehxA* positive STEC and diarrhoea. The seven strains from this study were identified as *ehxA*-positive using primer sequences previously described by Paton and Paton (1998a) were *eae* negative. However, the full length *ehxA* gene of five such isolates could not be amplified using the primers described in this study. This indicates that there may be further *ehxA* subtypes in addition to the six subtypes identified both in this study and that of Cookson et al. (2007b). Other studies have also reported the isolation of *E. coli* isolates only positive for *ehxA* (Boczek et al., 2006; Hornitzky et al., 2005). However, in the previous study of *E. coli* isolates from New Zealand cattle, sheep and humans only *eae* and/or *stx* positive isolates were examined for the presence of *ehxA* (Cookson et al., 2007b). Boczek et al. (2006) isolated 338 *E. coli* isolates from effluent of seven different waste water treatment plants located in different geographical areas. Of 338 isolates 336 were positive for *ehxA* gene only. These findings may indicate that *E. coli* isolates only positive for *ehxA* gene can survive better in waste water and that *ehxA* gene may be helpful in survival of *E. coli* isolates outside gastrointestinal tract. However, the exact role of *ehxA* in survival of *E. coli* isolates in waste water is not known.

In this study plasmid-associated genes were detected in STEC and aEPEC isolates in various combinations. Similar findings were reported by Brunder et al. (1999). They analysed 156 *E. coli* isolates obtained from human stool, bovine faeces, meat and raw milk for the presence of *ehxA*, *espP* and *katP* and identified seven combinations of plasmid-associated genes. The most commonly found plasmid-associated gene of STEC and aEPEC in this study was *ehxA*, followed by *espP*, *katP* and *etpD*. A previous study that included the analysis of 139 aEPEC isolates from RAMS of cattle and sheep of different ages detected *ehxA* (57.8%), *etpD* (53.5%) and *espP* (45.6%), but in contrast to this study *katP* was not identified from any of the 139 aEPEC isolates. The overall contrast in *katP* associated with aEPEC in this study and that of Cookson et al. (2010) is the targeted methodology (RT-PCR and immuno-magnetic separation) for isolation of O26 and O145 and the numbers of *katP* positive aEPEC identified as a result of these serogroup specific methods.

Collectively *E. coli* O26 and O145 isolates displayed three different haemolytic phenotypes (narrow or broad zone of haemolysis or non-haemolytic). In contrast other serogroups were associated with a single haemolytic phenotype. For example, serogroup O103 strains displayed only a broad zone of haemolysis, and the three O157 isolates were non-haemolytic on washed sheep blood agar plates. The α-*hly*-positive strains were associated with broad zone of haemolysis obtained on sheep blood agar. O26 strains that were α-*hly*-positive were also rhamnose fermenting strains, in contrast to those that were *stx*-positive or had STEC plasmid-associated virulence factors that were non-rhamnose fermenting indicating at least two separate O26 clades/pathotypes. O103 and O145 strains that were *ehxA* subtype F also displayed this phenotype, but were confirmed as α-*hly* negative. This could be due to the reason that various *E. coli* serotypes require different culture conditions for expression of haemolytic activity (Eklund et al., 2006; Schmidt and Karch, 1996).

In this study allelic profiling and PFGE were used to determine the diversity of *E. coli* isolates from calves. PERMANOVA analysis and an MDS plot of PFGE profiles and allelic profiles indicated that both methods showed similar diversity among *E. coli* isolates and there was considerable concordance between them. PFGE has been used extensively to study the diversity of *E. coli* isolates (Akiba et al., 2000a; Leotta et al., 2008) and is the basis for the PulseNet system that aims to provide rapid molecular epidemiological data for national or international disease outbreaks. *E. coli* O157 isolates (n = 73) from human patients in Argentina, Australia and New Zealand have been analysed by PFGE (Leotta et al., 2008). There were 46 different patterns by *XbaI* PFGE and no common *XbaI* PFGE pattern was observed in the isolates recovered from three countries. However, it is a time consuming, expensive and laborious method providing only the genomic profile of the isolates and does not give any information regarding specific genes or allelic variation. In contrast allelic profiling is less time consuming, easy to standardise and gives information about the presence or absence of specific alleles. Allelic profiling in this study was used to determine the diversity of the isolates on the basis of allelic differences. However, there are also some limitation associated with allelic profiling. For example, analysis of PFGE profiles indicated that STEC O26 isolates from two different geographical areas grouped in two different clusters; cluster A (isolates from bobby calves study) and cluster C (isolates from catchment study) whereas allelic profiling grouped all the STEC O26 isolates in one cluster irrespective of their geographical location.

E. coli serogroups and *eae* and *ehxA* subtypes observed in these serogroups have also been observed from human *E. coli* isolates indicating the public health significance of these isolates. However, these are the results of the small scale study and further studies are required from bovine and human *E. coli* isolates for better understanding the role of various *eae* and *ehxA* subtypes in virulence of *E. coli* isolates.

Table 5.1: Relationship between serogroups and *ehxA* subtypes in *E. coli* (n=121) isolates recovered from calves in the North Island of New Zealand.

Serogroup/Serotype	A	B	C	D	E	F	Untypable	Total
O26	0	0	64	0	0	0	0	64
O103	0	0	0	0	0	13	0	13
O145	0	0	7	0	0	7	0	14
O157	0	3	0	0	0	0	0	3
O68:H24	0	0	1	0	0	0	0	1
O71:HR	0	0	0	0	0	1	0	1
Unknown	4	1	7	1	1	6	5	25
Total	4	4	79	1	1	27	5	121

Table 5.2: Relationship between *ehxA* subtypes and zone of haemolysis on sheep blood agar plates in *E. coli* (n=121) isolates recovered from calves in the North Island of New Zealand.

ehxA subtype	No haemolysis	Narrow zone of haemolysis	Broad zone of haemolysis	Total
A	3	1	0	4
B	3	1	0	4
C	7	72	0	79
D	1	0	0	1
E	0	1	0	1
F	1	7	19	27
Untypeable	5	0	0	5
Total	20	82	19	121

Table 5.3: Relationship between serogroups and zone of haemolysis on sheep blood agar plates in *E. coli* (n=137) isolates recovered from calves in the North Island of New Zealand.

Serogroup/ Serotype	No haemolysis	Narrow zone of haemolysis	Broad zone of haemolysis	Total
O26	1	64	9	74
O103	0	0	13	13
O145	1	7	6	14
O157	3	0	0	3
O68:H24	0	1	0	1
O71:HR	1	0	0	1
Unknown	21	10	0	31
Total	27	82	28	137

Table 5.4: Relationship between *eae* subtypes and virulence profile of *E. coli* (n=129) isolates recovered from calves in the North Island of New Zealand.

eae PCR-RFLP subtype	Virulence profiles			Total
	(stx1, stx2, eae, ehxA)	*(eae, ehxA)*	*(eae)*	
δ/κ	0	1	1	2
β	41	29	12	82
γ	3	8	0	11
ϵ	0	31	3	34
Total	44	69	16	129

Table 5.5: Relationship between serogroups and *eae* subtypes in *E. coli* (n=129) isolates recovered from calves in the North Island of New Zealand.

Serogroup/Serotype	δ/κ	β	γ	ϵ	Total
O26	0	74	0	0	74
O103	0	0	0	13	13
O145	0	0	7	7	14
O157	0	0	3	0	3
O68:H24	0	1	0	0	1
O71:HR	0	1	0	0	1
Unknown	2	6	1	14	23
Total	2	82	11	34	129

Table 5.6: The relationship between various combinations of plasmid-associated genes *(ehxA, espP, etpD and katP)* and serotypes of *E. coli* isolates (n=137) recovered from bobby calves in the North Island of New Zealand.

Genotypes	Serogroups						
	O26	O103	O145	O157	O68:H24	O71:HR	Unknown
ehxA+, espP+,etpD-, katP+	62	0	7	0	0	0	1
ehxA+, espP-,etpD-, katP+	1	0	0	0	0	0	0
ehxA+, espP+,etpD-, katP-	1	0	0	0	0	0	11
ehxA-, espP-,etpD-, katP-	10	0	0	0	0	0	2
ehxA+, espP-,etpD+, katP-	0	13	7	0	0	0	6
ehxA+, espP+,etpD+, katP-	0	0	0	0	0	0	1
ehxA-, espP+,etpD-, katP-	0	0	0	0	0	0	4
ehxA+, espP-,etpD-, katP-	0	0	0	0	0	0	5
ehxA+, espP+,etpD+, katP+	0	0	0	3	1	0	1
Total	74	13	14	3	1	1	31

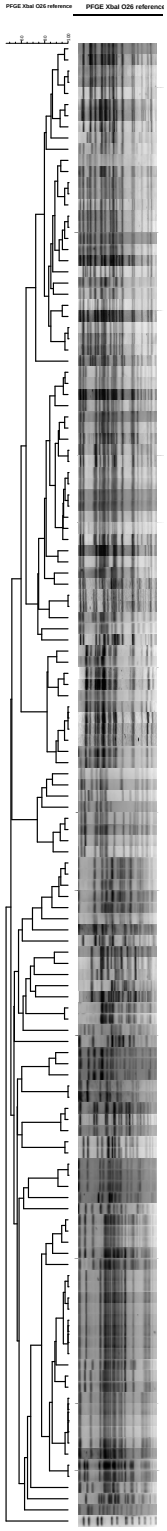
(+) = gene present
(-) = gene absent

Table 5.7: PERMANOVA analysis results indicating the contribution of allelic profile (8 genes) in the variation of PFGE profiles of *E. coli* isolates obtained from calves in the North Island of New Zealand. Variable serogroup was not included in the model to assess the contribution of *stx2* and *etpD* towards the model.

Variables	Degree of freedom	Mean square	P-value	Perms	Estimated component of variation (%)
stx1	1	13956	0.001	998	9.8
RFLP *ehxA*	6	1656.1	0.001	999	13
RFLP *eae*	4	3581.5	0.001	997	10.6
espP	3	1042.6	0.008	998	7.4
katP	1	4501.5	0.001	999	13.1
etpD	1	2777.2	0.001	999	20.3
stx2	1	2705	0.001	999	16.7
Residuals	113	369.8			8.6

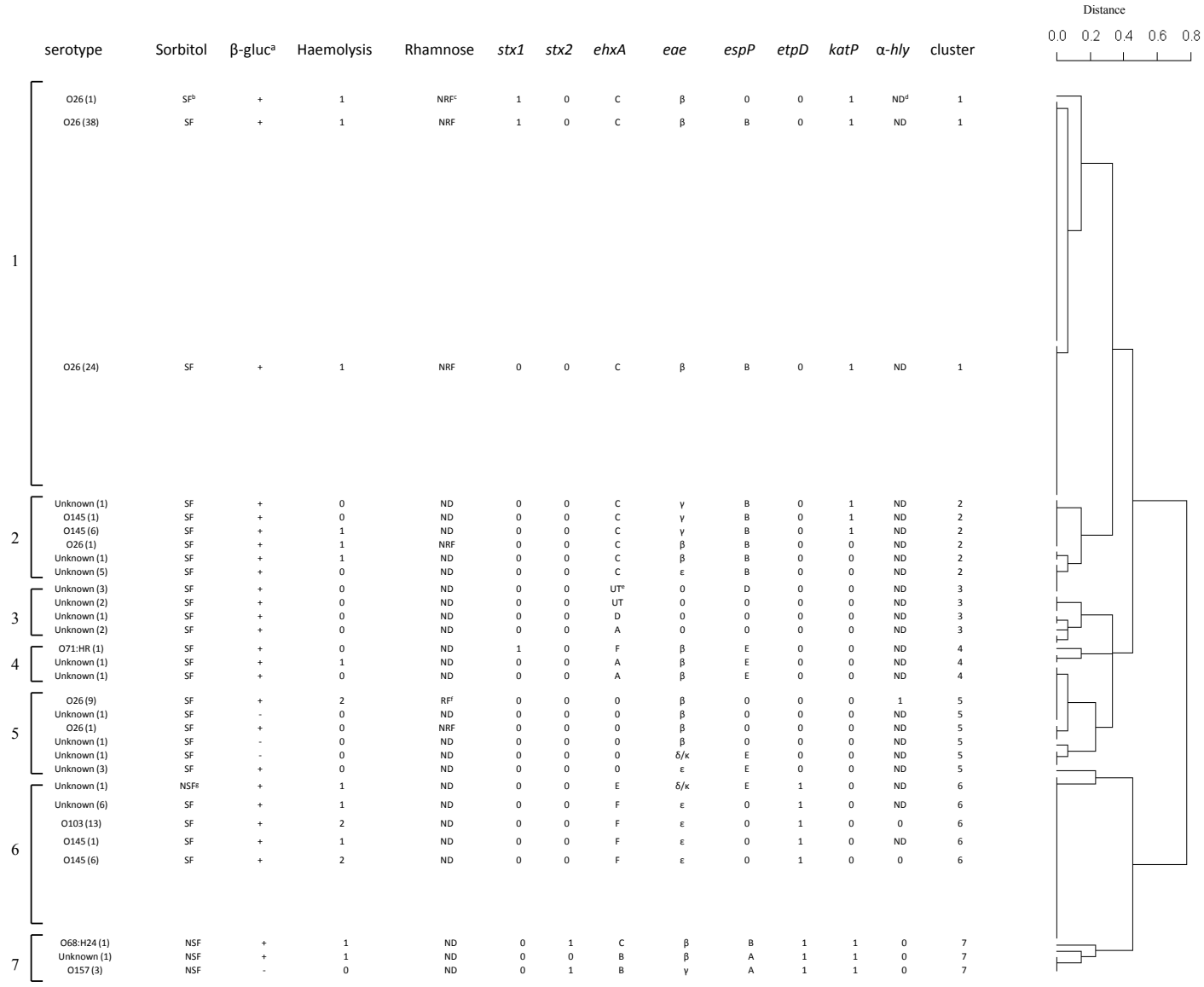
Table 5.8: PERMANOVA analysis results indicating the contribution of allelic profile (8 genes) in the variation of PFGE profiles of *E. coli* isolates obtained from calves in the North Island of New Zealand. Variables *stx2* and *etpD* were not included in the model to asses the contribution of variable serogroup towards the model.

Variables	Degree of freedom	Mean square	P-value	Perms	Estimated component of variation (%)
stx1	1	13989	0.001	999	14.9
RFLP *ehxA*	6	1499.1	0.001	999	17.8
RFLP *eae*	4	1976.9	0.001	998	19.3
espP	3	915.5	0.018	998	11.9
katP	1	1023.8	0.052	997	10.6
serogroup	6	1878.6	0.001	998	12.9
Residuals	109	322			12.4



Key	Type	stx1	stx2	PFGE group	Region	RFLP eltxA	RFLP eae HhaI	espP	etpD	katP	Sorbitol	BG	Haemolysis	a-hly
EcCa153a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa66a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa103a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa104a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa100b	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa67a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa68a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa85a	O26	1	0	A	Manawatu	C	beta	B	0	1	SF	BGP	1	ND
EcCa97a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa98a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa74a	O26	1	0	A	Taranaki	C	beta	0	0	1	SF	BGP	1	ND
EcCa80a	O26	1	0	A	Manawatu	C	beta	B	0	1	SF	BGP	1	ND
EcCa76a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa77a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa87a	O26	1	0	A	Manawatu	C	beta	B	0	1	SF	BGP	1	ND
EcCa106a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa107a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa304a	O26	1	0	A	Manawatu	C	beta	B	0	1	SF	BGP	1	ND
EcCa108a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa69a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa92b	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa47a	O26	1	0	A	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa94a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa64a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa71a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa101a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa102a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa105a	O26	1	0	A	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa110a	O26	0	0	A	Waikato	C	beta	B	0	1	SF	BGP	1	ND
EcCa100a	O26	0	0	B	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa75a	O26	0	0	B	Taranaki	C	beta	B	0	0	SF	BGP	1	ND
EcCa70a	O26	0	0	B	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa83a	O26	0	0	B	Taranaki	0	beta	0	0	0	SF	BGP	0	0
EcCa143a	O26	0	0	B	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa79a	O26	0	0	B	Manawatu	C	beta	B	0	1	SF	BGP	1	ND
EcCa65a	O26	0	0	B	Manawatu	C	beta	B	0	1	SF	BGP	1	ND
EcCa82a	O26	0	0	B	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa89a	O26	0	0	B	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa92a	O26	0	0	B	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa93a	O26	0	0	B	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa272a	O26	0	0	B	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa309a	O26	0	0	B	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa95a	O26	0	0	B	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa84a	O26	0	0	B	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa90a	O26	0	0	B	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa72a	O26	0	0	B	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa99a	O26	0	0	B	Taranaki	C	beta	B	0	1	SF	BGP	1	ND
EcCa109a	O26	0	0	B	Waikato	C	beta	B	0	1	SF	BGP	1	ND
EcCa51a	O26	0	0	B	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa54a	O26	0	0	B	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa55a	O26	0	0	B	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa45a	O26	0	0	B	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa36a	O26	0	0	B	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa86a	O26	0	0	B	Waikato	C	beta	B	0	1	SF	BGP	1	ND
EcCa41a	O26	1	0	C	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa44a	O26	1	0	C	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa38a	O26	1	0	C	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa39a	O26	1	0	C	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa42a	O26	1	0	C	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa43a	O26	1	0	C	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa46a	O26	1	0	C	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa48a	O26	1	0	C	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa53a	O26	1	0	C	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa49a	O26	1	0	C	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa52a	O26	1	0	C	Catchment	C	beta	B	0	1	SF	BGP	1	ND
EcCa116a	O145	0	0	D	Taranaki	C	gamma	B	0	1	SF	BGP	1	ND
EcCa117a	O145	0	0	D	Taranaki	C	gamma	B	0	1	SF	BGN	1	ND
EcCa115a	O145	0	0	D	Taranaki	C	gamma	B	0	1	SF	BGN	1	ND
EcCa355a	O145	0	0	D	Taranaki	C	gamma	B	0	1	SF	BGP	1	ND
EcCa118a	O145	0	0	D	Manawatu	C	gamma	B	0	1	SF	BGP	1	ND
EcCa284c	Unknown	0	0	D	Manawatu	C	gamma	B	0	1	SF	BGP	0	ND
EcCa119a	O145	0	0	D	Manawatu	C	gamma	B	0	1	SF	BGP	0	ND
EcCa44c	O145	0	0	D	Catchment	C	gamma	B	0	1	SF	BGP	1	ND
EcCa378b	Unknown	0	0	E	Taranaki	C	epsilon	B	0	0	SF	BGP	0	ND
EcCa412c	Unknown	0	0	E	Waikato	C	epsilon	B	0	0	SF	BGP	0	0
EcCa418a	Unknown	0	0	E	Waikato	C	epsilon	B	0	0	SF	BGP	0	ND
EcCa328a	Unknown	0	0	E	Taranaki	C	epsilon	B	0	0	SF	BGP	0	ND
EcCa296a	Unknown	0	0	E	Manawatu	C	epsilon	B	0	0	SF	BGP	0	ND
EcCa412a	Unknown	0	0	E	Waikato	C	beta	B	0	0	SF	BGP	1	ND
EcCa354a	Unknown	0	0	F	Manawatu	D	0	0	0	0	SF	BGP	0	ND
EcCa238a	O71:H8	1	0	G	Waikato	F	beta	E	0	0	SF	BGP	0	ND
EcCa327a	O26	0	0	H	Taranaki	0	beta	0	0	0	SF	BGP	2	1
EcCa96a	O26	0	0	H	Taranaki	0	beta	0	0	0	SF	BGP	2	1
EcCa78a	O26	0	0	H	Manawatu	0	beta	0	0	0	SF	BGP	2	1
EcCa366b	O6:H24	0	1	I	Taranaki	C	beta	B	1	1	NSF	BGP	1	ND
EcCa73a	O26	0	0	I	Taranaki	0	beta	0	0	0	SF	BGP	2	1
EcCa387a	Unknown	0	0	J	Waikato	A	beta	E	0	0	SF	BGP	1	ND
EcCa407a	Unknown	0	0	J	Waikato	A	beta	E	0	0	SF	BGP	0	ND
EcCa237a	O145	0	0	K	Taranaki	F	epsilon	0	1	0	SF	BGP	2	0
EcCa113a	O103	0	0	L	Taranaki	F	epsilon	0	1	0	SF	BGP	2	0
EcCa202c	O157	0	1	M	Taranaki	B	gamma	A	1	1	NSF	BGN	0	ND
EcCa260b	O157	0	1	M	Taranaki	B	gamma	A	1	1	NSF	BGN	0	ND
EcCa229b	O157	0	1	M	Taranaki	B	gamma	A	1	1	NSF	BGN	0	ND
EcCa280a	Unknown	0	0	N	Taranaki	A	0	0	0	0	SF	BGP	0	ND
EcCa398c	Unknown	0	0	N	Taranaki	A	0	0	0	0	SF	BGP	0	ND
EcCa318d	Unknown	0	0	O	Taranaki	0	delta/kappa	E	0	0	SF	BGN	0	0
EcCa353c	Unknown	0	0	O	Manawatu	0	beta	0	0	0	SF	BGN	0	0
EcCa416d	Unknown	0	0	O	Waikato	0	beta	0	0	0	SF	BGN	0	0
EcCa37a	O103	0	0	P	Catchment	F	epsilon	0	1	0	SF	BGP	2	0
EcCa40a	O103	0	0	P	Catchment	F	epsilon	0	1	0	SF	BGP	2	0
EcCa264a	Unknown	0	0	Q	Taranaki	UT	0	D	0	0	SF	BGP	0	ND
EcCa277a	Unknown	0	0	Q	Taranaki	UT	0	D	0	0	SF	BGP	0	ND
EcCa274a	Unknown	0	0	Q	Taranaki	UT	0	D	0	0	SF	BGP	0	ND
EcCa273a	Unknown	0	0	R	Taranaki	UT	0	0	0	0	SF	BGP	0	ND
EcCa419b	Unknown	0	0	S	Waikato	E	delta/kappa	E	1	0	NSF	BGP	1	ND
EcCa312a	Unknown	0	0	T	Taranaki	0	epsilon	E	0	0	SF	BGP	0	0
EcCa313a	Unknown	0	0	T	Taranaki	0	epsilon	E	0	0	SF	BGP	0	0
EcCa331a	Unknown	0	0	T	Manawatu	0	epsilon	E	0	0	SF	BGP	0	0
EcCa293a	Unknown	0	0	T	Manawatu	F	epsilon	0	1	0	SF	BGP	1	ND
EcCa322a	Unknown	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	1	ND
EcCa271a	O103	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	2	0
EcCa417a	O103	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	2	0
EcCa114a	O103	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	2	0
EcCa221a	O103	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	2	0
EcCa111a	O103	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	2	0
EcCa112a	O103	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	2	0
EcCa134a	O103	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	2	0
EcCa175a	O103	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	2	0
EcCa419a	Unknown	0	0	T	Waikato	F	epsilon	0	1	0	SF	BGP	1	ND
EcCa335a	O103	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	2	0
EcCa351a	O103	0	0	T	Manawatu	F	epsilon	0	1	0	SF	BGP	2	0
EcCa156a	O145	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	2	0
EcCa175b	O145	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	2	0
EcCa309c	O145	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	2	0
EcCa335c	O145	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	2	0
EcCa388a	O145	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	1	ND
EcCa237c	O145	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	2	0
EcCa285a	Unknown	0	0	T	Taranaki	F	epsilon	0	1	0	SF	BGP	1	ND
EcCa332a	Unknown	0	0	T	Manawatu	F	epsilon	0	1	0	SF	BGP	1	ND
EcCa270a	Unknown	0	0	U	Taranaki	F	epsilon	0	1	0	SF	BGP	1	ND
EcCa350b	Unknown	0	0	V	Manawatu	B	beta	A	1	1	NSF	BGP	1	ND
EcCa259a	Unknown	0	0	W	Taranaki	UT	0	0	0	0	SF	BGP	0	ND
PFGE XbaI O26 Ref.														

Figure 5.1: Clustering (UPGMA dendrogram) of the PFGE profiles of *E. coli* isolates from calves with >70% similarity cut off using *XbaI*. The column PFGE group refers to the cluster of *E. coli* isolates. The last lane is the *Salmonella* serotype Braenderup reference standard (H9812). The column BG (β glucuronidase) shows the BG activity of isolates. The column a-hly (α-*haemolysin*) shows the presence (indicated by 1) or absence (indicated by 0) of α-*hly* gene and ND (not done) means isolate was not analysed for presence or absence of α-*hly* gene



	serotype	Sorbitol	β-gluc[a]	Haemolysis	Rhamnose	*stx1*	*stx2*	*ehxA*	*eae*	*espP*	*etpD*	*katP*	α-*hly*	cluster
1	O26 (1)	SF[b]	+	1	NRF[c]	1	0	C	β	0	0	1	ND[d]	1
	O26 (38)	SF	+	1	NRF	1	0	C	β	B	0	1	ND	1
	O26 (24)	SF	+	1	NRF	0	0	C	β	B	0	1	ND	1
2	Unknown (1)	SF	+	0	ND	0	0	C	γ	B	0	1	ND	2
	O145 (1)	SF	+	0	ND	0	0	C	γ	B	0	1	ND	2
	O145 (6)	SF	+	1	ND	0	0	C	γ	B	0	1	ND	2
	O26 (1)	SF	+	1	NRF	0	0	C	β	B	0	0	ND	2
	Unknown (1)	SF	+	1	ND	0	0	C	β	B	0	0	ND	2
	Unknown (5)	SF	+	0	ND	0	0	C	ε	B	0	0	ND	2
3	Unknown (3)	SF	+	0	ND	0	0	UT[e]	0	D	0	0	ND	3
	Unknown (2)	SF	+	0	ND	0	0	UT	0	0	0	0	ND	3
	Unknown (1)	SF	+	0	ND	0	0	D	0	0	0	0	ND	3
	Unknown (2)	SF	+	0	ND	0	0	A	0	0	0	0	ND	3
4	O71:HR (1)	SF	+	0	ND	1	0	F	β	E	0	0	ND	4
	Unknown (1)	SF	+	1	ND	0	0	A	β	E	0	0	ND	4
	Unknown (1)	SF	+	0	ND	0	0	A	β	E	0	0	ND	4
5	O26 (9)	SF	+	2	RF[f]	0	0	0	β	0	0	0	1	5
	Unknown (1)	SF	-	0	ND	0	0	0	β	0	0	0	ND	5
	O26 (1)	SF	+	0	NRF	0	0	0	β	0	0	0	ND	5
	Unknown (1)	SF	-	0	ND	0	0	0	β	0	0	0	ND	5
	Unknown (1)	SF	-	0	ND	0	0	0	δ/κ	E	0	0	ND	5
	Unknown (3)	SF	+	0	ND	0	0	0	ε	E	0	0	ND	5
6	Unknown (1)	NSF[g]	+	1	ND	0	0	E	δ/κ	E	1	0	ND	6
	Unknown (6)	SF	+	1	ND	0	0	F	ε	0	1	0	ND	6
	O103 (13)	SF	+	2	ND	0	0	F	ε	0	1	0	0	6
	O145 (1)	SF	+	1	ND	0	0	F	ε	0	1	0	ND	6
	O145 (6)	SF	+	2	ND	0	0	F	ε	0	1	0	0	6
7	O68:H24 (1)	NSF	+	1	ND	0	1	C	β	B	1	1	0	7
	Unknown (1)	NSF	+	1	ND	0	0	B	β	A	1	1	0	7
	O157 (3)	NSF	-	0	ND	0	1	B	γ	A	1	1	0	7

Figure 5.2: Hierarchical clustering analysis of *eae* and/*ehxA* positive *E. coli* isolates obtained from calves of the North Island of New Zealand based on the data from eight PCR(-RFLP) analysis: *stx1*, *stx2*, *eae*, *ehxA*, *espP*, *etpD*, *katP* and α-*hly* genes. Superscripts: a, β-glucuronidase; b, sorbitol fermenting; c, non-rhamnose fermenting; d, not done; e, untypable; f, rhamnose fermenting; g, non-sorbitol fermenting

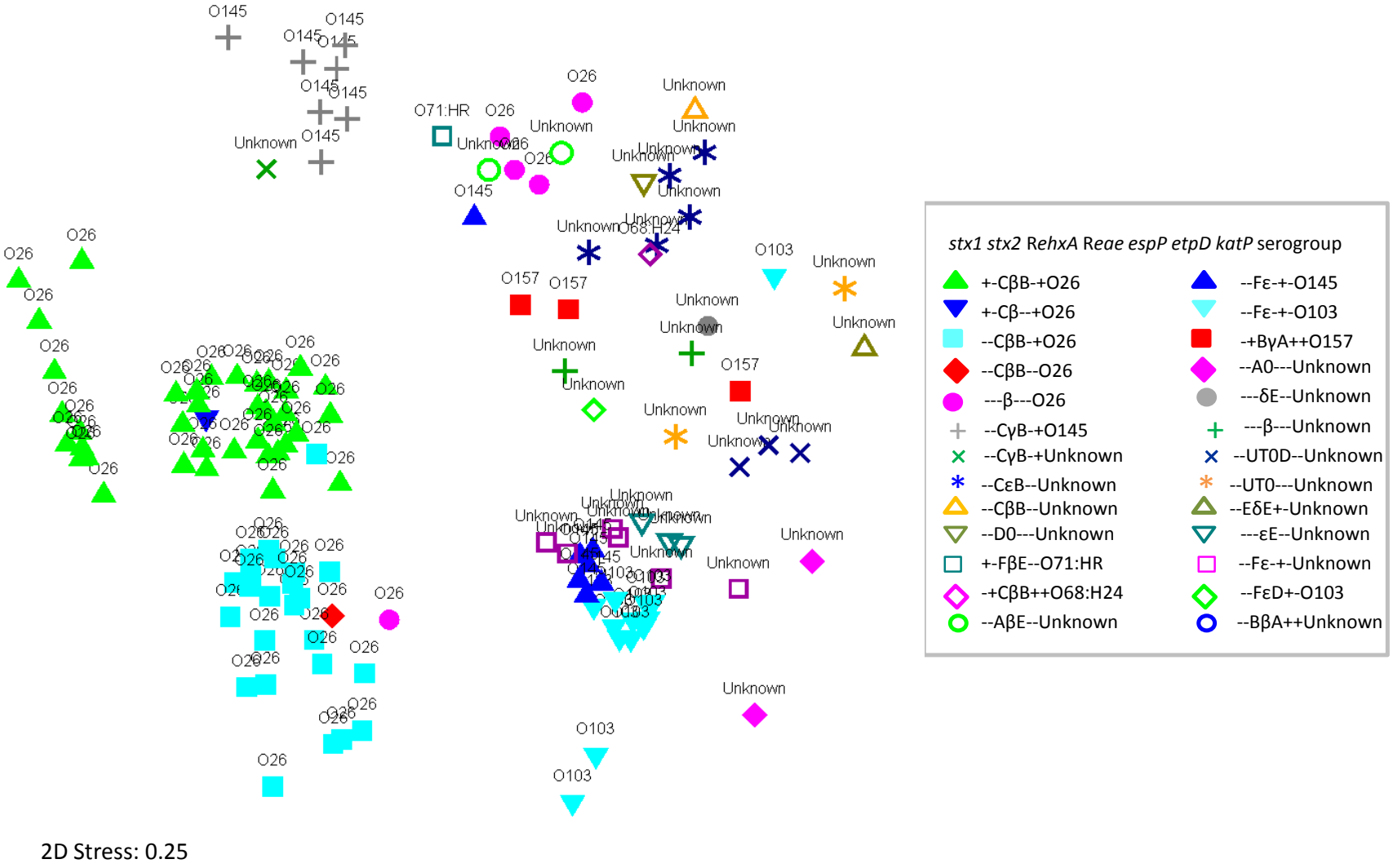


Figure 5.3: Multi dimensional scaling plot showing association between PFGE and allelic profiles (8 genes) of 137 *E. coli* isolates obtained from calves from the North Island of New Zealand

6 Chapter 6

General discussion

The purpose of these studies was primarily to understand the molecular epidemiology of *E. coli* serogroups O26, O103, O111, O145 and O157 of economic and public health concern in the calves of the North Island of New Zealand. Shiga toxin-producing *E. coli* (STEC) are considered emerging food-borne pathogens of global significance causing diarrhoea, haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (Bettelheim, 2007; Karmali et al., 2010). Cattle and sheep are considered important reservoirs of STEC (Bettelheim, 2007; Hancock et al., 1998). Cattle in particular are thought to play a major role in transmission of STEC to humans either through direct contact with infected animals or indirectly through ingestion of contaminated food or water (Armstrong et al., 1996; Renwick et al., 1993).

STEC O157 is considered the most virulent STEC serogroup causing approximately 73,500 cases annually in the USA as compared to 37,000 cases due to non-O157 (Kasper et al., 2010). However, the number of cases due to non-O157 may be under-estimated as no standard laboratory methods are available for detection of non-O157 and most laboratories test samples for O157 only (Kasper et al., 2010). Many of the non-O157 STEC strains are less virulent than O157 (Kasper et al., 2010) but two recent related European outbreaks due to enteroaggregative enterohaemorrhagic *E. coli* (EAEHEC) O104 have shown that non-O157 STEC may become highly virulent after acquiring additional virulence factors (Bielaszewska et al., 2011; Frank et al., 2011; Mariani-Kurkdjian et al., 2011). The most common non-O157 STECs associated with disease in the USA are O26, O45, O103, O111, O121 and O145 (Brooks et al., 2005; Gyles, 2006). The USA has a zero tolerance policy for STEC O157 in meat and because of the public health significance of non-O157 STEC, the USA has extended the list of meat adulterants by adding six (O26, O45, O103, O111, O121 and O145) non-O157 STEC serogroups (Food Safety and Inspection Service, 2011a). The dairy and meat industries are key contributors to the New Zealand economy (Cavanagh, 2003), therefore these changes have serious economic implications due to their impact on international trade. Very limited information is available regarding the epidemiology of STEC in New Zealand cattle and to our knowledge this is the first study that targets isolation and epidemiological investigation of the economically important non-O157 STEC serogroups O26, O103, O111 and O145 in New Zealand calves.

Faecal samples collected from calves in a catchment area (chapter 2) and recto-anal mucosal swabs (RAMS) samples collected from calves slaughtered at two abattoirs of the North Island of New Zealand (chapters 3 and 4) were analysed in different ways to understand the molecular epidemiology of non-O157 STEC serogroups including O26, O103, O111, O145 and O157. These cross-sectional studies were carried out over a short period of time, which provided a snapshot of the distribution of STEC. Data provided by cross-sectional studies are also helpful in examining risk factors for carriage, but it is not possible to study the temporal trends using this study design; and therefore, these studies were not able to estimate the possible seasonal trends. Previous work suggested a seasonal pattern of *E. coli* O157 (Hancock et al., 1997; Mechie et al., 1997; Ogden

et al., 2004) and non-O157 (Fukushima and Seki, 2004; Leung et al., 2001; Pearce et al., 2006). However, conflicting information about the seasonality of STEC is evident from the literature. Although this study provided information about the detection and distribution of various *E. coli* serogroups from different farms of the North Island of New Zealand, it is very difficult to form any conclusions about the transmission of various *E. coli* serogroups between different farms.

The study described in chapter 2 was conducted to understand the population structure, transmission dynamics and spatial relationship between genotypes of STEC and *C. jejuni* within and between predominantly dairy farms in a catchment area. This information can help with the design of appropriate control measures to limit the spread of these zoonotic pathogens in the environment, which in turn would reduce the spread to animals and humans. This study was conducted on a limited number of farms in a small catchment and the objective was to obtain the maximum number of representative isolates for genotyping from as many farms as possible given the resources available. Therefore, to enhance the recovery of pathogens of interest the method of pooling faecal samples from four different animals was adopted. Insufficient numbers of O103 and O145 isolates were obtained therefore only O26 isolates were further analysed using molecular epidemiological methods. These results indicated a trend for transmission of *E. coli* O26 between animals in the same farm but very limited transmission between animals from different farms. Due to the limited number of O26 isolates, further analysis of population structure and differentiation was not performed. However, for *C. jejuni* enough isolates were available to study the relationship between cattle and water genotypes using Minimum Spanning Trees and to understand population differentiation using analysis of molecular variance (AMOVA). It was observed that genotypes of *C. jejuni* common to both cattle and water were of zoonotic importance. Therefore water could be an important source of human infection (Mullner et al., 2009b). The AMOVA provided evidence of a high level of *C. jejuni* within farm transmission and some evidence of limited between farm transmission indicating surface run-off, direct contact between animals and sharing of equipment and personnel between farms may be important means of localised transmission of *C. jejuni*.

There were some differences in the sampling protocol between the catchment study described in chapter 2 and the bobby calf studies described in chapters 3 and 4. For example, in the catchment study composite faecal samples were collected from calves grazing on farms, whereas in the bobby calf studies RAMS were collected from calves at slaughter plants. Despite these differences the two studies generally showed a similar pattern in prevalence of O26, O103 and O145.

The focus of the study described in chapter 3 was to determine the distribution of *E. coli* strains harbouring STEC-associated virulence genes (*stx1*, *stx2*, *eae* and *ehxA*) from bobby calves in the North Island. Enrichment broth cultures of RAMS were directly inoculated onto two different media (CT-SMAC and TBX) and two colonies were randomly selected from each plate and analysed for the presence of virulence genes. Underestimation of the prevalence of both STEC and atypical enteropathogenic *E. coli* (aEPEC) by this method was likely as preliminary screening of the samples such as for *stx* and/or *eae* was not performed. However, the absence of preliminary screening of samples for *stx* and/or *eae*, the use of two different culturing media and the analysis of four randomly chosen colonies helped to minimise the bias towards subculture of *stx*-and/or *eae*-positive

isolates. This study was not focused on specific *E. coli* serogroups and therefore provided more insight into the presence of various *E. coli* serogoups and distribution of STEC-associated virulence factors (*stx*1, *stx*2, *eae* and *ehxA*) in these isolates. This study showed that calves harbour STEC and aEPEC serogroups of zoonotic importance such as O26, O103, O145 and O157 and therefore may represent an important source of environmental contamination and possible human infection.

STEC O157 and non-O157 STEC show diverse phenotypes on various media (Posse et al., 2008). Therefore, a single selective medium may not be efficient enough to detect different STEC and aEPEC. Various media used for isolation of STEC contain antibiotics to inhibit the growth of background bacteria but these antibiotics also inhibit the growth of some STEC strains. For example, potassium tellurite used in CT-SMAC inhibits the growth of some strains of O103 (Tillman et al., 2012). In order to overcome these limitations two different media, one with antibiotics (CT-SMAC) and the other without antibiotics (TBX) were used for random isolation of *E. coli* from RAMS (chapter 3). This study indicated that use of one selective media might result in underestimation of aEPEC and STEC. For example, a single *eae* negative STEC was obtained on TBX plates and a previous study from New Zealand (Cookson et al., 2006b) also showed that *eae* negative STEC were more commonly isolated on TBX plates as compared to CT-SMAC plates. Similarly, isolation of aEPEC was more frequent on CT-SMAC plates as compared to TBX plates.

In addition to STEC O157, STEC serogoups O26, O103, O111 and O145 have recently been described as adulterants of meat (Food Safety and Inspection Service, 2011a). Chapter 4 described the distribution of these STEC and their virulence factors in isolates obtained from bobby calves at slaughter originating from farms of different areas of the North Island of New Zealand. Samples were initially screened for specific serogroups using real time PCR (RT-PCR). This assisted in reducing the processing time of samples as only positive samples were further investigated. This method has also been used previously for detection of non-O157 STEC in food and faecal samples (Perelle et al., 2004; Sharma, 2002). The PCR prevalence of *E. coli* O26, O103 and O145 was higher in this study compared to previous New Zealand based studies (Cookson et al., 2006b,c). This difference is likely to be due to the use of sample pre-screening with specific PCR primers and it also highlights the use of initial screening method for determining prevalence of specific *E. coli* serogroups. RT-PCR positive samples were then subjected to isolation using specific media and immuno-magnetic separation beads (IMS) for better recovery of isolates from RT-PCR positive samples.

The isolation rate was higher for O26 compared to O103 and O145 (chapter 2 and 4). This may be due to availability of specific media for isolation of O26 whereas no specific media is available for the isolation of O103 and O145. This finding highlights the need for development of specific media for O103 and O145. Attempts have been made to develop a media for detection of non-O157 STEC; Posse et al. (2008) developed a media on which STEC O26, O103, O111 and O145 produced different colour colonies. However, it has been reported that colony colour varies with incubation time, number of colonies on plate and sample from which they are isolated (Mathusa et al., 2010). Genome sequencing of additional non-O157 isolates may provide useful

information to identify genes responsible for fermentation of different sugars to distinguish various STEC serotypes using different media technologies. This information could then be used for the development of new media for isolation and identification of non-O157 STEC.

Not a single sample was positive for O111 in the studies described in chapters 2, 3 and 4 and to our knowledge, STEC O111 has never been isolated from New Zealand cattle. STEC O111 is considered an important cause of HUS. For example in the USA STEC O111 is the second most common cause of HUS (Brooks et al., 2005). Similarly, in Australia STEC O111 is the second most common cause of STEC infection after STEC O157 (Vally et al., 2012). STEC outbreaks have been reported from the USA (Brooks et al., 2004), Australia (Vally et al., 2012), Japan (Tanaka et al., 1989) and Europe (Caprioli and Tozzi, 1998). Due to the public health significance of STEC O111 the USA has included O111 in the list of meat adulterants (Food Safety and Inspection Service, 2011a). The reason for the absence of STEC O111 in New Zealand cattle is not known, however it can be speculated that the diversity of microbial pathogens in New Zealand is generally lower than that observed elsewhere due to its unique geographic location and relatively recent contact to exotic animals and humans (Crump et al., 2001). Therefore, further studies are required to confirm whether O111 is absent from New Zealand and, if so, why given the introduction and dissemination of other STEC.

RT-PCR is a fast and sensitive method for detection of non-O157 STEC but isolates could not be obtained from all the RT-PCR positive samples. This raises the issue of both the sensitivity and specificity of RT-PCR and the sensitivity of culturing. The performance of RT-PCR primers for O26, O103, O111 and O145 was assessed in three different ways (described in chapter 4) and there was no indication of interaction of primers with each other. In contrast, a recent study has shown that *rfbE* O157 primers interact with *rfbE* gene of *Escherichia fergusoni* (Fegan et al., 2006). Therefore further studies may be required to evaluate the sensitivity and specificity of O26, O103, O111 and O145 primers. Another possible explanation for low recovery of isolates could be low sensitivity of culture of frozen RAMS enriched in BPW in this study (chapter 4). Freezing can cause irreversible damage to bacterial cells including *E. coli* (Ignatov et al., 1981). This damage may have serious impact in recovery of *E. coli*. Studies have shown differences in survival of STEC O157 depending upon the method of freezing and thawing, the strain of STEC O157 and the origin of samples (Bollman et al., 2001; Rocelle et al., 1995; Sage and Ingham, 1998). Dead *E. coli* of interest may therefore be present in samples, and these *E. coli* are detectable by RT-PCR but not able to be isolated. The variation in prevalence of O26, O103 and O145 in calves from this study compared to other New Zealand studies (Cookson et al. 2006a) may have been due to difference in sample size and age of sampled animals.

Age is considered an important risk factor for the prevalence of *E. coli* O157 (Garber et al., 1995; Nielsen et al., 2002). The studies described in chapter 3 and 4 were focussed on calves of fewer than seven days of age. The gastro-intestinal tract (GIT) of calves is not fully developed and may be more susceptible to colonisation with certain *E. coli* serogroups such as O26, O103 and O145. The fully developed rumen of adult calves produces a high concentration of volatile fatty acids which leads to low pH and inhibition of growth of certain STEC (Rasmussen et al., 1993).

This may also be due to variation in diet of the weaned and un-weaned calves. The role of feed in attachment and effacement of bacteria to GIT has been investigated, for example, Neef et al. (1994) showed the association between feed and formation of attachment and effacement lesions in the large intestine of pigs. Change in diet may lead to change in ruminal microflora which may provide resistance in attachment of some micro-organisms to epithelial lining of intestine. A study has shown significant reduction in colonisation of chicks by O157:H7 if administered with an adult type microflora (Stavric et al., 1992). Moreover, age dependent reduction in infection by enteric pathogens may also be associated with development of intestinal flora (Nakagawa et al., 1969; Nurmi and Rantala, 1973). Stress is considered an important factor in onset and development of infection (Peterson et al., 1991). Bobby calves are only 4-7 days old. Therefore they are very prone to stress during loading on truck, transportation to abattoir and holding in lairage for long times. Stress may lead to changes in GIT microflora and reduced immunity which may provide suitable conditions for colonisation and growth of STEC.

Studies have shown that the host specificity and tissue tropism displayed by different STEC may be influenced by the various *eae* types (Fitzhenry et al., 2002, 2003; Phillips and Frankel, 2000; Phillips et al., 2000). *E. coli* O26 was isolated more frequently than O103 and O145 (chapters 2 and 4). This could be attributable to the pre-ruminant gut environment being more suitable for colonisation by O26 which have *eae* subtype β.

E. coli isolates obtained from calves (chapters 2, 3 and 4) were genotyped using an array of genotyping methods including PFGE, SBI typing and allelic profiling (based on 8 virulence genes). PFGE is considered to have high discriminatory power and reproducibility (Barrett et al., 1994; Izumiya et al., 1997) but it is a time consuming, expensive and laborious method and does not provide information about the specific alleles. PFGE was therefore, compared with allelic profiling (based on 8 genes) using PERMANOVA and multidimensional scaling plot and the results (described in chapter 5) indicate that the discriminatory power of allelic profiling was similar to PFGE. In addition allelic profiling is relatively quick, easy to standardise and provide information about the presence or absence of specific genes. The results also indicate that six of the eight genes explain much of the variation evident in PFGE profiles and of these six the major contributors were *eae* and *ehxA*. These results may provide a basis for the development of a new genotyping method for *E. coli* isolates having high discriminatory power and ease of performance and standardisation. However, these results are based on the analysis of limited number of *E. coli* isolates obtained from one source i.e. calves from a limited geographical area therefore a large set of *E. coli* isolates from different sources and geographical areas should be analysed to further assess the genotyping ability of allelic profiling. SBI typing has been used for genotyping of *E. coli* O157 isolates (Besser et al., 2007; Irshad et al., 2012). To our knowledge this is the first study to report the genotyping of O26 isolates using SBI typing. However, SBI typing gave only two genotypes among *E. coli* O26 isolates indicating low discriminatory power of this method for this serogroup.

Spatial data analysis can be used to identify geographical areas with different disease intensities (Haine et al., 2004; Sanchez et al., 2005), to select areas for further study (Graham et al., 2005) and to hypothesise means and routes of introduction of disease (Vigre et al., 2005). K function analysis

was used (chapter 4) to estimate the second order clustering of positive and negative farms. The intensity of spatial point patterns depends upon the location in space, for example farms are mostly located away from urban areas and close to localities which can provide specific inputs such as feed supply and market access. Therefore, the advantage of using this technique is that it does not require the underlying process to be stationary i.e. the intensity of the process is independent of the location in space. K function analysis did not show any second order clustering of the farms for *E. coli* O26, O103 and O145 and therefore provided no evidence of localised transmission of these serogroups.

Bobby calves are slaughtered within seven days of birth therefore passive immunisation through colostrum feeding appeared to be the best option to control carriage and shedding of STEC in bobby calves. Previous studies have reported higher levels of antibodies in sera of the calves that were fed colostrum (Rabinovitz et al., 2012; Widiasih et al., 2004b). Therefore, it was hypothesized that carriage of non-O157 STEC may be associated with the concentration of maternally derived immunity (chapter 4). However, this study did not find any association between the concentration of the maternally derived IgG in the sera of calves that were RT-PCR positive or negative for *E. coli* O26, O103 and O145. This work also indicated that STEC are widely distributed in calves in the North Island of New Zealand. Therefore, intervention strategies such as vaccination at farm level may not be economical, even if an effective vaccine was available. Intervention strategies at the slaughter plant may be both more effective and economical in controlling the contamination of meat with hazardous micro-organisms including STEC of commercial importance, thereby avoiding economic losses and reducing human disease.

This is the first study in New Zealand describing the molecular epidemiology of *E. coli* O157 and non-O157 of economic and public health significance in calves using the targeted diagnostic methods RT-PCR and IMS, and the genotyping methods PFGE, allelic profiling and SBI typing. *E. coli* O26 was found to be more prevalent, compared to O103 and O145 and these *E. coli* serogroups were widely distributed in the important dairy producing areas of the North Island. Some STEC (O68 and O71) of lesser zoonotic importance were also isolated from the calves and their public health significance should not be underestimated, as the recent EAEHEC O104:H4 outbreak in Europe indicated the evolution of less virulent STEC to highly virulent strains by the acquisition of additional virulence factors (Brzuszkiewicz et al., 2011). There was no association between maternally derived immunity and calves positive with *E. coli* O26, O103 and O145 indicating that colostrum feeding may not be effective in reducing carriage of *E. coli* O26, O103 and O145. This work also indicated that allelic profiling (based on 8 genes) may be used for genotyping of *E. coli* isolates but its effectiveness should be further investigated using larger and more diverse set of *E. coli* isolates.

7 Appendix

7.1 Appendix 1

Figures describing the results of multiple correspondence analysis (chapter 3)

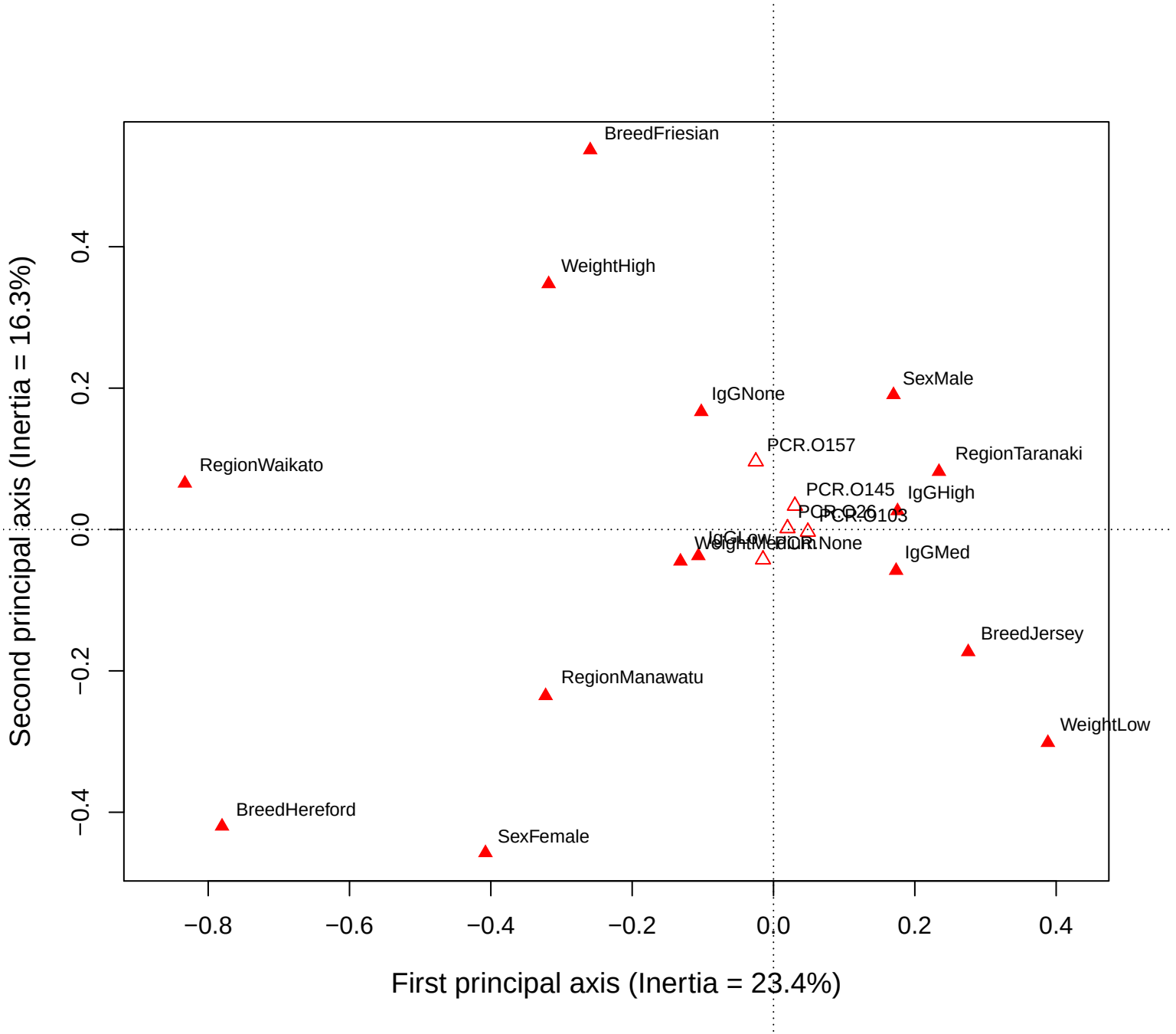


Figure 7.1: Two dimensional plot showing the results of multiple correspondence analysis model of RT-PCR results (O26, O103, O145 and O157), weight, sex, breed and concentration of IgG of samples obtained from 299 bobby calves in the North Island of New Zealand. The variable PCR was used as supplementary variable (Δ)

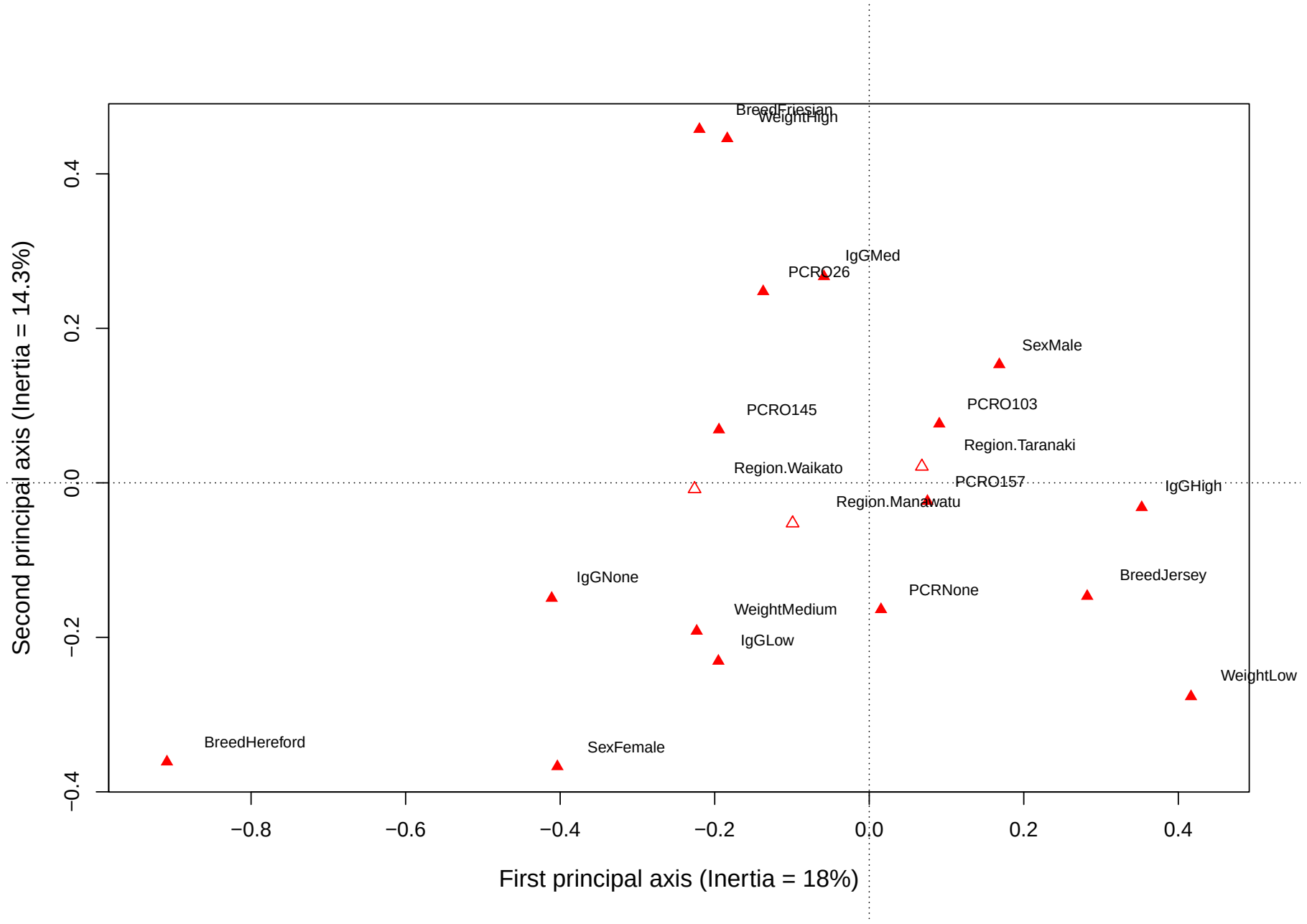


Figure 7.2: Two dimensional plot showing the results of multiple correspondence analysis model of RT-PCR results (O26, O103, O145 and O157), weight, sex, breed and concentration of IgG of samples obtained from 299 bobby calves in the North Island of New Zealand. The variable region was used as supplementary variable (Δ)

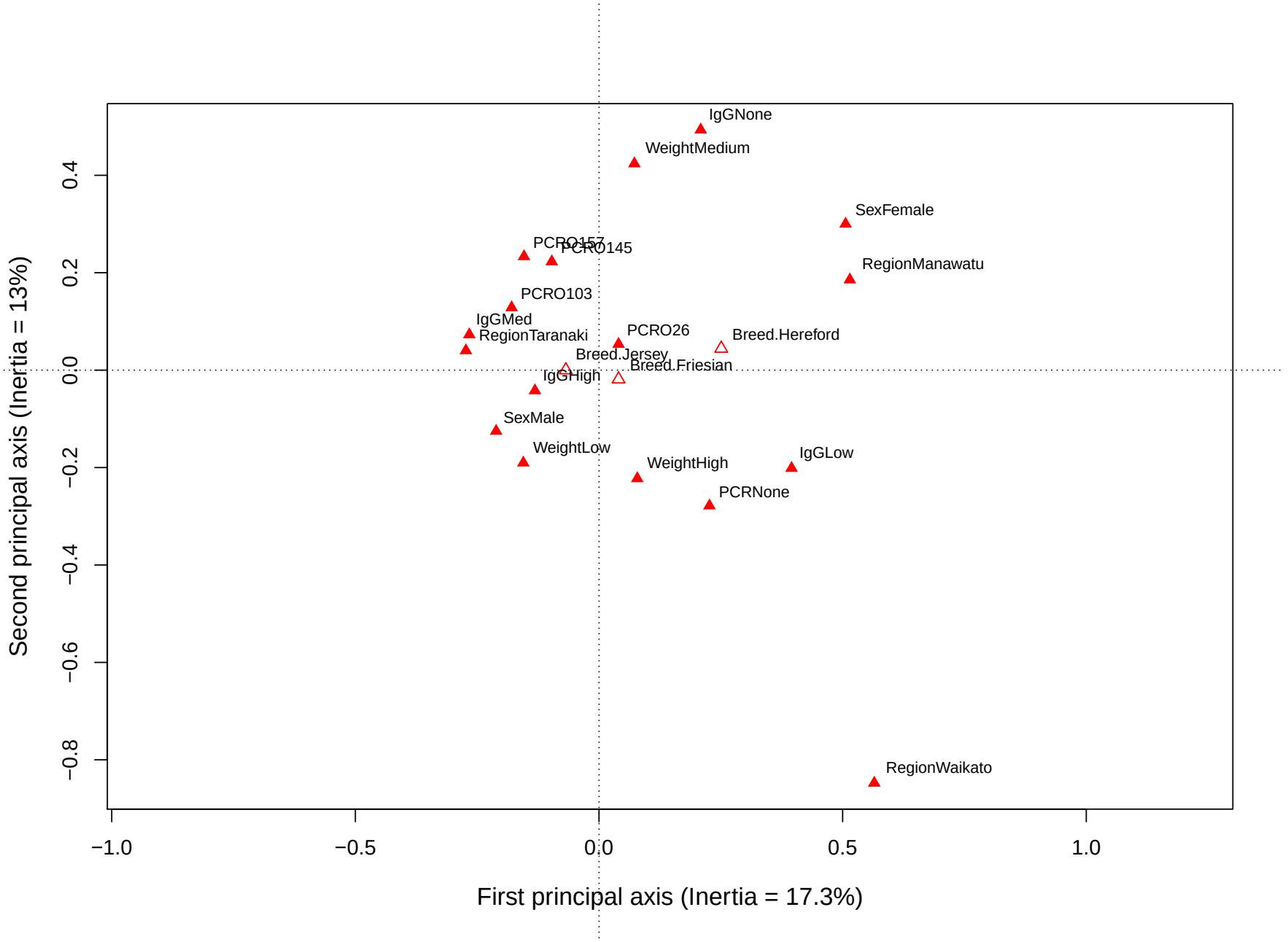


Figure 7.3: Two dimensional plot showing the results of multiple correspondence analysis model of RT-PCR results (O26, O103, O145 and O157), weight, sex, breed and concentration of IgG of samples obtained from 299 bobby calves in the North Island of New Zealand. The variable breed was used as supplementary variable (Δ)

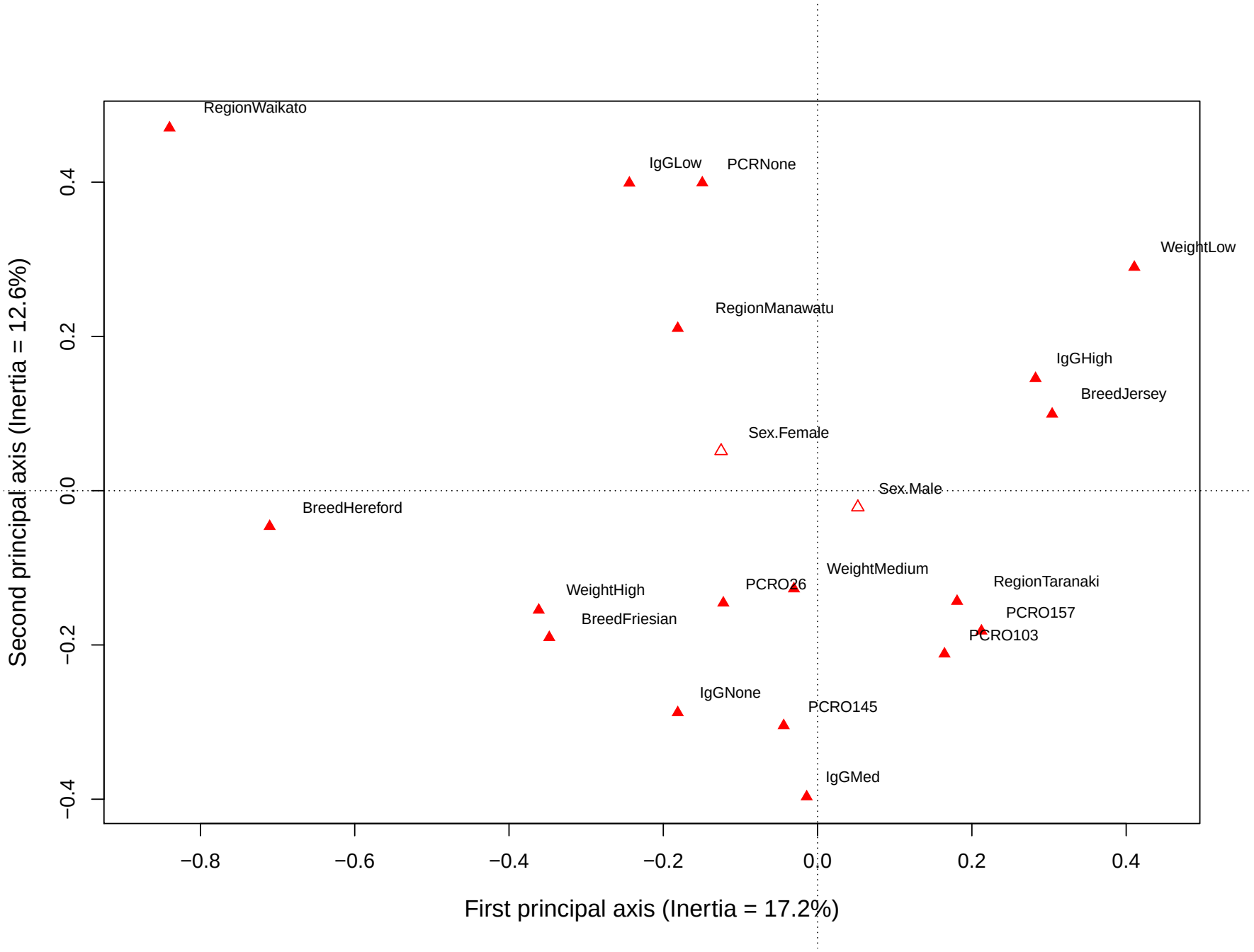


Figure 7.4: Two dimensional plot showing the results of multiple correspondence analysis model of RT-PCR results (O26, O103, O145 and O157), weight, sex, breed and concentration of IgG of samples obtained from 299 bobby calves in the North Island of New Zealand. The variable sex was used as supplementary variable (Δ)

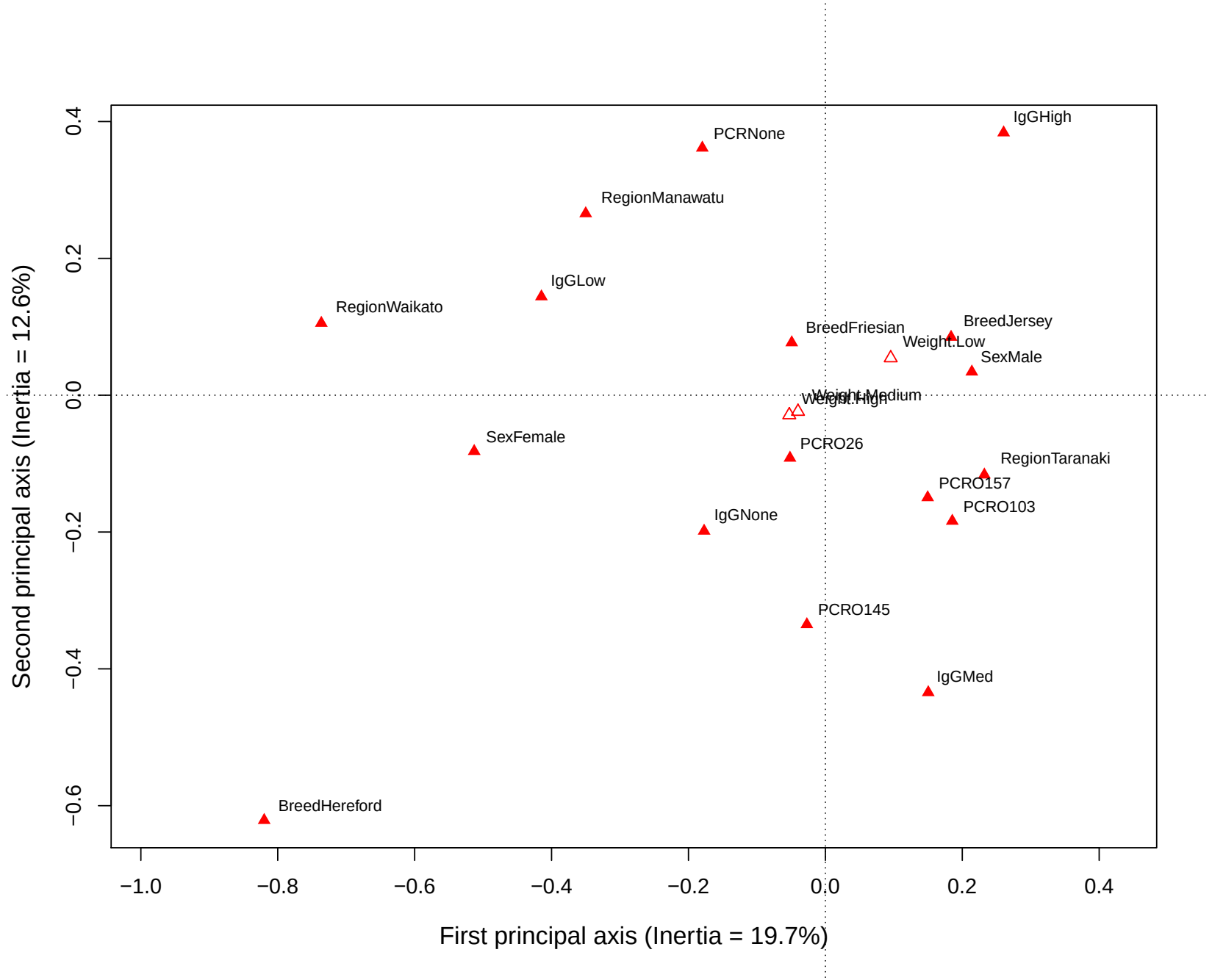


Figure 7.5: Two dimensional plot showing the results of multiple correspondence analysis model of RT-PCR results (O26, O103, O145 and O157), weight, sex, breed and concentration of IgG of samples obtained from 299 bobby calves in the North Island of New Zealand. The variable weight was used as supplementary variable (Δ)

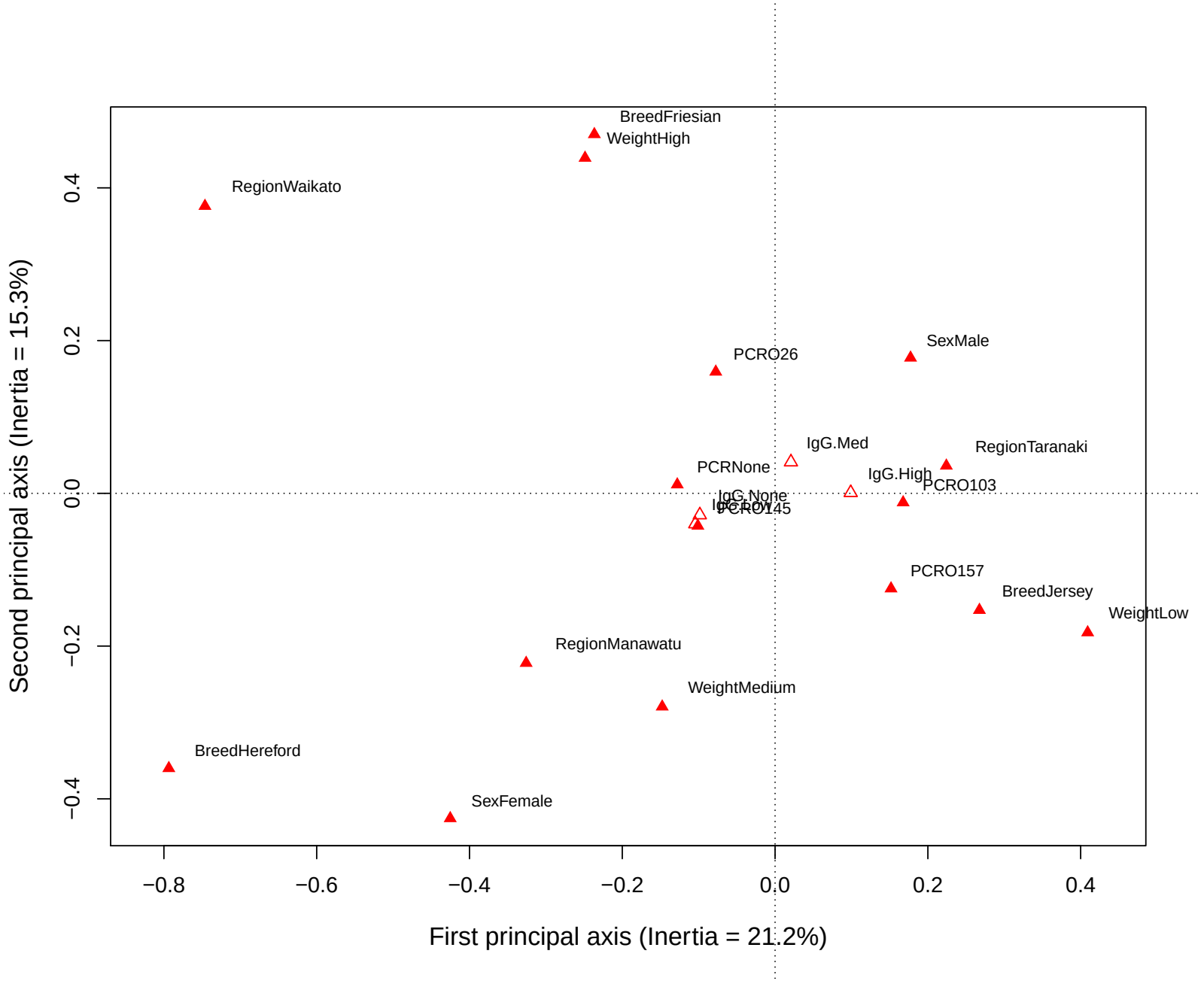


Figure 7.6: Two dimensional plot showing the results of multiple correspondence analysis model of RT-PCR results (O26, O103, O145 and O157), weight, sex, breed and concentration of IgG of samples obtained from 299 bobby calves in the North Island of New Zealand. The variable IgG was used as supplementary variable (Δ)

7.2 Appendix 2

PERMANOVA analysis results of chapter 5 excluding *E. coli* isolates from catchment study

Table 7.1: PERMANOVA analysis results indicating the contribution of allelic profile (8 genes) in the variation of PFGE profiles of *E. coli* isolates obtained from bobby calves (excluding catchment isolates) in the North Island of New Zealand.

Variables	Degree of freedom	Mean square	P-value	Perms	Estimated component of variation (%)
stx1	1	9438.2	0.001	999	14.1
stx2	0	0	0	0	0
RFLP *ehxA*	5	1450.5	0.001	999	20.6
RFLP *eae*	4	1930.6	0.001	999	19.6
espP	3	910.6	0.011	999	12.8
etpD	0	0	0	0	0
katP	1	1027.9	0.025	999	11
serogroup	4	1657.9	0.001	999	11
Residuals	89	213.73			10.1

Table 7.2: PERMANOVA analysis results indicating the contribution of allelic profile (8 genes) in the variation of PFGE profiles of *E. coli* isolates obtained from bobby calves (excluding catchment isolates) in the North Island of New Zealand. Variable serogroup was not included in the model.

Variables	Degree of freedom	Mean square	P-value	Perms	Estimated component of variation (%)
stx1	1	9418	0.001	999	9.2
RFLP *ehxA*	6	1631.8	0.001	999	13.3
RFLP *eae*	4	3259.7	0.001	998	10.7
espP	3	1057.9	0.005	997	8
katP	1	4263.8	0.001	999	13
etpD	1	2658.6	0.001	998	20.4
stx2	1	2767.3	0.001	999	17.3
Residuals	93	275.8			7.5

Table 7.3: PERMANOVA analysis results indicating the contribution of allelic profile (8 genes) in the variation of PFGE profiles of *E. coli* isolates obtained from bobby calves (excluding catchment isolates) in the North Island of New Zealand. Variables *stx2* and *etpD* were not included in the model.

Variables	Degree of freedom	Mean square	P-value	Perms	Estimated component of variation (%)
stx1	1	9438.2	0.001	999	14
RFLP *ehxA*	6	1497.8	0.001	997	18.4
RFLP *eae*	4	1930.6	0.001	999	19.5
espP	3	910.6	0.009	999	12.8
katP	1	1027.9	0.035	999	11.4
serogroup	6	1854.3	0.001	999	13.4
Residuals	89	213.7			10

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