Epidemiological investigations of Shiga toxin-producing *Escherichia coli* (STEC) O157 and STEC O26 in New Zealand slaughter cattle, and the source attribution of human illness

A thesis presented in partial fulfilment of the requirements for the degree
of

Doctor of Philosophy
in
Veterinary Science

at Massey University, Palmerston North,
New Zealand.

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2014
(Submitted March 31, 2014)

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Abstract

Shiga toxin-producing *Escherichia coli* O157:H7 (STEC O157) and related non-O157 STEC strains are enteric pathogens of significant public health concern worldwide, including New Zealand, causing clinical diseases ranging from diarrhoea and bloody diarrhoea to the life-threatening haemolytic uraemic syndrome. Cattle are considered the principal hosts and have been shown to be a source of STEC infection for both foodborne and environmental outbreaks of human diarrhoeal disease overseas. A series of observational studies were conducted to gain knowledge on the epidemiology of STEC O157 and STEC O26 in New Zealand slaughter cattle and assess the relative importance of cattle as a source of domestically-acquired STEC infections in humans.

A repeated cross-sectional study conducted on four selected New Zealand beef slaughter plants provided detailed data on the prevalence and concentration of faecal shedding of STEC O157 and STEC O26 in 695 very young calves (4–7 days-old) and 895 adult cattle post-slaughter, identifying calves as more prevalent carriers of STEC. Findings of a subsequent cohort study, the first of its kind, provided evidence that for the 60 calves examined, transportation and lairage was not associated with increase of faecal shedding of *E. coli* O157 and O26 (STEC and non-STEC) but increase of cross-contamination of hides and carcasses post-slaughter.

In a national prospective case-control study, 113 STEC cases and 506 random controls were interviewed for risk factor evaluation. The study findings implicate that environmental and animal contact, but not food, as significant exposure pathways for sporadic STEC infections in humans in New Zealand, and suggest ruminants as the most important source of infection. The molecular analysis of bovine and human STEC O157 isolates provided evidence for the historical introduction of a subset of the globally-circulating STEC O157 strains into New Zealand and ongoing localised transmission of STEC between cattle and humans.

These findings will contribute to the development of a risk management strategy for STEC, similar to those already implemented for *Campylobacter*, *Salmonella*, and *Listeria*, which

pose a high risk to public health and New Zealand's access to international markets. Furthermore, risk factors identified in the case-control study will contribute to the design of public health interventions to reduce the incidence of STEC infections in New Zealand.

Acknowledgements

It was five years ago, April 2009, when I set sail to a long but adventurous journey, called a PhD. It was a life-changing journey loaded with numerous rewarding challenges, fulfilling experiences, and valuable friendships I have made. There are a number of people to whom I express my deep gratitude for their considerable help and contribution to this rather large research project and thesis.

First and foremost, to my excellent supervisors, Professor Nigel French, Dr Adrian Cookson, Dr Deborah Prattley, Dr Donald Campbell, and Dr Steve Hathaway, thank you for all your knowledge, inputs, and inspiring guidance during the study and writing of my thesis.

Thanks must go to the New Zealand beef industry, particularly the managers of the four beef slaughter plants, who consented to participate in the nationwide prevalence study, and their staff, who were equally helpful and accepted my presence during sampling without complaint. Special thanks must also go to the dairy farmers in the Waikato, who participated in the transport and lairage study and allowed me to sample their calves during the busy time of calving.

Angie Reynolds deserves special appreciation. I could not have asked for a better laboratory technician to help me with the processing of samples. Her assistance was so precious to me, especially during the weeks of intensive sampling for the transport and lairage study, when the laboratory workload was gigantic. Angie – you are a fabulous and hard-working technician with great organisational skills. Thanking you is not enough.

I am also indebted to several other ^{m}EpiLab staff members; Dr Anne Midwinter and Errol Kwan for providing valuable advice on microbiological and molecular laboratory techniques; and Sarah Moore, Rukhshana Akhter, Dr Julie Collins-Emerson, Rebecca Pattison, and Lynn Rogers for providing their help in the laboratory when needed. I would also like to acknowledge Dr Jonathan Marshall for his contribution on the statistical analysis of data; Simon Verschaffelt for helping with any type of software- or computer-related issues, and

Christine Cunningham for dealing with all other administrative and contract-related issues of my research project.

I also thank my fellow students, colleagues and friends from the *m*EpiLab and EpiCentre, especially Barbara Binney, Hamid Irshad, Fang Fang, Kruno Bojanic, Zoë Grange, Anja Friedrich, Rima Shresta, Ben Phiri, Anou Dreyfus, Naomi Cogger, Jackie Benschop, and Charlotte Bolwell for their kind support throughout my study.

Jaimie Hunnam and Lesley Stringer, you were an inspiration for me. Thank you!

My sincere thanks also to people from other organisations for their collaboration and participation in different studies of this research project; the Food Microbiology and Safety Team from AgResearch (Ruakura, Hamilton, New Zealand), the Health Intelligence Team at the Institute of Environmental & Science Research Ltd (ESR) (Porirua, New Zealand), staff from UMR Research Ltd (Wellington, New Zealand), Muriel Dufour from the Enteric Reference Laboratory (ERL) (Upper Hutt, New Zealand), and Brent Gilpin from ESR (Christchurch, New Zealand).

I would also like to acknowledge the financial support of the Meat Industry Association of New Zealand (Inc) (Wellington, New Zealand) and the Ministry for Primary Industries (MPI) (Wellington, New Zealand), who generously funded this research project; Massey University for awarding me a Doctoral Scholarship, and the Institute of Veterinary, Animal and Biomedical Sciences for financial support to attend several national and international conferences.

Most of all I would like to express my special thanks and love to my parents in Switzerland and Bryce for continued moral support, encouragement and love throughout my PhD.

Declaration

Studies presented in this thesis are written in manuscript style prepared for publication in peer-reviewed journals, hence there is some repetition, particularly in the introductions and methodologies. Two chapters have been formatted in style required of the journals, to which they have been submitted. Co-authors of the papers have made their contributions to the research and/or manuscripts, however, my input was the greatest as I designed the studies, conducted all the fieldwork; entered and analysed all data, and wrote the manuscripts. Angie Reynolds, a designated laboratory technician from the ^{m}EpiLab, helped me with the processing of samples.

Nomenclature

A/E lesions	Attachment and effacement lesions
AIC	Akaike information criterion
AMOVA	Analysis of molecular variance
BPW	Buffered peptone water
CFU	Colony forming unit
CI	Confidence interval
CT-RMAC	Cefixime-tellurite rhamnose MacConkey agar
CT-SMAC	Cefixime-tellurite sorbitol MacConkey agar
DNA	Deoxyribonucleic acid
EHEC	Enterohaemorrhagic *Escherichia coli*
ERL	Enteric Reference Laboratory
ESR	Institute of Environmental Science & Research Ltd
HUS	Haemolytic uraemic syndrome
IMS	Immunomagnetic separation
MPN	Most probable number
OR	Odds ratio
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
rRNA	Ribosomal ribonucleic acid
SBI	Shiga toxin-encoding bacteriophage insertion
STEC	Shiga toxin-producing *Escherichia coli*
Stx	Shiga toxin
TSB	Tryptic (or tryptone) soy broth
UPGMA	Unweighted pair group method with arithmetic mean
US or USA	United States of America

Glossary – New Zealand specific descriptions and terminology

Pastoral farming

New Zealand's beef and dairy production systems are pasture-based with year-round grazing (supplemented with hay, baleage, maize silage, or palm kernel during winter), outdoor housing, and seasonal calving in late winter/early spring (July/August to September/October). In 2013, 3.7 million beef cattle and 6.6 million dairy cattle (including bobby calves) were recorded [1].

Bobby calves

Bobbies are milk-fed calves, which are not reared for dairy replacement stock or the dairy beef market, but are slaughtered at 4–7 days of age and exported as veal to overseas markets (mainly the US).

Cattle slaughter system

Slaughter cattle in New Zealand comprise bobby calves and adult cattle. Adult slaughter cattle consist of dairy and beef animals, which are classified according to sex and maturity as bulls, steers, heifers, and cows [2]. Bulls are entire male cattle, compared to steers, which were castrated at a young age; heifers and cows are female cattle with ≤4 and >4 permanent incisors, respectively.

Beef slaughter plant

In 2013, there were 55 beef slaughter plants actively processing adult cattle (and bobby calves) including slaughter and dressing and/or further processing of carcasses at primary industry level. Of those 55 beef plants, 42 were listed on the National Microbiological Database (NMD) as undertaking STEC-testing of beef as required for overseas market access.

Transportation and lairage of slaughter cattle

Adult cattle are usually transported as a mob in a livestock transporter from a farm to a slaughter plant where the animals are kept in separate holding pens in the lairage area until

slaughter. Adult cattle of the same mob are slaughtered consecutively as a line. In contrast, bobby calves are collected by a regional livestock transporter, picking-up calves from local dairy farms on a pre-determined route, and transported collectively to a slaughter plant where the calves are kept in large holding pens until slaughter. Bobby calves are slaughtered as large groups from different regions.

National Microbiological Database (NMD)

NMD is a mandatory food safety programme for New Zealand primary processors of meat, poultry, game and ratites, which receive live animals for slaughter, dressing or other processing to produce food suitable for human consumption [3]. The microbiological monitoring programme is controlled by the Ministry for Primary Industries (MPI, Wellington, New Zealand) and aims to minimise the incidence of foodborne pathogens. It ensures common microbiological standards for food sold on the domestic market and to meet the export requirements set by destination countries.

Human disease notification system

General practitioners/medical centres/hospitals notify cases of STEC infections to regional Public Health Units. Any notified case is then investigated by a Public Health Officer/Medical Officer, who completes a case report form and enters epidemiological data into a national surveillance database (EpiSurv) used for notified cases of communicable and other diseases. EpiSurv is held by the Institute of Environmental Science & Research Ltd (ESR). Most of STEC cases are confirmed by culture isolation of STEC from clinical specimen submitted to medical laboratories or the Enteric Reference Laboratory (ERL).

*m*EpiLab

Unless stated otherwise, laboratory testing of samples and isolates used in studies presented in this thesis was conducted at the Molecular Epidemiology and Public Health Laboratory (*m*EpiLab), which is located within the Hopkirk Research Institute at Massey University in Palmerston North, New Zealand.

List of Publications

Jaros P, *et al*. (2014) Geographic divergence of bovine and human Shiga toxin-producing *Escherichia coli* O157:H7 genotypes, New Zealand. *Emerging Infectious Diseases*, doi: 10.3201/eid2012.140281 (e-published ahead of print).

Jaros P, *et al*. (2014) Geographic divergence of bovine and human Shiga toxin-producing *Escherichia coli* O157:H7 genotypes in New Zealand. (Australasian Epidemiological Association (AEA) Annual Scientific Meeting, 8–10 Oct 2014; Auckland, New Zealand). *Australasian Epidemiologist*, Vol. 21 (3), p 65.

Jaros P, *et al*. (2014) International divergence of bovine and human Shiga toxin-producing *Escherichia coli* O157:H7 genotypes. (International Association for Food Protection (IAFP) 2014 Annual Meeting, 3–6 Aug 2014; Indianapolis, Indiana, USA). *Journal of Food Protection* Suppl. A, Vol. 77, p 161.

Jaros P, *et al*. (2014) Between- and within-island divergence of bovine and human Shiga toxin-producing *Escherichia coli* O157:H7 genotypes in New Zealand. In: *Proceedings of the Food Safety, Animal Welfare & Biosecurity Branch of the New Zealand Veterinary Association (NZVA)* (16–20 Jun 2014; Hamilton, New Zealand), pp 127-128.

Jaros P, *et al*. (2013) A prospective case-control and molecular epidemiological study of human cases of Shiga toxin-producing *Escherichia coli* in New Zealand. *BMC Infectious Diseases*, doi:10.1186/1471-2334-13-450.

Jaros P, *et al*. (2013) Source attribution for human cases of Shiga toxin-producing *Escherichia coli* in New Zealand. (IAFP 2013 Annual Meeting, 28–31 Jul 2013; Charlotte, North Carolina, USA). *Journal of Food Protection* Suppl. A, Vol. 76, p 58.

Jaros P, *et al*. (2013) Source attribution for human cases of Shiga toxin-producing *Escherichia coli* in New Zealand. In: *Proceedings of the Food Safety, Animal Welfare & Biosecurity Branch of NZVA* (3–4 Jul 2013; Palmerston North, New Zealand), p 11.

Jaros P, *et al*. (2012) An analytical source attribution study on human cases of Shiga toxin-producing *Escherichia coli* in New Zealand. In: *Book of Abstracts of the Annual meeting of the New Zealand Microbiological Society (NZMS)* (26–29 Nov 2012; Dunedin, New Zealand), A2.5 (p 50).

Jaros P, *et al*. (2012) Molecular epidemiology: a tool for source attribution investigation of *Escherichia coli* O157:H7 infections in New Zealand. In: *Proceedings of the 13th International Symposium on Veterinary Epidemiology and Economics (ISVEE)* (20–24 Aug 2012; Maastricht, The Netherlands), p 452.

Jaros P, *et al*. (2012) Population dynamics of *E. coli* O157 and O26 – the effect of transport and lairage on faecal shedding and carcase contamination of very young calves in New Zealand. In: *Proceedings of the 8th International Symposium on Shiga Toxin (Verocytotoxin) Producing Escherichia coli Infections, VTEC 2012* (6–9 May 2012; Amsterdam, The Netherlands), pp 70-71.

Jaros P, *et al*. (2012) Molecular epidemiology of human and bovine *Escherichia coli* O157:H7 isolates in New Zealand. In: *Proceedings of the 8th International Symposium on Shiga Toxin (Verocytotoxin) Producing Escherichia coli Infections, VTEC 2012* (6–9 May 2012; Amsterdam, The Netherlands), p 99.

Jaros P, *et al*. (2011) Shedding of *Escherichia coli* O157:H7 and O26 STEC by slaughter cattle in New Zealand. In: *Book of Abstracts of the Annual meeting of NZMS* (23–25 Nov 2011; Palmerston North, New Zealand), p 86.

Jaros P, *et al*. (2010) Prevalence of faecal shedding of *E. coli* O157:H7 and non-O157 STEC in New Zealand slaughter cattle. In: *Book of Abstracts of the Annual meeting of NZMS* (30 Nov–3 Dec; Auckland, New Zealand), p 129.

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Preface

"Learn from yesterday, live for today, hope for tomorrow.
The important thing is not to stop questioning."

Albert Einstein

Chapter 1

Introduction

1.1 General background

Escherichia coli O157:H7 and related non-O157 Shiga toxin-producing *E. coli* (STEC) strains are recognised as important zoonotic pathogens globally. They can cause clinical diseases ranging from diarrhoea and bloody diarrhoea to the life-threatening haemolytic uraemic syndrome (HUS); young children and the elderly are at higher risk of developing HUS [4]. Ruminants, including cattle, are considered to be an important reservoir of STEC, shedding the pathogen via faeces [5-7].

Possible exposure pathways of STEC infection in humans are via STEC-shedding animals, faecally-contaminated food, the environment, and infected humans. Food products of animal and plant origin, contaminated directly or indirectly with STEC containing ruminant faeces before or after processing, have been confirmed as vehicles of disease transmission in STEC outbreaks and sporadic STEC infections. Implicated food vehicles included raw milk [8, 9], unpasteurised cheese [10], uncooked cookie dough [11], leafy green vegetables [12-16], sprouted seeds [17, 18], unpasteurised juice including cider [19-21], sausages [22, 23], and undercooked hamburgers [24-26].

STEC O157 (*E. coli* O157:H7) first emerged as a foodborne pathogen in 1982, causing two large outbreaks of disease associated with undercooked meat patties in patrons of restaurants of a fast-food chain in the United States (US) [26]. Throughout the 1980s, this pathogen was associated with several other foodborne illnesses/outbreaks in the US, which often implicated improperly cooked ground beef as the vehicle for transmission. A large outbreak in the western US in 1992-1993, causing more than 700 clinical cases of STEC infections and four deaths [25], prompted regulatory actions aimed at reducing the public health risks associated with STEC O157 in ground beef [27]. The Food Safety and Inspection Service of the United States Department of Agriculture (USDA FSIS) declared STEC O157 as an adulterant of raw ground beef in 1994 [28]. Furthermore, to stimulate industry testing and other actions to

reduce the presence of STEC O157 in ground beef, the USDA FSIS started a microbiological testing programme on 17 October 1994 [29].

Over recent decades, other non-O157 STEC serogroups have been recognised as causal pathogens of STEC infections in humans in the US; most frequently STEC serogroups O26, O45, O103, O111, O121, and O145. Hence, on 4 June 2012, the USDA FSIS extended its testing programme for raw ground beef to these six non-O157 STEC serogroups, in addition to STEC O157, and to raw beef manufacturing trimmings intended for use in raw, non-intact products [30].

1.2 STEC in New Zealand

To provide assurance of an STEC O157-free product, and to meet market access requirements for beef exports to the US, in 1997 New Zealand's meat industry agreed to a programme for microbiological process control, the National Microbiological Database (NMD) programme. Since 2005, the NMD programme has also been applied to domestic products under specifications of the Animal Products Act 1999.

The extended testing requirements for STEC O157 and the six non-O157 STEC have become a costly aspect of international trade access conditions for New Zealand. A failure to restrict the contamination of these pathogens to extremely low levels in exported meat is a market access risk with devastating consequences for the international trade of New Zealand; losses in verification credibility and product values, and intensified monitoring at greater cost to the meat industry would be expected. To enable the implementation of tailor-made and cost-effective controls that significantly reduce the level of contamination of red meat (beef and veal) produced in New Zealand, data are needed on the basic epidemiology of STEC O157 and non-O157 STEC in slaughtered cattle and the levels of STEC entering the food chain.

In addition to the market access risks associated with these pathogens, STEC poses a public health threat to New Zealand's population. New Zealand's annual incidence rate of 4.6 reported STEC cases/100,000 population (2013) [31] is, along with Scotland [32], Ireland, Denmark, and Sweden [33], among the highest in the world. STEC infections in New Zealand appear as sporadic cases or small clusters, with outbreaks of STEC infections being rare, suggesting that food is not a significant exposure pathway. However, little is known

about the relative importance of cattle as a reservoir, or the relative contribution of different exposure pathways to human cases of STEC in New Zealand, highlighting the need for research to better understand the epidemiology and source attribution of human illness in New Zealand.

The Ministry for Primary Industries (MPI), formerly New Zealand Food Safety Authority (NZFSA), has developed risk management strategies for major foodborne pathogens prevalent in New Zealand, including *Campylobacter*, *Salmonella*, and *Listeria*, which pose a high risk to public health and New Zealand's access to international markets. However, there is insufficient understanding of the epidemiology of STEC in New Zealand and a similar 'farm-to-fork' risk management strategy for STEC O157 and related non-O157 STEC cannot yet be implemented. Therefore, a hazard reduction science programme for STEC has been established [34], supporting various research projects, which represent interests of the New Zealand meat industry and the public health sector. For example, work presented in this thesis was conducted as part of a research project funded by MPI and the New Zealand meat industry as a contribution to the development of the risk management strategy for STEC.

1.3 Thesis aims and structure

The primary aims of the thesis were to provide detailed knowledge on the epidemiology of STEC in New Zealand slaughter cattle, and to investigate the source attribution of human STEC infections in New Zealand.

Three key research questions were posed, namely:

a) What is the genotype-specific prevalence (and concentration) of STEC O157 and non-O157 STEC strains in New Zealand dairy and beef populations at the time of slaughter, including very young calves?

b) What are the risk factors associated with high prevalence (and concentration) of STEC O157 and non-O157 STEC strains in/on animals at the time of slaughter, including the impact of transportation from farm to the slaughter premises on carcass contamination of very young calves?

c) What are the risk factors associated with STEC infections in humans in New Zealand?

To answer these questions, a series of epidemiological field-based studies were conducted as illustrated in Figure 1.1. It shows the order studies are presented in this thesis, and how data generated by one study were also used in other areas of the PhD project. All but one of the studies were conducted in collaboration with other New Zealand organisations, such as AgResearch (Hamilton), the Institute of Environmental Science & Research Ltd (ESR, Porirua), and MPI (Wellington).

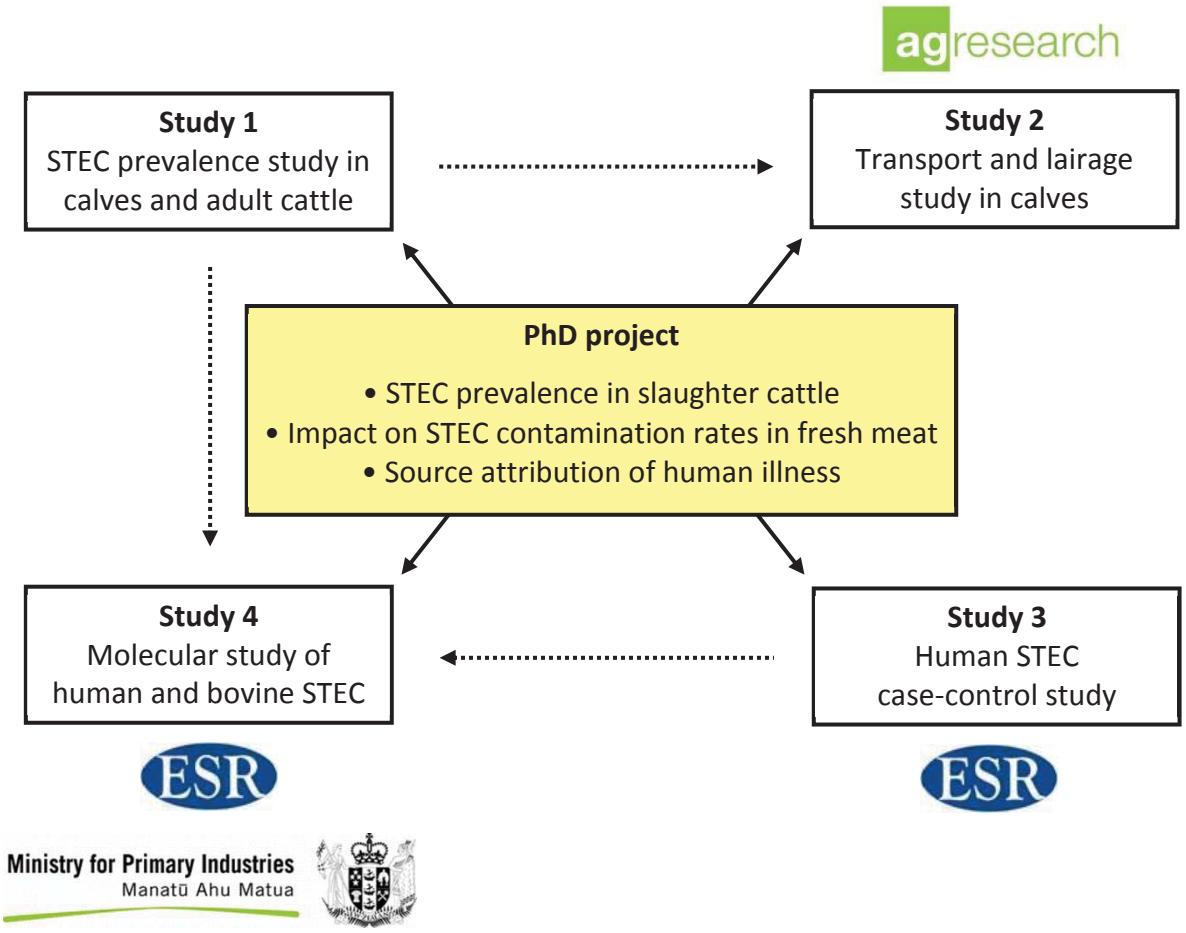

Figure 1.1: General outline of PhD research showing the order of epidemiological field-based studies presented in this thesis, and how study outcomes and data were used in other studies of the project (dashed lines).

The scientific work of the individual studies is described in Chapters 3 to 6. A brief overview of each study is given below.

Chapter 2: Literature review

The first chapter of this thesis reviews the current knowledge on the epidemiology of STEC in humans and cattle, with particular focus on STEC serogroups O157 and O26, and highlights areas where more scientific work is needed; with respect to red meat production, food safety, and veterinary public health in New Zealand.

Chapter 3: Prevalence study (Study 1)

The primary objective of the first study was to gain detailed epidemiological knowledge on the prevalence and concentration of STEC O157 and STEC O26 in New Zealand slaughter cattle nationwide. A cross-sectional study was chosen as the most suitable observational study design for this task, being ideal to measure prevalence, as exposure and outcome status of study subjects are obtained at the same point in time. To gather data on the nationwide prevalence of STEC in slaughter cattle, four large beef slaughter plants licenced for beef and veal export markets were selected from across the country. To assess the seasonality of faecal shedding of STEC in adult cattle and very young calves, each plant was visited repeatedly over a study period of two years, resulting in a repeated cross-sectional study design.

Chapter 4: Transport and lairage study (Study 2)

Epidemiological information gathered in the first year of Study 1 provided information essential to the design of the second observational study. A cohort study was chosen to evaluate the impact of transportation and lairage on the rectal carriage and carcass contamination with STEC O157 and STEC O26 in very young calves at slaughter. Cohorts of animals destined for slaughter were followed from their farm of origin to a slaughter plant to investigate risk factors associated with the prevalence (and concentration) of *E. coli* strains in/on very young calves at the time of slaughter. This study was conducted in collaboration with AgResearch staff experienced in the collection and laboratory analysis of carcass samples.

Chapter 5: Human case-control study (Study 3)

A prospective case-control study was designed to investigate the attribution of STEC O157 and non-O157 STEC in cattle populations to disease in humans in New Zealand, examining different exposure pathways such as food, animals, environment, and people. Over a period of 12 months, any confirmed STEC case notified to the regional public health units across the country, together with a random selection of controls representative of the national demography, were interviewed for risk factor evaluation. This observational study was executed in close collaboration with an experienced team from ESR Ltd.

Chapter 6: Molecular epidemiological study (Study 4)

The last study was conducted to compare the population structure and geographical distribution of genotypes of STEC O157 isolated from cattle (Study 1) and human cases (Study 3) in New Zealand, using molecular typing methods. With the addition of isolates from other sources, the study assessed evidence for localised transmission of STEC from cattle to humans in New Zealand and provided molecular findings to support results of the source attribution study of human illness (Study 3). Furthermore, New Zealand genotypes of STEC O157 were compared with isolates from Australia and the United States to illustrate the international divergence of STEC O157 genotypes.

Chapter 7: General discussion

The last chapter of this thesis discusses the outcomes of the PhD research and its implications to the New Zealand meat industry and the public health sector, and the understanding of the epidemiology of STEC in New Zealand and overseas, highlighting areas for further research.

Chapter 2

Literature review

2.1 Introduction

Since the first outbreak of human disease associated with Shiga toxin-producing *Escherichia coli* (STEC) was reported in 1982 [26], STEC have emerged as pathogens of significant importance to public health worldwide; causing life-threatening illnesses such as haemorrhagic colitis and haemolytic uraemic syndrome (HUS), affecting particularly young children and the elderly [4]. The epidemiology of STEC has been a focus of research in different parts of the world, but there remain some major gaps in our understanding of the transmission of infection and the determinants of disease in humans. Currently, ruminants, particularly cattle are considered an important reservoir of STEC and have been identified as a source of foodborne and environmental outbreaks of STEC [35, 36] and sporadic STEC infections in humans [37, 38]. STEC serogroups frequently associated with the causation of human disease are O157, O26, O103, O111, and O145 [39]. The importance of STEC O157 and related non-O157 STECs in New Zealand's slaughter cattle population and the risk to public health are unknown and serve as objectives for our studies presented in this thesis, with the main focus on STEC O157 and STEC O26.

2.2 *Escherichia coli* – the organism

2.2.1 Discovery

The bacterium *Escherichia coli* (*E. coli*) was first discovered by Theodor Escherich, a German-Austrian paediatrician and bacteriologist, in 1885, whilst investigating the cause of fatal intestinal diseases in children [40, 41]; Escherich considered this bacterium as a commensal of the infant gastrointestinal tract and named it *Bacterium coli commune*; the bacterium was later renamed *Escherichia coli* in his honour.

2.2.2 Classification of *E. coli*

E. coli belongs to the family of Enterobacteriaceae (genus *Escherichia*), and is, in general, classified as a harmless commensal of the lower intestine in humans and animals. There are two distinct approaches to classify *E. coli* strains systematically. Firstly, serology is used to classify *E. coli* by its O and H antigen, where the O antigen is part of the lipopolysaccharide (LPS) in the outer membrane of the bacterium, and the H antigen the bacterial flagellum; 181 O antigens and 56 H antigens of *E. coli* have been described [42]. Secondly, *E. coli* can also be classified into groups of pathovars, according to their pathogenicity and clinical symptoms they cause [43, 44]. The six currently recognised pathovar groups are [42, 45]:

(1) Entero-pathogenic *E. coli* – EPEC,
(2) Entero-toxigenic *E. coli* – ETEC,
(3) Entero-invasive *E. coli* – EIEC,
(4) Entero-aggregative *E. coli* – EAEC,
(5) Diffusely adherent *E. coli* – DAEC, and
(6) Shiga toxin-producing *E. coli* – STEC.

STEC are defined through the production of at least one of two antigenically distinct cytotoxins referred to as Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) [46]. In humans, these toxins can affect the host's intestine and kidney tissues and cause haemorrhagic colitis or HUS leading to potentially life-threatening kidney failures [26]. Since 1982, when *E. coli* O157:H7 emerged as a new pathogen and caused two large outbreaks of human disease in the United States (US) [26], this serotype has become the most important STEC strain (STEC O157). In addition to *E. coli* serotype O157:H7, more than 100 different serotypes of *E. coli* have been identified as STEC strains causing human illness [47]. STEC strains considered of public health importance include *E. coli* serogroups O26, O45, O91, O103, O111, O113, O121, and O145 [48, 49]. A commonly used classification divides all STEC strains, from humans and animals, into two groups: STEC O157 and STEC non-O157.

Most of *E. coli* pathogens can be grouped into the above pathovars. Nonetheless, some *E. coli* strains can represent pathogenic characteristics of two pathovar groups, for example the outbreak strain O104:H4 in Germany/France in 2011. This strain was a hybrid pathotype of EAEC and STEC [50, 51].

2.2.3 History of STEC (VTEC)

The cytotoxic effect of Shiga toxin (Verotoxin) produced by STEC was described for the first time by Konowalchuk *et al.* [52] in the late 1970s. They reported that certain strains of *E. coli* produced a heat-labile toxin antigenically different to the known heat-labile enterotoxin of *E. coli* and had a cytotoxic effect on Vero cells[1]. O'Brien *et al.* [53] verified in 1983 that isolates of *E. coli* O157:H7 produce a cell-associated cytotoxin for HeLa and Vero cells, which could be neutralised by antiserum to purified Shiga toxin[2], and concluded that Shiga-like toxin and Vero cytotoxin were the same toxins. In 1985, Scotland *et al.* [54] detected a toxin with cytotoxic effects on Vero cells distinct to that described in 1983. The authors adopted the nomenclature and suggested naming the toxins Verotoxin 1 (VT1) and Verotoxin 2 (VT2), hence the name Verocytotoxin-producing *E. coli* – VTEC. Strockbine *et al*. [55] designated the same cytotoxins as Shiga-like toxin I (SLT-I) and Shiga-like toxin II (SLT-II), which resulted in naming Shiga toxin-producing *E. coli* – STEC. In 1996, Calderwood *et al*. [56] proposed to call the Shiga toxins Stxs, which are encoded on genes (*stx*1 and *stx*2) of the bacterial chromosome, originally acquired from bacteriophages [57]. Since then, the names STEC and VTEC have been used interchangeably, as well as the terms Shiga toxin and Verotoxin [46].

2.3 Epidemiology of STEC in humans

2.3.1 STEC O157

The clinical importance of STEC as a causal pathogen of human disease became apparent in the early 1980s. The first reports of *E. coli* O157:H7 infections in humans were described in the US by Uyeyama [58] in 1982. They reported the identification of *E. coli* O157:H7 isolates from specimens of four patients with bloody diarrhoea, designated as haemorrhagic colitis. These STEC cases occurred sporadically but were similar to two separate outbreaks of *E. coli* O157:H7 infections in Oregon (26 cases) and Michigan (21 cases) earlier in 1982, associated with the consumption of undercook beef patties from a national chain of fast-food restaurants [26]. Riley *et al.* [26] established that haemorrhagic colitis in affected patients was due to the infection with *E. coli* O157:H7. Although it is believed that *E. coli* O157:H7 emerged as a new pathogen in 1982, it was previously isolated from a sporadic case of

[1] A cell lineage of epithelial kidney cells originally isolated from an African green monkey.
[2] The bacterium *Shigella dysenteriae* type 1 produces a cytotoxin called Shiga toxin.

haemorrhagic colitis in a 50-year-old California woman in 1975, showing acute, self-limiting, and grossly bloody diarrhoea (cited by [26]).

At the same time, Karmali *et al.* [59] reported the isolation of Verocytotoxin-producing *E. coli* from eight stool samples of 15 sporadic cases with HUS. Affected HUS patients were children of one month to 12 years of age with a history of having had bloody diarrhoea for between 4–10 days before the diagnosis of HUS. Some children required life-saving treatments such as dialysis and/or blood transfusions. Major central nervous system diseases like seizures and prolonged coma were observed in four children; two deaths were reported. STEC isolated from stool samples included *E. coli* serogoups O26, O111, O113, and the serotype O157:H7, providing strong evidence of STEC as the aetiological agent for HUS.

Following its recognition as a causal pathogen of haemorrhagic colitis and HUS in the early 1980s, *E. coli* O157:H7 has become the most recognised STEC serotype. Numerous outbreaks of gastrointestinal illness due to STEC O157 have been reported from overseas including the US [11, 16, 25, 60, 61], Scotland [62], England [63, 64], and Japan [18, 65]; the majority of them being foodborne. A US study [66] reviewed 350 STEC O157 outbreaks (defined as ≥2 cases confirmed with *E. coli* O157:H7 infection and a common epidemiologic exposure) reported to the Centres for Disease Control and Prevention (CDC) from 1982 through 2002 and highlighted that 183 (52.3%) outbreaks were foodborne, 50 (14.3%) due to person to person transmission, 31 (8.8%) waterborne, and only 11 (3.1%) due to animal contact (74/350 (21.1%) of unknown source). Ground beef and contaminated produce were identified as the food vehicle for 75 (41.0%) and 38 (20.8%) of foodborne outbreaks, respectively.

Although intense media and scientific research interest is associated with STEC outbreaks, most reported STEC cases do not occur as part of outbreaks (e.g. foodborne or due to other common sources) but as sporadic infections. For example, although Scotland has reported two large foodborne outbreaks of STEC O157 in 1994 [67] and 1996 [62], it has one of the highest annual incidence rates of STEC O157 infections worldwide (4.5 per 100,000 in 2012 [32]), with sporadic STEC O157 infections accounting for the majority of cases. Different pathways of exposure are suggested for sporadic STEC cases as compared to outbreaks [68].

2.3.2 STEC O26

Although most outbreaks and sporadic cases of STEC infections in humans in the past have been caused by strains of STEC serotype O157:H7, an increase in non-O157 STEC cases has been observed in the US [69] and the European Union (EU) [33] over the past decade. For example in the US, the incidence of non-O157 STEC infections increased from 0.12 per 100,000 population in 2000 to 0.95 per 100,000 in 2010 [48], with the most commonly reported non-O157 STEC serogroups being O26, O103, O111, O121, O45, and O145 [48]. STEC O26 is the most frequently isolated non-O157 STEC strain in association with human disease in the US [70] and the EU [71-73]. This overall increase of non-O157 cases could be attributable to the improved laboratory methods for isolation of non-O157 STEC serogroups and the additional laboratory testing of faecal specimens for non-O157 STECs.

STEC O26 has emerged as one of the most clinically relevant non-O157 STEC associated with human disease [43, 74]. Sporadic cases and outbreaks of diarrhoea or HUS due to STEC O26 have been reported in the US [75], Europe [22, 76-78], and in other parts of the world such as South America [79], Australia [80], New Zealand [81], and Japan [82], showing the worldwide distribution of this pathogen.

O26:H11 and O26:H$^-$ are the most frequently isolated serotypes from clinical STEC O26 cases [45, 48, 83] and can possess *stx*1 and/or *stx*2 genes. *stx*1 is detected in most STEC O26 strains isolated from clinical cases [22, 70], but there are increasing reports from Europe of STEC O26 strains harbouring *stx*2 [71, 83].

A distinctive feature of diarrheagenic *E. coli* O26 strains is the diversity of virulence factors that they possess. Some strains found in humans as well as in domestic animals of different species share STEC-associated virulence factors, but do not possess *stx* genes, which can be acquired by horizontal gene transfer [84]. Often these *stx*-negative strains possess the *eae* gene that codes for an outer membrane protein, intimin; a virulence gene profile characteristic of EPEC. However, another characteristic of EPEC absent from *E. coli* O26 is the presence of bundle forming pili (*bfp*) [85]. Therefore, these *bfp*-negative EPEC are described as atypical EPEC (aEPEC), a heterogeneous assortment of serotypes associated with prolonged mild diarrhoea in humans and carried by several bird and mammal species [86]. The mobile nature of *stx*-encoding bacteriophage has been noted on a number of previous occasions. For

example, during an outbreak in Germany in 1999, STEC O26:H11 strains (*stx*2 positive) were isolated from initial stool samples of three children and aEPEC O26:H11 (*stx*2 negative) were detected in their follow-up samples [87], suggesting the modification of an STEC O26 to an aEPEC during infection through loss of the *stx* gene. Similarly, another study in Germany investigated sequentially collected stool samples from 210 HUS patients between 1996 and 2003 for the presence of *E. coli* strains possessing *stx* and/or *eae* genes [88] and reported the loss of *stx* genes in 6 of 21 STEC serotypes O26:H11 but in none of 61 STEC serotype O157:H7, providing supporting evidence that this phenomenon does occur in STEC O26. The loss or acquisition of *stx*-encoding bacteriophage and/or an *stx* gene during infection might have clinical implications. For example, the loss of the *stx* gene at an early stage of infection (before the production of Stx) might prevent the development of HUS, while the loss at a later stage (after production of Stx and tissue damage) could confound diagnostic methods applied and lead to misdiagnosis [87].

2.3.3 Annual incidence rates and seasonality of STEC

STEC infections are considered as a serious public health risk and are classified as notifiable diseases in many countries around the globe. Despite the worldwide spread of STEC, annual incidence rates of STEC infections vary considerably between countries, which might be due to differences in reporting and health surveillance systems. An overview of annual STEC incidence rates in developed countries is presented in Table 2.1.

Table 2.1: Annual incidence rates (per 100,000 population) of confirmed STEC infections listed by countries.

Country	Year	Incidence rate	Number of confirmed cases	Reference
New Zealand	2013	4.6	207	[31]
Australia	2011	0.4	95	[89]
Austria*	2011	1.43	120	[90]
Belgium*	2011	0.91	100	[90]
Bulgaria*	2011	0.01	1	[90]
Canada	2012	1.39[a]	485[a]	[91]
Cyprus*	2011	0	0	[90]
Czech Republic*	2011	0.07	7	[90]
Denmark*	2011	3.87	215	[90]
Estonia*	2011	0.30	4	[90]
EU total	2011	1.93	9,485	[90]
Finland*	2011	0.50	27	[90]
France*	2011	0.34	221	[90]
Germany*	2011	6.80	5,558	[90]
Greece*	2011	0.01	1	[90]
Hungary*	2011	0.11	11	[90]
Iceland	2011	0.63	2	[90]
Ireland*	2011	6.14	275	[90]
Italy*	2011	0.08	51	[90]
Latvia*	2011	0	0	[90]
Lithuania*	2011	0	0	[90]
Luxembourg*	2011	2.74	14	[90]
Malta*	2011	0.48	2	[90]
Netherlands*	2011	5.07	845	[90]
Norway	2011	0.96	47	[90]
Poland*	2011	0.01	5	[90]
Romania*	2011	0.01	2	[90]
Slovakia*	2011	0.09	5	[90]
Slovenia*	2011	1.22	25	[90]
Spain*	2011	0.04	20	[90]
Sweden*	2011	4.96	467	[90]
Switzerland	2011	0.90	71	[90]
UK*	2011	2.41	1,509	[90]
United States	2012	1.12 (1.16)[a]	531 (551)[a]	[92]

[a] STEC incidence rate presented for serogroup O157, and if applicable for non-O157 (in brackets).
*Member states of the European Union (EU).

The high annual incidence rate in Germany was associated with a large outbreak of enteroaggregative STEC O104:H4 in May to July 2011 [50], affecting 3,816 individuals, of which 845 developed HUS and 54 cases died [90]. The outbreak was centred in northern Germany and only a very small percentage of cases (2.7% (87/3,241)) with a history of travel to Germany were reported from 15 other countries[3]. Independently of the large O104 outbreak in Germany, EU-countries such as Denmark, Ireland, Sweden, and the Netherlands have reported high STEC incidence rates for 2011.

STEC incidence is reported to follow a regular, cyclical pattern with seasonal peaks in summer as shown in a recent multinational systematic review of seasonality in human zoonotic enteric diseases [93]. The study illustrated that annual STEC incidence rates in Canada, the UK, and Europe peak in summer (July/August) and in the US in autumn (September). Similar seasonal patterns have also been observed in countries of the Southern hemisphere. Vally *et al.* [80] reported that a large proportion of STEC cases in Australia from 2000 to 2010 occurred in the summer months of December to February. In New Zealand, a bimodal seasonal pattern with a higher peak in late summer/early autumn (January to March) and a second, lower peak in spring (August to October) has been apparent [94].

It is likely that the seasonal patterns of STEC disease (and other human zoonotic enteric diseases) are influenced by environmental effects on the pathogen's occurrence and pathogen-host interactions, as well as characteristics and behaviour of the population at risk [93].

2.3.4 Transmission pathways and risk factors for STEC infection

Humans can acquire STEC infections from various sources as illustrated in Figure 2.1. Ruminants, particularly cattle are considered the main host of STEC, shedding the pathogen via faeces [5-7]. The primary and most common transmission pathways of human infection are: (i) faecally-contaminated food, (ii) direct or indirect contact with STEC-shedding animals, or (iii) faecally-contaminated drinking water or recreational water. The secondary transmission pathway from person to person is particularly common in households [95], day care [77] and nursing homes [61, 96, 97].

[3] Number of STEC cases from other countries associated with the O104 outbreak in Germany can be viewed on the last update of the outbreak on: http://www.euro.who.int/en/health-topics/disease-prevention/food-safety/news/news/2011/07/outbreaks-of-e.-coli-o104h4-infection-update-28.

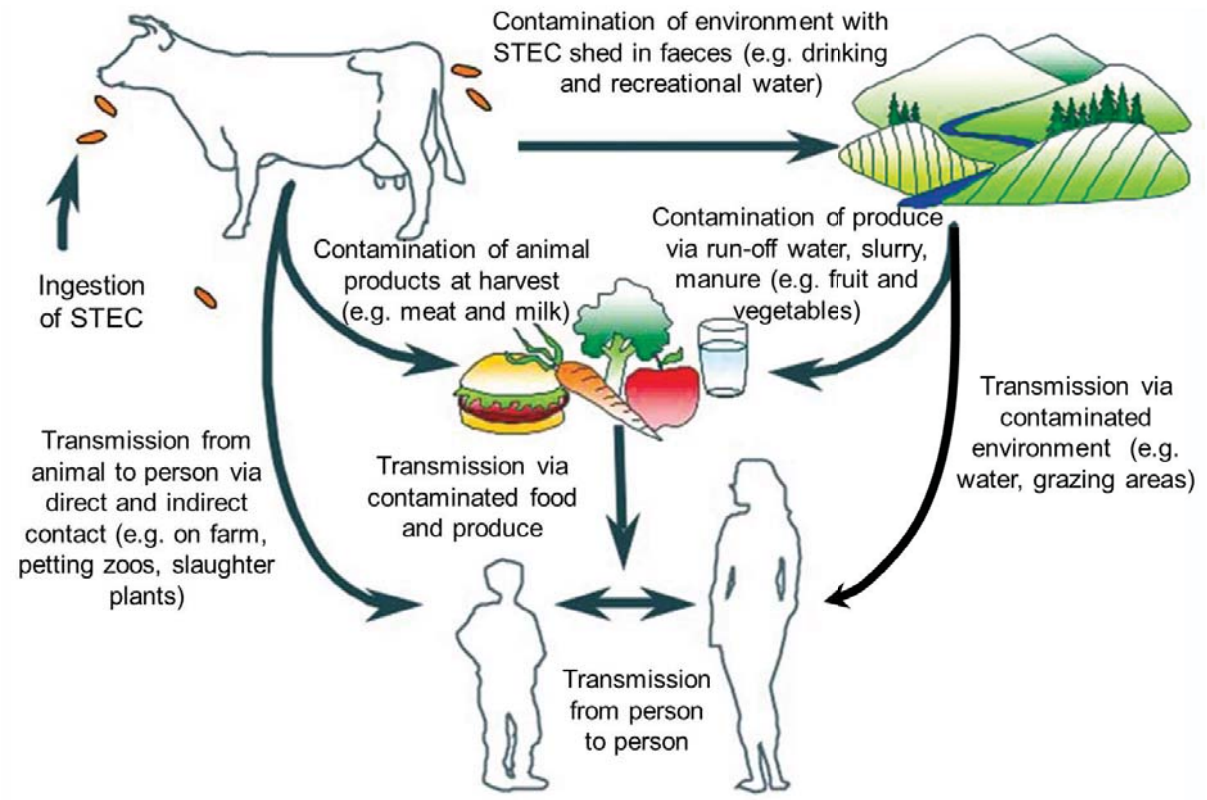

Figure 2.1: Possible routes of STEC contamination of environment, animal products, and food and produce, and transmission pathways of STEC infection in humans (modified from website of the *Escherichia coli* Laboratory (www.ecl-lab.ca)).

A large variety of food products of animal and plant origin have been confirmed as vehicles of STEC transmission in outbreaks and sporadic infections. These included raw milk [8, 9, 38], unpasteurised cheese [10], undercooked hamburgers [24-26], sausages [22, 23], leafy lettuce [16], sprouts [98], and unpasteurised apple cider [20], which were most commonly contaminated directly or indirectly with ruminant faeces containing STEC before or after processing. STEC transmission via direct or indirect contact with STEC-shedding animals has been well documented [99-101]. Faecally contaminated recreational waters and water supplies have been also implicated as likely sources of STEC transmission to humans [102-104]. Surface water contaminated with *E. coli* O157:NM (non-motile; harbouring *stx*1 and *stx*2) caused a large outbreak of bloody diarrhoea in Swaziland, southern Africa, in 1992 [105]. During October to December, over 15,000 children (<5 years-old) and 40,000 individuals (≥5 years-old) were affected with diarrheal disease and sought medical care. *E.*

coli O157 was isolated from environmental samples collected from surface water of a large sugar plantation, various other water sources (borehole, standpipe, river), and cattle faeces at the plantation, and showed indistinguishable molecular typing (pulsed-field gel electrophoresis) patterns. Although the simultaneous occurrence of cholera in some areas might have affected the magnitude of the *E. coli* O157 outbreak, it was believed that severe drought, presence of *E. coli* O157-shedding cattle, and heavy rains with contamination of surface water led to this large environmental outbreak.

Farming environments have been also reported as risk factors of sporadic STEC infections, particularly for young children, suggesting exposures to livestock faeces as the most likely sources of infection. Werber and colleagues [38] conducted a matched case-control study in Germany to identify risk factors for sporadic STEC infections. They reported that children aged <3 years had the highest disease risk when having touched a ruminant, and raw milk was the only food significantly associated with disease. In contrast to this, only food items were identified as risk factors for disease in ≥10 year-olds, indicating that risk factors for sporadic STEC infections are age-specific. Similarly, Rivas *et al.* [37] also described in their case-control study that sporadic STEC infections in children in Argentina were associated with exposures such as living in or visiting a place with farm animals, eating undercooked beef, and having contact with a child <5 years of age with diarrhoea.

2.3.5 Clinical disease

Infections associated with STEC O157 can be asymptomatic but also show a broad spectrum of clinical symptoms in humans as non-bloody diarrhoea, haemorrhagic colitis, HUS, and thrombotic thrombocytopenic purpura [4]. Most patients experience severe abdominal cramps, have low or no fever, and develop bloody diarrhoea within three to four days after onset of disease. In general, the disease is self-limiting and clinical symptoms disappear within about a week, however, the infection can progress to HUS and affect other extra-intestinal organs in association with HUS and be fatal. HUS is characterised by haemolytic anaemia, thrombocytopaenia, and acute renal failure, leading to kidney dysfunctions, seizures, coma, and death. In particular, young children (<4 years) and the elderly (>65 years) are at higher risk of developing HUS [4]. The mortality rate due to HUS is 3% to 5% (cited in [106]).

Clinically, STEC O157 cannot be distinguished from non-O157 infections because of the wide spectrum of symptoms these pathogens can cause. For example, like STEC O157, STEC O26 can cause severe disease including bloody diarrhoea and HUS [107, 108], however, it has been documented that children infected with STEC O26 are more likely to have neurological complications and develop diabetes mellitus [107]. The severity of the STEC infection depends on the patient's age but also on the virulence of the infecting STEC serogroup. A recent retrospective cohort study conducted in Germany [109] analysed data from 8,400 patients diagnosed with community-acquired gastroenteritis or HUS due to STEC infection from January 2004 through to December 2011, to assess difference in disease severity between STEC serogroups using hospitalisation and death as proxies for severity of disease. The study concluded, based on their population-based and age-adjusted data, that STEC O157 shows an exceptionally high virulence in relation to non-O157 STEC serogroups (other than the outbreak strain O104).

The large *E. coli* O104:H4 outbreak in Germany in 2011 was caused by an unusual strain of STEC; a hybrid of EAEC and STEC called EAEHEC. The outbreak strain possessed genes typical of EAEC such as *attA*, *aggR*, *aap*, *aggA*, *aggC* but no other STEC virulence genes than *stx*2 (*stx*1, *eae*, and *ehx* genes were missing) [50]. This strain was exceptionally virulent and affected 3,816 individuals, of which 845 developed HUS and 54 cases died [90]. When compared to previously reported large outbreaks of STEC infection [15, 25, 60], characteristic features of this outbreak were: (i) a larger percentage of cases (22.1% (845/3,816)) developed HUS, (ii) most of HUS cases were adults rather than children (89%, median age: 43 years), and (iii) the estimated median incubation time of 8 days was longer than expected for STEC O157 (3–4 days) [50]. Outbreak investigations implicated organic fenugreek sprouts as the source of this outbreak [98, 110]. Closely related STEC O104:H4 strains were previously isolated from HUS cases in Germany (1 case; strain also *stx*2-positive) [111] and South Korea (1 case) [112]. No clinical case of EAEHEC O104:H4 or STEC O104:H4 infection has ever been reported in New Zealand, but STEC O104:H- and O104:H7 (both *stx*1-positive) have been detected in New Zealand sheep meat [113].

2.3.6 Infectious dose

For pathogenic *E. coli*, the probability of infection and disease is high at low doses based on outbreak investigations and modelling of epidemiological data. Willshaw *et al.* [114] have

reported on an outbreak of STEC O157 in Britain, where an infectious dose of probably 10 organisms was enough to cause haemorrhagic diarrhoea in humans after consumption of contaminated beef burgers. Tilden *et al.* [115] evaluated the infectious dose of STEC O157 in an outbreak of disease associated with the consumption of dry fermented salami in the US in 1994. Based on their calculations, the infectious dose was suggested to have been fewer than 50 STEC O157 organisms. A similarly low dose (4–24 organisms) was estimated to have caused disease in a study by Strachan *et al.* [116], investigating an environmental outbreak of *E. coli* O157 in a scout camp in Scotland. These findings were supported by Hara-Kudo *et al.* [117]. They conducted microbiological risk analysis on existing data of *E. coli* outbreaks occurring in Japan between 2004 and 2006 and reported an infectious dose of 2 to <9 organisms in an *E. coli* O157 outbreak among three members of a household; raw beef liver was the food item associated with this outbreak. In contrast, a STEC O145:H28 outbreak in Belgium suggested that the ingestion of approximately 400 organisms had caused disease (HUS) in five children after eating contaminated ice cream [118].

2.3.7 Pathophysiology

Figure 2.2 illustrates schematically the pathophysiology of STEC infection in humans.

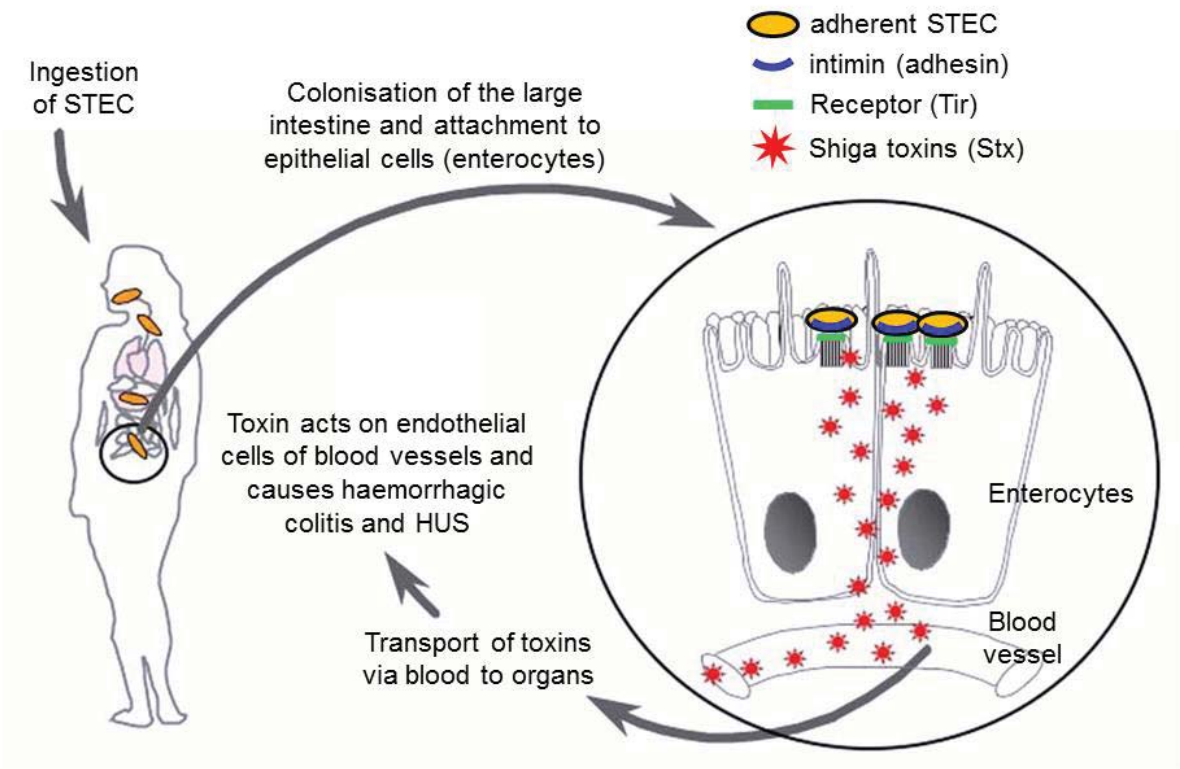

Figure 2.2: Pathophysiology of STEC infection in humans (adopted from website of the *Escherichia coli* Laboratory (www.ecl-lab.ca)).

After ingestion of STEC, the bacteria colonise the host's large intestine by attaching to receptors of epithelial cells (enterocytes) of the mucosal gut tissue. The bacterial adherence (STEC produce intimin, which mediates the attachment to enterocytes) and following effacement of enterocytes causes tissue damage and diarrhoea. STEC produce Shiga toxins, which are transported across enterocytes to blood vessels and distributed systemically to susceptible host organs such as kidneys where they damage endothelial cells and cause renal microvascular thrombosis, clinically apparent as HUS [119]. Microvascular thrombosis can also affect the brain tissue and be clinically evident as encephalopathy. The neurological manifestation of STEC infection is the most common cause of death in children with HUS [120]. In general, the incubation period for *E. coli* O157 is 3–5 days but can range from 1–9 days (cited in [121]), and up to 14 days (cited in [68]). Ryan *et al.* [97] reported a median incubation period of eight days for an STEC O157 outbreak that occurred in a nursing home in the US in 1984. It was much longer compared to the incubation periods described for the two large foodborne outbreaks in Oregon (3.9 days) and Michigan (3.8 days) in 1982 [26].

2.3.8 Pathogenesis and bacterial virulence factors

STEC's characteristic virulence factors are located on the bacterial chromosome and on plasmids. Important virulence genes of STEC are (i) the attachment and effacement gene *eae*, (ii) the *ehx*A gene, and (iii) the Shiga toxin genes *stx*1 and *stx*2, which play a crucial role in the pathogenesis of STEC infection in the host. Table 2.2 summarises the main virulence factors of STEC, their genomic location in the bacterium, microbial structures and functions.

Table 2.2: A summary of main virulence factors of STEC including their genomic location, bacterial structure, and functions in the pathogenesis of STEC infections.

Virulence factor	Gene	Genomic location	Microbial structure	Function in STEC pathogenesis
Intimin	*eae*	Chromosome, LEE[a]	Outer membrane protein	Mediating intimate attachment of STEC to enterocytes
EspA	*esp*A	Chromosome, LEE	Secretory protein	Forming needle-like structure for injection of EspB, EspC, EspD, and Tir into enterocytes (part of T3SS[b])
EspB, EspC, EspD	*esp*B, *esp*C, *esp*D	Chromosome, LEE	Secretory proteins	(part of T3SS)
Tir	*tir*	Chromosome, LEE	Secretory protein	Receptor for intimin in cell membrane of enterocytes (part of T3SS)
Entero-haemolysin	*ehx*A	Plasmid (pO157)	Structural protein	Still unknown
Serine protease (EspP)	*esp*P	Plasmid (pO157)	Enzyme	Probably contributing to mucosal haemorrhage
Catalase-peroxidase (KatP)	*kat*P	Plasmid (pO157)	Enzyme	?
T2SS[c]	*etp*	Plasmid (pO157)	Secretory protein	?
Shiga toxin (Stx)	*stx*	Bacteriophage	Toxin	Inhibiting enterocyte's protein synthesis, exact role still not fully understood

[a] LEE = Locus for enterocyte effacement.
[b] T3SS = Type III secretion system.
[C] T2SS = Type II secretion system.

Attachment and effacement gene (*eae*)

The attachment and effacement gene *eae* and other important virulence genes are responsible for the formation of attachment and effacement (A/E) lesions on intestinal epithelial cells. Characteristics of A/E lesions are the intimate attachment of STEC to enterocytes, the effacement of microvilli and pedestal-like formation of enterocytes [43]. The *eae* gene encodes a bacterial outer membrane protein, intimin, which mediates the intimate attachment of STEC to the enterocytes [43]. It is located on a large chromosomal pathogenicity island known as the 'locus for enterocyte effacement' (LEE) [122], which harbours also other important virulence genes including *esp*A, *esp*B, *esp*C, *esp*D (encoding for *E. coli* secretory proteins EspA to EspD) and *tir* (encoding for translocation intimin receptor Tir) [123], which are part of the type III secretion system (T3SS). EspA forms a filamentous structure through which EspB, EspC, EspD, and Tir are injected into the cytoplasm of the enterocyte [124]; Tir is then inserted as a receptor for intimin in the cell membrane of the enterocyte [123]. The typical A/E lesions are formed when intimin attaches to Tir [125].

Enterohaemolysin gene (*ehx*A)

The enterohaemolysin gene *ehx*A, also known as *hly*A, is an important virulence marker of STEC and has been associated with clinical disease in humans; however its precise role in the complex mechanism of STEC pathogenesis is still not fully understood. *ehx*A encodes for the structural enterohaemolysin protein, which causes haemolysis of washed sheep erythrocytes [126-128]. *In vitro* studies have indicated that enterohemolysin from an STEC O128:H12 isolate increased the levels of proinflammatory cytokine interleukin-1β from human monocytes [126]. The *ehx*A gene is located on a plasmid, an extrachromosomal DNA structure (92 kb to 104 kb) capable of replicating independently from the DNA chromosome. Toth *et al.* [129] named this plasmid pO157, which is carried by almost all *E. coli* O157:H7 strains. Enterohaemolysin was the first described virulence factor of the pO157 plasmid [130, 131]. In addition to *ehx*A, pO157 also harbours other virulence factors involved in the pathogenesis of STEC including serine protease (*esp*P) [132], catalase-peroxidase (*kat*P) [133], and type II secretion system (T2SS) (*etp*) [134].

Shiga toxin genes (*stx*)

stx is the most important virulence marker of STEC. STEC can produce two types of Shiga toxins (Stx) referred to as Stx1 and Stx2. The toxins are encoded on two separate *stx* genes (*stx*1 and *stx*2), which are associated with separate bacteriophage and acquired by the bacterial host through transduction [57, 135]. An STEC strain can possess the *stx*1 or *stx*2 gene, or both genes expressing Stx. Stx1 and Stx2 are serologically different Stxs, showing a homology of only 58% and 56% at the amino acid and nucleotide levels, respectively [135, 136]. Allelic variants have been described for both types of Stx, with three genetic subtypes for Stx1 (Stx1, Stx1c, and Stx1d) and more than 10 subtypes for Stx2, including Stx2, Stx2a Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g [137]. Stx2 is more frequently associated with haemorrhagic colitis and HUS in clinical cases compared to Stx1 [138, 139]. Furthermore, data suggest that clinical manifestations of STEC infection in humans depend on the *stx* genetic subtype of the infecting strain. For example, Friedrich *et al.* [140] observed a significant association between genetic variants *stx*2d and *stx*2e (gene variants of *stx* encoding for Stx2d and Stx2e) and diarrhoea, while *stx*2c was the only variant associated with HUS. Stxs contain a structure consisting of one A subunit (A1) and five identical receptor-binding B subunits (B1 to B5). To enter the enterocyte, B5 subunit binds the toxin to a specific receptor (globotriaosylceramide or Gb_3) on the surface of the enterocyte [43]. After binding to the cell, Stx (A1B5) is endocytosed and the A subunit is translocated through the cytoplasm to the Golgi apparatus and endoplasmic reticulum to inhibit the cell's protein synthesis [141]. After uptake and translocation of Stxs by enterocytes, Stxs are distributed systemically and bind to susceptible cells in other organs, i.e. kidneys, where Gb_3 receptors are present at high concentrations [142]. The exact role of Shiga toxins in mediating intestinal disease, HUS, and neurological disorder is still not fully understood.

2.4 Epidemiology of STEC in animals and environment

Implications of undercooked ground beef (hamburgers) [26] and raw milk [7] as sources of foodborne STEC O157 outbreaks in the early 1980s have led to investigations of cattle as a reservoir of this pathogen. Strategies to reduce the risk of STEC infections in humans require an understanding of the ecology of STEC in cattle and other potential reservoirs. Over 435 and 470 different STEC serotypes have been isolated from cattle and humans, respectively, with great overlap. However, fewer than 10 STEC serotypes cause the majority of clinical disease in humans [143]. These STEC serotypes commonly include serogroups O26, O91,

O103, O111, O113, O121, O145 and O157 [49], which have also been isolated from animals [121, 144]. Despite intensive research over the past decades to identify putative reservoirs of STECs in animals and the environment, the epidemiology of this important zoonotic pathogen is still not fully understood.

2.4.1 Cattle as reservoir

STEC O157 had been prevalent in cattle, years before it emerged as a 'new' pathogen of foodborne outbreaks in Oregon and Michigan (US) in 1982 [26]. Ørskov *et al.* [145] identified STEC O157 strains in cattle faeces, which were collected from 1–3 months-old calves in Argentina in 1977 and were the first to suggest cattle as a reservoir of this zoonotic pathogen. As further outbreaks of STEC O157 infections were associated with products of bovine origin, it became more evident that cattle are the principal host of STEC O157 [146]. With increasing numbers of non-O157 STEC-related outbreaks and sporadic infections being reported globally over the past decade, research has been extended to gain further knowledge of the epidemiology of non-O157 STECs in cattle. Cattle are now considered as the main reservoir of STEC serogroups O26, O103, O111, O145, and O157 [45, 121].

Although STEC-infected adult cattle are generally asymptomatic carriers of the organism, there have been frequent reports of STEC causing clinical disease in calves [145, 147]. In a study of 78 diarrhoeic calves (aged <30 days), Blanco *et al.* [148] reported the isolation of STEC strains belonging to serogroups O2, O103, O104, O128, O153, and O157. Similarly, Sherwood *et al.* [149] isolated 13 different STEC strains from nine calves (1–3 days old) with diarrhoea, including STEC O26 and O111, which have previously been detected in patients with diarrhoea [59, 150]. STEC may cause diarrhoeal disease in calves but not in adult cattle due to the less well developed protective microbiota associated with the naïve neonatal gastrointestinal tract compared to adult cattle. Wray *et al.* [151] described diarrhoeal disease in 1–17 day-old calves experimentally inoculated with bovine Stx2-producing STEC strains. Compared to colostrum-fed animals where no obvious signs of disease were noted, colostrum-deprived calves developed diarrhoea and showed severe lesions in the small intestine, which confirmed previous findings of resistance to neonatal diarrhoeal disease [152, 153].

Several studies have reported *E. coli* O26-associated diarrhoeal disease in calves and older animals. Iijima *et al.* [154] described A/E lesions in the large intestine of an 8-day-old calf with diarrhoea and the associated isolation of an STEC O26 strain. Similarly, Pearson *et al.* [155] observed A/E lesions in the large intestine of an 8-month-old heifer with bloody diarrhoea, which were caused by *E. coli* O26:K60, possessing genes characteristic of STEC (*stx*1, *stx*2, *eae*, *hly*A).

2.4.2 Colonisation and shedding pattern in cattle

To better understand the biology of STEC in cattle, experimental inoculation studies have been used as models to investigate the faecal shedding patterns and the gastrointestinal colonisation sites of STEC O157 in cattle. Grauke *et al.* [156] in their study of 18 adult cattle (1–5-year-old) suggested the colon as the proliferation site of STEC O157. Following Grauke's observations, another experimental study [157] identified the recto-anal junction (RAJ) as the principal site of STEC O157 colonisation in cattle, describing a defined area of lymphoid follicle-dense mucosa (extending up to 5 cm proximally from the RAJ) as the target region of STEC O157. Due to STEC O157 adhering to epithelial cells of the RAJ, pathogens were detected predominantly on the surface of collected stool samples. Furthermore, Baines *et al.* [158] also reported the colonisation of STEC O157 and associated pathological changes in the tissue of the small and large intestine, for example, focal petechiae and mucosal haemorrhages in the jejunum, ileum, caecum, and ascending colon of STEC O157 shedding cattle.

Several studies have noted contrasting levels of faecal shedding associated with STEC O157 excretion [159-162]. Based on shedding patterns observed, three distinct types of STEC O157 shedders have been described. Firstly, passive shedders excreting the pathogen for only a few days and therefore being transiently culture positive; these animals are considered unlikely to be colonised at the RAJ. Secondly, colonised short-term shedders excreting pathogens in average for one month but usually not longer than two months; and thirdly, colonised long-term shedders excreting bacteria from three to 12 months or longer. Colonised cattle shedding STEC O157 for longer than two months are considered rare. Cray and Moon [162] also noted in their inoculation study that calves shed STEC O157 for longer compared to adult cattle, despite being dosed with the same inoculum level.

In addition to persistent shedding, cattle can shed STEC intermittently. This has been described in two short-term cohort studies of 14 and 16 calves (6–11 months of age) naturally infected with *E. coli* O157 [163]. Faecal samples were collected frequently from voided faeces over a period of between five and 15 days and tested for the prevalence of *E. coli* O157 using culture isolation. All calves shed the pathogen at some point during both cohort studies. Persistent shedders of *E. coli* O157 were identified in both cohorts of calves, however, the majority of animals shed the pathogen intermittently.

Calves and adult cattle shed *E. coli*/STEC at different levels and concentrations. Smith and Crabb [164] reported *E. coli* concentrations of 10^2–10^6 colony forming unit (CFU)/g faeces in adult cattle, while calves had *E. coli* levels of 10^9–10^{10} CFU/g early in life, which decreased to 10^6 CFU/g at the age of six weeks. In a longitudinal study on three Australian dairy farms, Cobbold and Desmarchelier [165] assessed faecal shedding rates of STEC from young calves to older heifers and noted that weanling calves (of 7–14 weeks of age) shed STEC significantly more often than the herd in general. Others have described higher shedding rates of STEC O157 in weaned compared to pre-weaned calves [166, 167].

In association with persistent shedding and the colonisation of the RAJ with STEC O157, several studies have provided evidence of so-called 'supershedders' (high shedders). These animals shed STEC O157 at much higher concentrations ($>10^3$ to $\geq 10^4$ CFU/g faeces) than others but are present as a low proportion of total herd numbers [168]. Low *et al.* [169] assessed the rectal carriage and concentrations of faecal shedding of STEC O157 in 267 Scottish slaughter cattle. Intact rectum samples were collected post-slaughter and faecal material and mucosal surfaces cultured for isolation of STEC O157 using direct and enrichment methods. Only 3.7% (95% confidence interval (CI) 1.8% – 6.8%) of all animals were identified as high shedders compared to 11.7% (95% CI 8.1% – 16.1%) culture-positive animals. In another Scottish study, Omisakin *et al.* [170] assessed the prevalence and concentrations of *E. coli* O157 shed in rectal faecal samples of 589 cattle at slaughter. They reported that 7.5% (44/589; 95% CI 5.4% – 9.6%) of cattle were culture-positive for *E. coli* O157, of which 9.1% (4/44) were classified as high shedders ($>10^4$ CFU/g faeces).

Although supershedders constitute a small proportion of cattle, their presence on farms has been reported to have a substantial impact on the within-group or on-farm prevalence of STEC. As estimated by Omisakin *et al.* [170], supershedders can excrete >96% of all *E. coli*

O157 bacteria shed by infected cattle and be a source of infection for other animals. Cobbold *et al.* [171] studied the population dynamics of STEC O157 in feedlot cattle in Alberta, Canada, and noted that animals penned with supershedders had a significantly greater mean faecal prevalence and RAJ colonisation with STEC O157 compared to non-penned cattle, providing evidence of supershedders affecting group-level excretion parameters. It is suggested that supershedders maintain the presence of STEC in cattle populations, hence, the identification and potential for removal through, for example, culling of supershedders on-farm would assist in the reduction of STEC transmission within and between herds and could be used to develop intervention strategies to mitigate the risks of contaminating produce of bovine origin. However, many of the studies were conducted as cross-sectional studies providing insufficient evidence for a subset of cattle being persistent supershedders, which could be identified for targeted control on a farm at any point in time.

2.4.3 Prevalence in cattle

Estimates of STEC prevalence in cattle have been determined by field studies in slaughterhouses and farms in different countries across the globe. The reported rates vary considerably, depending on study design, sensitivity of detection and isolation methods, age of animals, and season [172]. Some studies assessed prevalence using culture isolation with or without the use of immunomagnetic separation (IMS), while others applied PCR screening methods to detect *stx* genes in faecal samples. Prevalence estimates can also be affected by different sampling methods applied. For example, Meichtri *et al.* [173] compared STEC prevalence in young beef cattle in a slaughterhouse study in Argentina, using two different sampling methods (caecum stool sample *vs.* rectal swab sample). They reported a significantly lower prevalence of STEC in stool samples than rectal swab samples (34.3% vs. 87.7%), although this could have been due to different enrichment media they have used for each sampling method and the season when the samples were collected. Similarly, Cobbold *et al.* [171] noted differences in prevalence estimates for STEC O157 among feedlot cattle using two different sampling methods. The STEC O157 prevalence in recto-anal mucosal swab samples was 11.0% (95% CI 9.9% – 12.1%) and significantly higher compared to prevalence in freshly passed manure (6.6%; 95% CI 5.6% – 7.6%).

Acknowledging differences in methodologies, prevalence estimates of STEC in cattle can also be affected by animal or environmental factors. For example, intermittent shedding of STEC

(as described above) can lead to an underestimation of the true STEC prevalence in a cattle population, while the phenomenon of seasonal prevalence of STEC should also be considered. Seasonality of STEC prevalence in cattle is well documented, with greater faecal shedding of STEC in warmer seasons (late spring and summer) having been reported [174, 175]. A 12-month survey of 118 slaughterhouses was conducted in Great Britain assessing the faecal carriage of STEC O157 in cattle (3,939 rectal samples) and sheep [176]. The study reported an annual prevalence of 4.7% (95% CI 4.1% – 5.4%) for cattle, with highest prevalence observed in summer (5.5%; 95% CI 4.2% – 7.1%). Similarly, in another 12-month slaughterhouse survey in the UK, the risk of faecal carriage of STEC O157 in cattle was associated with the summer season [177]. The phenomenon of seasonality was also observed in the southern hemisphere as reported in a study on five dairy farms in Argentina [178]. A significant difference in faecal prevalence (rectal swab) of *stx*-positive cows was observed between warm and cold seasons, with a prevalence of 44% and 56% in spring and summer, respectively, compared to 22% and 28% in autumn and winter, respectively. In contrast to this, Alam and Zurek [179] did not observe a seasonal difference in prevalence of STEC O157 in 891 faecal samples collected from feedlot cattle during a 6-month study in Kansas (US) and reported the highest monthly prevalence of 18.1% in February (later winter). Similar contradictory evidence was provided in a Scottish study by Ogden *et al.* [180], reporting a higher faecal prevalence of STEC O157 in slaughter cattle in cooler months (11.2%; 95% CI 8.4% – 13.9%) compared to warmer months (7.5%; 95% CI 5.4% – 9.6%) (χ^2 test, $p = 0.035$). Interestingly though, they observed a six fold increase in average concentration of STEC O157 shed in the warmer season (1,932 CFU/g faeces) compared to the cooler season (330 CFU/g faeces), and suggested that this could explain to some extent the increased incidence of STEC infections in humans over the summer period. The reason for seasonality of STEC in cattle is still not understood, but it is hypothesised that other environmental factors (e.g. weather, climate, length of day-light) could play a role.

Considering animal, methodological and environmental factors as discussed above, longitudinal studies provide generally more accurate prevalence estimates in cattle populations compared to cross-sectional studies as the latter are equivalent to ‘snap-shot’ assessments of prevalence distributions.

STEC O157

Meyer-Broseta *et al.* [181] evaluated previously published surveys in North American cattle (conducted between 1986 and 1994) and reported that herd prevalence rates for STEC O157 ranged from 0% to 22% for dairy farms, and up to 61% for feedlot farms, whereas herd prevalence rates in European cattle (surveys conducted between 1992 and 1998) were 0% to 3%. However, other European studies showed higher herd prevalence estimates. A study conducted among 75 dairy herds in England and Wales in 1999 reported a herd prevalence of 38.5% (95% CI 28.1% – 50.4%) [182]. Similarly, Bonardi *et al.* [174] detected STEC O157 in 16.6% (37/223) of feedlot cattle and in 16.1% (22/137) of dairy cull cows sampled at three slaughter plants in northern Italy. They estimated a herd prevalence for STEC O157 of 28.3% (15/53) and 21.7% (13/60) in feedlot and dairy farms, respectively.

Several studies have also assessed within-herd prevalence rates of STEC O157, observing higher prevalence rates in animals from weaning (2 months-old) to two years of age as compared to younger calves or older cattle [165, 183, 184]. In the US, for example, Hancock *et al.* [185] described a within-herd prevalence for STEC O157 of less than 1.5% in calves <8-weeks-old, while animals aged eight weeks to four months showed a prevalence from 1.8% [186] to 5% [187]. A study on heifers of four to 24 months of age observed a mean within-herd prevalence for STEC O157 of 2.3% [188]. In contrast to this, a within-herd prevalence estimate of <1% was described for adult dairy cows [185].

STEC O26

A number of studies have also reported the prevalence of STEC O26 in cattle. Chase-Topping *et al.* [189] described in a national survey of 338 Scottish cattle farms a herd prevalence of 18.6% (63/338) and a prevalence of 4.0% in 6,086 faecal pats. A similarly low intestinal prevalence for STEC O26 was reported in a slaughterhouse study in northern Italy including 182 slaughter cattle (dairy cows and feedlot cattle) [190]. Compared to an overall STEC prevalence of 4.9% (9/182 animals), STEC O26 was detected in the caecal contents of only 1 (0.5%) animal. In contrast to this, Lee *et al.* [191] assessed the faecal prevalence of STEC O26 (and STEC O111) in 442 beef and dairy calves of <16 weeks of age from 115 farms in Korea. They observed a prevalence of 14.4% (37/257) and 7.6% (14/185) in diarrhoeic and non-diarrhoeic calves, respectively, providing evidence for cattle being a potential source of STEC O26 infections in humans.

2.4.4 Other animals

Cattle are considered the main reservoir of STEC however, this pathogen has also been isolated from faeces of other ruminants such as sheep, deer, and goats [192-194]. There are a few reports where STEC have been detected in other species including horses [195, 196], dogs [196, 197], and cats [198], identifying them as sources of sporadic STEC infections in humans. However, pigs could represent another potential reservoir of STEC O157 as shown in a transmission study by Cornick *et al.* [199], where STEC could be transmitted from an infected animal to naïve pigs via both direct contact and contaminated aerosol. Feral pigs were identified as vectors of STEC O157 in an outbreak in California (US) in 2006, which was associated with the consumption of contaminated fresh spinach grown on crop fields accessible to feral pigs [200, 201]. A few studies have also shown that rabbits are both vectors and reservoir hosts of STEC [202-204]. Currently, Dutch belted rabbits serve as natural and experimental animal models for enterohaemorrhagic *E. coli*- and Stx-induced diseases [205-207].

Studies of wildlife shedding STEC have been conducted in Europe and North America. Wahlström *et al.* [208] examined 791 samples collected from Canada geese, roe deer, hares, moose, wild boar and gulls shot by 27 hunters in Sweden over a period of one year in 1998/1999 and isolated STEC O157 from only one wild boar (*Sus scrofa*). In a wildlife survey in Galicia, northwest of Spain, Mora *et al.* [209] detected 40 different STEC serotypes in rectal swab samples collected from 262 wild boars (*Sus scrofa*), 179 roe deer (*Capreolus capreolus*), and 260 foxes (*Vulpes vulpes*) killed by hunters during hunting seasons from 2009 to 2010. Of these STEC serotypes 21 were classified as pathogenic for humans, which included O157:H7, O26:H11, O121:H19, and O145:H28. In contrast, Jijón *et al.* [210] tested 71 faecal samples collected from various wildlife (e.g. opossums (*Didelphis virginiana*), ray squirrels (*Sciurus carolinensis*), American kestrels (*Falco sparverius*), and red-tailed hawks (*Buteo jamaicensis*)) admitted to two rehabilitation centres in Ohio (US) in 2004 but they did not detect STEC O157 in any samples.

STEC have also been detected in birds. Hancock *et al.* [188] collected faecal samples from various domestic and wild animals on 12 cattle feedlot farms in Idaho, Oregon, and Washington (US) and found STEC O157 in only one of 200 pooled bird samples (0.5%). A study conducted in Italy examined individual stool samples from 649 trapped pigeons in three

different squares in Rome for the presence of Stx using the vero cell assay [211]. STEC serotype O45:H- was the most frequently found STEC strain in a total of 70 (10.7%) samples positive for Stx. Kauffman and LeJeune [212] identified starlings (*Sturnus vulgaris*) as vectors and a potential reservoir of STEC O157. They challenged starlings with various infectious doses and observed that when exposed to doses of $>10^{5.5}$ CFU the pathogen was shed for more than three days in 50% of the birds. A within- and between-species pathogen transmission was observed within 24 h after STEC O157-positive starlings shared biocontainment rooms with culture-negative birds or two 12-week-old calves. Vice versa, when exposed to STEC O157-shedding calves, previously culture-negative starlings became infected with STEC O157 within 24 h. A 3-year study conducted on 150 different dairy farms in northern Ohio (US) tested faecal samples from 9,000 cattle and 430 captured European starlings (*Sturnus vulgaris*) for the presence of STEC O157 [213]. The molecular results of bovine and avian isolates in this study provided evidence that these birds contributed to the transmission of STEC O157 between dairy farms, highlighting the complex ecology of agricultural animals and wildlife in the epidemiology of STEC O157.

2.4.5 Environment

STEC O157 is able to replicate and survive for long periods of time in environmental niches. Jiang *et al.* [214] demonstrated that STEC O157 can survive in manure-amended autoclaved soil at 5°C, 15°C, and 21°C for 77, >226, and 231 days, respectively. Islam *et al.* [215] determined the occurrence and persistence of STEC O157 in soil plots fertilised with STEC O157-contaminated poultry and bovine manure compost and on parsley and lettuce grown on these plots in the natural environment. After planting seedlings of parsley and lettuce, STEC O157 was detected on lettuce and parsley for up to 77 and 177 days, respectively; however, STEC O157 persisted for much longer (154–217 days) in soils amended with contaminated fertilisers.

It is hypothesised that the environment plays an important role in the persistence and dissemination of STEC O157 on farms. LeJeune *et al.* [216] studied the survival of STEC O157 in sediments of experimental microcosms simulating cattle water troughs and observed that infectious STEC O157 survived in the microcosm sediments for at least 245 days. Four 10-week-old calves became infected and shed STEC O157 after being exposed to water from the microcosms, demonstrating that cattle water troughs can serve as a reservoir of STEC

O157 on farm and be a source of infection for other animals. Avery *et al.* [217] studied the survival of *E. coli* in faeces from livestock such as cattle, sheep, and pigs and indicated that it can survive on pastures for at least 5–6 months, being able to spread and contaminate the surrounding environment including water and pasture and pose a risk of infection for other livestock; hence, sustaining the presence of STEC on a farm.

2.5 Laboratory methods for *E. coli* isolation

2.5.1 Enrichment

To increase the rate of *E. coli* isolation from samples, different enrichment methods have been developed to enhance the growth of *E. coli* (O157 and non-O157) in samples prior to culturing on selective media. This is of particular importance when the pathogen is present in low densities compared with the background microflora in samples (e.g. faecal samples or minced beef). Commonly used enrichment media are buffered peptone water (BPW), tryptic soy broth (TSB), and *E. coli* broth, which can be modified with antibacterial additives, for example BPW with vancomycin, cefsulodin and cefixime (BPW + vcc) [218], TSB with novobiocin [219] or vancomycin, cefsulodin and cefixime [220], or *E. coli* broth with novobiocin (mEC + n) [221], to suppress the growth of competitive natural bacteria in samples.

Several studies have evaluated the efficacy of these media on various types of samples, but often providing conflicting results. For example, Seo *et al*. [222] reported that modified BPW with casaminoacids was the most effective enrichment for STEC O157 in ground beef, whereas Heuvelink *et al.* [223] found mEC + n to be the most efficacious media for selective enrichment of STEC in minced beef. Sanderson *et al.* [224] assessed the efficacy of enrichments for STEC O157 in bovine faeces and found no statistically significant differences between TSB modified with cefixime, vancomycin and tellurite, and TSB modified with cefixime and vancomycin. In contrast, Foster *et al.* [225] reported that BPW without antibacterial additives was a superior enrichment to isolate STEC O157 from bovine faecal samples, compared to BPW + vcc. Hara-Kudo *et al.* [226] noted that mEC + n at 42°C was the most effective enrichment method for isolation of both *E. coli* O157 and O26 from inoculated ground beef and radish sprout samples, whereas Catarame *et al.* [227] observed

optimum enrichment for *E. coli* O26 in beef samples using TSB modified with cefixime, vancomycin, and potassium tellurite at 41.5°C.

2.5.2 Culture

Based on the reported inability of most *E. coli* O157 to ferment sorbitol, sorbitol-MacConkey agar (SMAC) has been developed as a selective medium to differentiate *E. coli* O157 from other *E. coli* serogroups and to advance the isolation of *E. coli* O157:H7 from faecal samples by direct culture [36]. The selectivity of SMAC agar has been further improved by the supplementation with antibiotics cefixime [228] and potassium tellurite [229], creating cefixime-tellurite sorbitol-MacConkey agar (CT-SMAC) [230]. Cefixime suppresses the growth of *Proteus* species, while tellurite suppresses most gram-negative organisms. CT-SMAC culture plates are commonly used in diagnostic laboratories for culture isolation of *E. coli* O157 from faecal samples, however, despite the supplementation with cefixime and tellurite, the test specificity of these plates is not 100% and false-positive isolates are still detected. Furthermore, sorbitol fermentation does not exclude the carriage of *stx* genes in *E. coli* O157 as reported by Gunzer *et al.* [231]. They isolated sorbitol-fermenting, Stx2-producing *E. coli* O157:H$^-$ from patients with HUS and diarrhoea, highlighting the need to screen also sorbitol-fermenting colonies for potentially pathogenic STEC O157, which otherwise could be missed. In addition, Karch *et al.* [232] indicated that some sorbitol-fermenting toxin-producing *E. coli* O157:H$^-$ are sensitive to the antibacterial supplements in CT-SMAC agar, providing evidence of reduced test sensitivity of CT-SMAC plates.

Similarly, STEC O26 was reported to be unable to ferment rhamnose, in contrast to many other *E. coli* and some *stx*-negative O26 strains. Hence, to differentiate STEC O26 from other STEC serogroups, a rhamnose-MacConkey agar (RMAC) has been developed and supplemented with cefixime and potassium tellurite to create CT-RMAC agar [233]. CT-RMAC has been tested as an optimum agar for recovery of STEC O26 [227, 234]. However, Evans *et al.* [235] observed a test sensitivity of 97% for CT-RMAC, when used in combination with tryptone bile X-glucuronide (TBX) agar to recover STEC O26 from ovine and bovine faecal samples, indicating that some STEC O26 strains could be sensitive to the antibacterial supplements in CT-RMAC agar. Furthermore, they also tested a collection of 96 bovine *E. coli* O26 strains (previously screened for the presence of virulence genes *stx*1, *stx*2, *eae*, and *ehx*A) for rhamnose fermentation and noted that 5.0% (2/40) and 19.2% (5/26) of

stx-positive and *stx*-negative *E. coli* O26 isolates, respectively, fermented rhamnose. Their findings highlighted the importance to screen all colony types, as rhamnose fermentation does not exclude the carriage of *stx* genes in *E. coli* O26, which could lead to some STEC O26 being overlooked. Rhamnose fermentation is also not a phenotypic characteristic of aEPEC O26 strains as shown in a study by Aktan *et al.* [236]. They observed rhamnose-fermenting and non-fermenting strains among ovine and bovine aEPEC O26 isolates from the UK, providing further evidence of the phenotypic diversity of aEPEC O26 strains prevalent in ruminants.

Culture isolation of *E. coli* may also be affected by cells being viable but non-culturable when exposed to natural stress factors [237]. This phenomenon has been observed for *E. coli* and STEC and a large number of other bacterial pathogens such as *Campylobacter* spp., *Helicobacter pylori, Legionella pneumophila, Listeria monocytogenes, Mycobacterium tuberculosis, Pseudomonas aeruginosa, Salmonella* and *Shigella* spp., and *Vibrio cholera* [237].

2.5.3 Immunomagnetic separation (IMS)

IMS is a technique applied to increase the sensitivity of *E. coli* isolation from enriched samples and is likely to occur through the concentration of *E. coli* relative to the background microbiota, which may inhibit growth of STEC on selective culture media. IMS is based on the immunological binding of the target organism followed by physical separation from the mixed enrichment culture, resulting in a concentration of the target organism. In brief, magnetic beads coated with polyclonal antibodies against surface antigens of *E. coli* O157 or O26 are added to the enriched sample to bind with *E. coli*. Using external magnets, magnetic beads are then separated from other sample material and plated on selective medium. Several studies have shown that IMS is a sensitive method for isolation of *E. coli* O157 and other non-O157 serogroups from artificially mixed bacterial cultures, inoculated and naturally contaminated samples of meat and bovine faeces [238-242].

2.6 Molecular typing methods of STEC

A variety of molecular typing methods have been used to advance the understanding of the epidemiology of STEC in animals as well as humans, providing data to support and develop

intervention strategies. These methods include, among others, polymerase chain reaction (PCR), pulsed-field gel electrophoresis (PFGE), and *stx*-encoding bacteriophage insertion typing (SBI).

2.6.1 PCR

PCR technique is a rapid, automated method with high test sensitivity and specificity for detecting microbial pathogens [243]. PCR is widely used in studies associated with the isolation of *E. coli* from a broad type of samples (i.e. food, faeces, water, and soil) and is used to detect the presence/absence of target genes in isolated *E. coli* strains (i.e. virulence genes *stx*, *eae*, and *ehx*A), describing their genotype or virulence profile. Numerous studies have reported the use of multiple primers in one PCR assay. For example, Meng *et al.* [244] designed primers for a multiplex PCR method to detect the presence of STEC O157, *stx*1, and *stx*2 simultaneously, using it as a confirmation method for the identification of isolated STEC O157 strains. Similarly, Paton and Paton [245] published two multiplex PCR methods to detect and genetically characterise *E. coli* strains isolated from faecal samples or foodstuffs. Their first assay described the detection of virulence genes *stx*1, *stx*2 (including genetic variants of *stx*2), *eae*A, and *ehx*A (*hly*A) of enterohaemorrhagic *E. coli*, while their second assay used two specific primer pairs to detect portions of the *rfb* regions (encoding for the O antigen of *E. coli*) of *E. coli* serogroups O157 and O111. The test sensitivity of the second primers was evaluated by spiking an STEC-negative faecal sample with serial dilutions of STEC culture strains O157 and O111. The highest dilution detectable by PCR was equivalent to approximately 10^3 CFU per assay, indicating that the test sensitivity of these primers was not 100%. In comparison, the test specificity of these primers was described as 100% and no PCR amplicons were observed indicating to cross-reactions between the two primers. However, no further tests (e.g. sequencing) were used to confirm the PCR amplicons were of the correct size and sequence.

2.6.2 PFGE

PFGE was developed over 30 years ago to subtype a variety of organisms including yeast and other bacterial pathogens [246], generating DNA fingerprints of organisms. Its value was first demonstrated in a large foodborne outbreak of STEC in the US in 1992/1993, when human illness was linked to the consumption of STEC-contaminated, undercooked beef patties from

a national chain of fast-food restaurants [25, 247]. Since then, scientists at the Centres for Disease Control and Prevention (CDC) (US) have refined and optimised the PFGE methodology, so other laboratories within the US and other countries across the globe can reproduce PFGE patterns and compare them electronically, for example, to rapidly track clinical cases associated with outbreak strains [248-250].

PFGE offers higher reproducibility and discriminatory power compared to other rapid PCR-based methods [247, 251-253] and has been applied in many outbreak investigations (STEC O157 or non-O157) to discriminate between outbreak-associated, sporadic, or unrelated STEC infections and to identify sources of infection [15, 17, 254, 255]. For example, Bender *et al.* [256] reported the usefulness of PFGE in the identification of additional outbreaks in Minnesota (US) during 1994 to 1995, which otherwise would have remained undetected by traditional surveillance methods. Another US study reported the use of PFGE in a prolonged outbreak of STEC O157 infections in Oregon (US) from January 1992 to June 1994, to establish a link between 14 STEC cases and the consumption of raw milk products from an STEC-infected dairy farm [255]. Although three different PFGE patterns were observed among STEC O157 isolated from dairy cattle, they were indistinguishable from those of patients reported to have consumed dairy products from this farm, providing sufficient evidence for causal association.

PFGE has been applied in the earlier studies to provide supporting molecular evidence for cattle being an important reservoir of STEC O157 and a source of human disease [257, 258], but PFGE has also been used to investigate the molecular diversity of STEC in humans. For example, Leotta *et al.* [259] compared the phenotypic and genotypic characteristics of 73 STEC O157 isolates from clinical cases in Argentina (n = 35), Australia (n = 20), and New Zealand (n = 18) occurring between 1993 and 1996. From 73 strains digested with restriction enzyme *Xba*I, 37 could be grouped into 10 PFGE clusters, while the remaining 36 strains showed unique patterns. Interestingly, no common PFGE pattern could be observed among isolates from these three countries, indicating a global diversity of STEC O157 isolates in humans. Similarly, Zhang *et al.* [253] applied PFGE on 55 STEC O26:H11 and O26:H- strains isolated from clinical cases in Germany and the Czech Republic between 1965 to 1999 and observed a remarkable heterogeneity among the isolates, highlighting the importance of subtyping STEC O26 to provide adequate strain discrimination.

PFGE has also been widely used in studies investigating the ecology of STEC on cattle farms. For example, Cobbold and Desmarchelier [260] compared 136 STEC O157 and STEC O26 isolates collected during a longitudinal study on three dairy farms in Australia and observed four and five distinguishable restriction patterns among bovine STEC O157 and STEC O26 isolates, respectively. A greater within-farm than between-farm similarity of isolates was noted, particularly for STEC O26 strains, which was consistent with observations in a prevalence study of 70 dairy farms in Wisconsin (US) in 1994 [186]. This US study also revealed that PFGE patterns between STEC O157 strains from consecutive samples of the same animal can be different, indicating to genetic changes or high clonal turnover [261]. Similarly, Pearce *et al.* [262] used PFGE in their cohort study to investigate the faecal shedding of *E. coli* O157 and O26 (including O103, O111, and O145) in 49 beef calves (and their dams) from birth to five months of age on a Scottish sheep and beef farm. *E. coli* O157 was rare but *E. coli* O26 was detected in 17.3% (115/664) of collected calf samples, of which 95.7% (110/115) were identified as STEC O26. PFGE analysis of O26 isolates revealed seven distinguishable patterns, including one dominant pattern, which represented the majority (>80%) of STEC O26 isolates (*stx*1-positive, *stx*2-negative, *eae*-positive).

*Xba*I is the most commonly used restriction endonuclease enzyme to digest chromosomal DNA because it is considered the most discriminatory restriction enzyme for PFGE [263, 264]. However, to further distinguish strains identified by *Xba*I, second or third restriction enzymes (e.g. *Bln*I, *Avr*II or *Spe*I) are required. For example, Rice *et al.* [265] described the PFGE analysis of 376 STEC O157 isolates collected from 41 different cattle farms in the Pacific Northwest of the US to determine the level of diversity of subtypes within and between herds and their geographical distribution in cattle herds. Use of *Xba*I revealed 81 subtypes in total and up to 11 different subtypes were observed per farm. Using *Not*I as a second restriction enzyme on subtypes present on more than one farm, revealed 23 additional subtypes.

PFGE is considered as a molecular typing method of high discriminatory power; however, as restriction enzymes cut at enzyme specific nucleotide sequences of 6–8 base pairs within the bacterial genome, thereby recognising only a small fraction of the whole genome, PFGE can only provide an indication of genomic relatedness but no other molecular data such as the presence/absence of virulence genes. Furthermore, to generate PFGE profiles using software

programmes, restriction band patterns on a PFGE gel are subjected to visual assessment, which can result in misclassification bias [266].

2.6.3 SBI

SBI genotyping is a multiplex PCR-based method, screening specific regions of the STEC O157 chromosome (*argW*, *wrbA*, *yehV*, and *sbcB*) as known bacteriophage insertion sites for presence or absence of inserted *stx* genes (*stx*1, *stx*2, and subtype *stx*2c) [267, 268]. This method has been used to study the genetic diversity of bovine and human STEC O157 strains as described by Besser *et al.* [267]. Sixteen different genotypes were observed among 282 clinical and 80 bovine isolates sourced from North America. A broad genetic diversity of genotypes was noted among bovine isolates with only 51.3% of isolates belonging to genotype 1, 2, or 3, as compared to 90.5% of clinical isolates, suggesting that some bovine genotypes of STEC O157 might be less virulent or transmissible to humans. In 2012, a new SBI coding system was proposed [269] to represent the characteristics of SBI genotypes (insertion sites and *stx* type), e.g. previous SBI genotype designation 1 or 3 are now equivalent to SBI genotypes AY2a or WY12a, respectively. SBI genotyping was also used in a recent study by Mellor *et al.* [270] to describe the geographic divergence of bovine and clinical STEC O157 isolates from Australia and the US. They observed a greater SBI diversity among US than Australian isolates, with genotype ASY12c predominant among Australian isolates (49%) and genotype WY12 among US isolates (55%), showing a genotypic diversity of bovine and human STEC O157 strains between both continents.

2.6.4 Other typing methods

In addition to PCR, PFGE, and SBI, there are a variety of other typing methods, which have been used to study the molecular epidemiology of STEC. These methods include phage typing [271], restricted fragment length polymorphism (RFLP) [272, 273], multilocus sequence typing (MLST) [274], single nucleotide polymorphism (SNP) [275, 276], locus specific polymorphism analysis (LSPS) [277, 278], and whole genome sequencing [279-281].

2.7 Animal processing and control of STEC contamination

The consumption of STEC contaminated food is a possible pathway of STEC transmission to humans. Undercooked and cross-contaminated meat and meat products, most likely faecally contaminated at slaughter, have been identified as sources of STEC infection in several outbreaks of human disease overseas [22, 23, 25, 26]. To prevent and control the contamination of carcasses at slaughter, numerous studies have been conducted to assess the risk factors along the farm-to-fork continuum, including stages such as farm, transportation, lairage, and slaughter.

Hadley *et al.* [282] demonstrated that the microbial load of dressed carcasses was significantly affected by the degree of soiling of live animals, stressing the importance to present animals in a clean condition for slaughter at the start of the meat supply chain. Further studies established that hides are the major source of STEC O157 contamination on beef carcasses at the time of slaughter [283-286], as the bacterial loads on hides are correlated with those found on carcasses after dehiding [287-289]. Hence, reducing the prevalence and level of hide contamination has been the objective of many pre- and post-harvest research studies.

2.7.1 Pre-harvest interventions and risk factors

Different approaches have been taken as control measures to reduce the proportion of slaughter cattle carrying STEC and the level of STEC O157 in faeces of slaughter cattle. These measures include the use of probiotics, bacteriophage, and vaccination, aiming to lower the load of STEC in faeces, which decreases the cross-contamination of hides with STEC and consequently reduces the carcass contamination rates. Younts *et al.* [290] evaluated the effect of probiotics (*Lactobacillus acidophilus*) in finishing beef cattle and observed a significant reduction of faecal shedding of STEC O157 ($p < 0.01$). Similar reducing effects of probiotics on faecal shedding of STEC O157 and O111 were observed in weaned calves (8–10 weeks of age) ($p < 0.05$), however this was not apparent for STEC O26 [291].

Other studies assessed the efficacy of bacteriophage treatment in cattle. Sheng *et al.* [292] applied bacteriophage on the mucosa of the recto-anal junction in steers and observed a significant decrease in the levels of faecal shedding of STEC O157 (10^6 CFU/swab to $10^{1.4}$ CFU/swab, $p < 0.05$). Callaway *et al.* [293] also found a significant ($p < 0.05$) decrease in

numbers of STEC O157 in faeces of sheep, which were experimentally inoculated with a cocktail of phages isolated from commercial cattle faeces.

Peterson *et al.* [294] tested the effect of a vaccine in feedlot cattle, which contained *E. coli* type III secreted proteins. At slaughter, they observed that vaccinated cattle were 98.3% less likely to be colonised by STEC O157 in mucosal cells of the terminal rectum. Similarly, McNeilly *et al.* [295] treated cattle with a vaccine containing the variable C-terminal end of intimin, EspA and Tir proteins and observed a significant reduction of faecal shedding after oral challenge with STEC O157.

Despite reporting significant reductions of faecal shedding of STEC in cattle, some of the above methods are not applied because, either they are still being developed to be used in a practical form on a farm (e.g. of phages), being too expensive, or not equally effective on all serogroups of STEC. However, vaccines have proven to be the most effective of these interventions. Currently, one fully licensed vaccine is available in Canada, and two vaccines with restricted licences in the US, permitting only limited use [296, 297]. Whether vaccines will be widely used in the future will mostly depend on the costs of vaccines and the number of prevented clinical cases and deaths [296].

Other researchers have studied the effect of transport and lairage on the faecal shedding of STEC O157 in cattle and the risk of hide contamination. Transportation of only a few hours appears to have very minimal impact on the prevalence of faecal shedding of *E. coli* O157 in cattle. As reported by Minihan *et al.* [298], 1.5 h and 6 h transportation of adult feedlot cattle to slaughter plants had no effect on the prevalence of *E. coli* O157 in faeces, which was consistent with findings of Bach *et al.* [299]. They also observed no increased faecal shedding of STEC O157 in calves after 3 h transportation to a feedlot (with or without preconditioning of animals (vaccinating and weaning 29 and 13 days, respectively, prior to hauling)), whereas long-haul transportation of 15 h significantly increased the number of calves test-positive for *E. coli* O157 ($p < 0.005$).

Commercial transportation has been identified as a (borderline) significant risk factor for increased cross-contamination of hides at slaughter [300]; while Arthur *et al.* [301] observed that hide contamination in the lairage area accounts for a larger proportion of hide and carcass contamination than the hide contamination occurring on farm. Similarly, Dewell *et al.* [302]

reported that holding animals in faecally contaminated lairage pens at slaughter was associated with higher risks of hide contamination in finished beef cattle.

2.7.2 Post-harvest interventions

Apart from slaughter practices, which have a large effect on the microbiological contamination of red meat carcasses [303], different hide and carcass treatments have been shown to reduce microbial contamination (at variable levels) on hides and carcasses post-slaughter.

Decontamination treatments of cattle hides (applied prior to hide opening) include hot water spraying, steam, acetic and lactic acids, and cetylpyridinium chloride [304]. Arthur *et al.* [305] evaluated the efficacy of a hide wash cabinet (water wash with a final chlorine spray) on the prevalence and levels of STEC O157 on hides of cattle. They observed a reduction of prevalence from 35.1% to 13.2% of 288 hides harbouring STEC O157 at levels of ≥40 CFU/100 cm^2 (detection limit). In another study, Bosilevac *et al.* [306] assessed the efficacy of a hide wash on the slaughter chain using a sodium hydroxide wash and a chlorinated (1 ppm) water rinse. They could show that the prevalence of *E. coli* O157 on hides after passage through the cabinet was reduced from 44% to 17%, and the aerobic plate counts and *Enterobacteriaceae* counts were reduced by 2.1 and 3.4 log CFU/100 cm^2, respectively. When the wash cabinet was used, they observed a reduction of prevalence from 17% to 2% on pre-evisceration carcasses contaminated with *E. coli* O157, while the aerobic plate counts and *Enterobacteriaceae* counts were both reduced by 0.8 log CFU/100 cm^2.

Antibacterial efficacies of different decontamination treatments of carcasses at pre- and post-evisceration, which include hot water spraying, steam, steam vacuuming, air chilling, and the application of organic acids, have also been studied. For example, Kalchayanand *et al.* [307] assessed the efficacy of hot water spraying (85°C) on the bacterial decontamination of beef carcass surfaces using inoculated sections of cutaneous trunci muscles (flank tissue) and beef hearts. They observed a reduction of between 3.0 and 4.1 log CFU/cm^2 in *Enterobacteriaceae* and between 1.8 and 2.3 log CFU/cm^2 in STEC O157, which was consistent with previous findings by Marshall *et al.* [308]. Although having applied hot water spraying at higher temperature (90°C), they described a 1.2 log CFU/cm^2 reduction of STEC O157 on inoculated carcass tissue samples (cutaneous trunci and adipose carcass trims).

Organic acids such as acetic, citric and lactic acid are commonly used chemical treatments of post-evisceration carcasses on cattle slaughter plants in the US and Canada and have been shown to reduce the bacterial load on carcasses ranging from 0.7 to 4.9 log CFU/cm^2 [304]. King *et al.* [309] reported a 2.7 log CFU/cm^2 reduction of STEC O157 on inoculated carcass surface samples after treatment with 4% L-lactic acid at 55°C. A slightly lower efficacy of lactic acid on STEC O157 was noted in a study by Arthur *et al.* [310], reporting a 0.65 and 1.15 log CFU/cm^2 reduction on inoculated cutaneous trunci muscle samples treated with 2% acetic acid and 2% DL-lactic acid, respectively.

2.7.3 Risk assessments

Data on efficacy of pre- and post-harvest intervention methods, as well as prevalence and concentration of pathogens in faeces and on carcasses have been used in quantitative microbiological risk assessments and simulation models to identify stages of increased risk for foodborne disease in the farm-to-fork continuum. Cassin *et al.* [311] modelled the human health risk associated with the consumption of STEC O157 contaminated ground beef burgers. In their first model, they used data to describe the behaviour of STEC O157 in the hamburger starting from the point of production, processing, handling, until consumption, to predict the human exposure, which was then used in a dose–response model to estimate the health risks associated with consumption of a contaminated hamburger. Their model predicted probabilities of 3.7×10^{-6} and 1.9×10^{-7} for HUS and mortality, respectively, per meal for very young children. Similarly, Ebel *et al.* [312] drafted a risk assessment for STEC O157 in ground beef and the associated risk of disease in humans in the US. Farm, slaughter and preparation factors have been considered in their model, which influence the probability of humans consuming servings of ground beef contaminated with STEC O157 and the number of STEC O157 cells in a contaminated serving of ground beef. With their model, they predicted that on average 0.018% and 0.007% of ground beef servings consumed in summer (June to September) and the rest of the year, respectively, contain ≥1 cell of STEC O157.

To allow the assessment of potential control measures, Jordan *et al.* [313] designed a Monte Carlo simulation model to assess the quantity of microbial hazards of STEC O157 and *Salmonella* spp. on contaminated beef carcasses considering different pre-slaughter

management regimens. The great advantage of simulation models is to test the effectiveness of interventions without the difficulties and expenses associated with quantifying slaughter outcomes like in conventional methods. However, these models require a good understanding of the dynamics of pathogens at all stages of the meat production to simulate the contamination of carcasses as accurately as possible.

2.8 Epidemiology of STEC infections in humans in New Zealand

2.8.1 Epidemiological facts

In 1980, Wilson and Bettelheim [150] reported New Zealand's first STEC cases, isolating STEC serotypes O26:H11 and O39:H8 from patients with diarrhoea. However, the first case of STEC O157 was not confirmed until 1993 [314]. Since then, the number of annual STEC notifications has increased steadily [315] [31] (Figure 2.3).

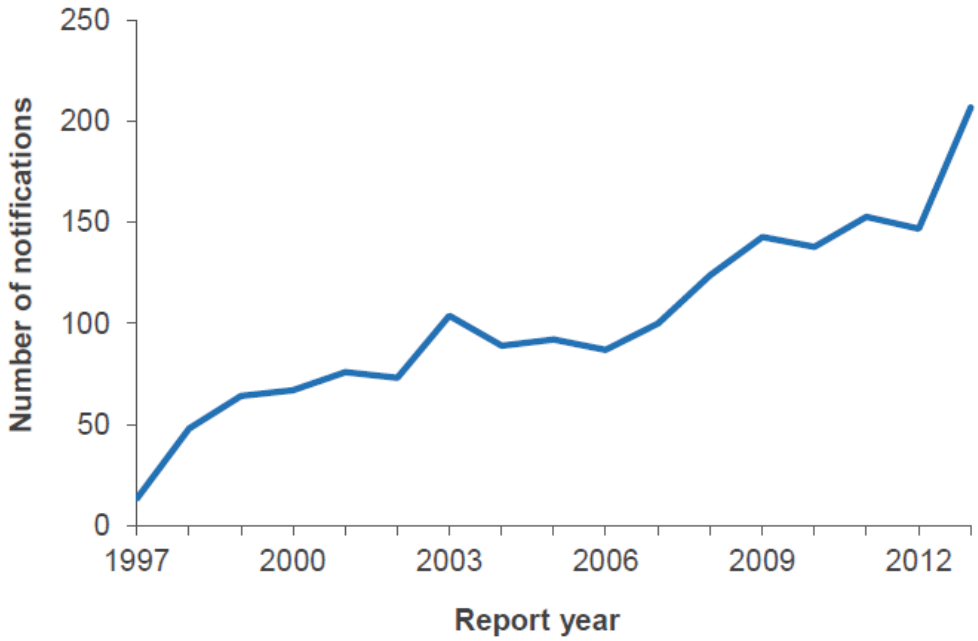

Figure 2.3: STEC notifications in New Zealand by year, 1997–2013 (sourced from ESR's Annual Surveillance Report 2014 [31]).

In 2013, there were 207 confirmed STEC cases, which is equivalent to an annual incidence rate of 4.6 STEC cases per 100,000 population [31]. Compared to the US (1.1 per 100,000 in 2012 [92]) or Australia (0.4 per 100,000 in 2011 [89]), New Zealand has, along with Ireland

(6.1 per 100,000 in 2011[90]), Sweden (5.0 per 100,000 in 2011 [90]), and Scotland (4.5 per 100,000 in 2012 [32]), one of the highest annual incidence rates for STEC infections reported in the world.

Although the majority of reported STEC cases in New Zealand are caused by serotype O157:H7 (89.4%, 194/217 in 2013 [316]), the percentage of non-O157 STEC cases has increased steadily over the past six years from 1.6% in 2008 to 10.6% in 2013. The most commonly reported non-O157 STECs included serogroups O26, O84, O103, O121, O128, O176, and ONT (O serogroup not typable) [81].

STEC cases appear across the whole of New Zealand. In 2013, the highest annual notification rates were reported for Waikato (11.8 per 100,000 population; 44 cases), Taranaki (8.1 per 100,000; 9 cases), and Northland (6.9 per 100,000; 11 cases) District Health Boards in the North Island [31]. With a notification rate of 26.6 per 100,000 (66 cases), the age group of 1–4 year-old children was affected the most, followed by the group of children <1 year of age (20.0 per 100,000; 12 cases). Among notified STEC cases, the hospitalisation status was recorded for 84.1% (174/207) of cases, of which 42.0% (73/174) were hospitalised and 15 cases developed HUS; no deaths due to STEC infection were reported.

Compared to observations from previous years, a higher STEC notification rate was observed in females (5.3 per 100,000 population; 121 cases) than males (3.9 per 100,000; 86 cases) in 2013, and there were more STEC cases reported in people of European or Other ethnicity (5.8 per 100,000 population; 172 cases) compared to Māori ethnicity (3.1 per 100,000; 21 cases) [31]. In line with data of previous case investigations, contact with pets, farm animals, or animal manure, and consumption of contaminated food (raw fruit or vegetables, dairy products, chicken or poultry, beef or beef products) were reported as the most likely sources of STEC infection [31]. However, these were based solely on case data, and no source attribution or case-control studies were reported to support these putative sources of infection or to identify other possible risk factors for sporadic STEC infections in New Zealand.

STEC cases in New Zealand appear as sporadic cases or small clusters. With no large outbreaks of STEC being reported to date, the spatial distribution of sporadic STEC cases suggests that infections might be associated with farming [31, 317]. STEC infections in New

Zealand display seasonal patterns, with peaks during spring and summer/early autumn [318], similar to observations from overseas [319].

2.8.2 Under-reporting

The number of confirmed cases reported through the STEC surveillance system is likely to be an underestimation of the true prevalence of human STEC infections in New Zealand. This is because firstly, asymptomatic or mild cases do not consult medical practitioners and remain therefore undetected, and secondly, not all stool samples received at diagnostic laboratories are routinely tested for *E. coli* O157:H7 and non-O157 STECs, overlooking some infections. In addition, the majority of diagnostic laboratories in New Zealand test stool samples of STEC cases for *E. coli* O157:H7 only due to limited resources for routine screening for non-O157 STEC [320].

Considering the increasing trend of annual STEC incidence rates in New Zealand, studies are required to identify risk factors associated with sporadic STEC infections and develop interventions to reduce or minimise the occurrence of disease.

2.9 Epidemiology of STEC in New Zealand cattle

Only very limited data are available on the epidemiology of STEC in New Zealand's livestock population. Over the past decades, only a few research studies have been conducted to investigate the prevalence of STEC in New Zealand cattle and sheep. In 1997, Buncic *et al.* [321] investigated the prevalence of *E. coli* O157:H7 in healthy dairy cows at a slaughter plant in the Waikato region of the North Island, but could find only two of 371 tested animals (0.5%) to be faecal carriers of this pathogen; no attempt was made to collect data on the prevalence of other non-O157 STEC. Cookson *et al.* [322] estimated the prevalence of STEC (including non-O157 STECs) in faecal samples collected from forage-fed cattle and sheep at four farm locations in the lower North Island, reporting a lower STEC prevalence in cattle (27.3%, 51/187) compared to sheep (65.9%, 87/132), and described the variety of STEC serotypes isolated from faeces of cattle and sheep [323]. They detected the presence of STEC serotypes O84:H-, O26:H11, O5:H-, O91:H- and O128:H2 in cattle and sheep, which have been isolated from clinical STEC cases in New Zealand. A more recent survey at two

slaughter plants in the lower North Island reported an STEC O157 prevalence of 3.2% (10/309) in very young (bobby) calves [324].

Findings of these studies clearly indicate that cattle (and sheep) are asymptomatic reservoirs of STEC in New Zealand, which might represent a significant source of STEC infection for humans, however further epidemiological studies are required to fill the gaps of basic knowledge on the epidemiology of STEC O157 and other non-O157 STECs in New Zealand cattle at a national level.

2.10 Economic costs of STEC in New Zealand

2.10.1 Meat industry

The red meat sector is New Zealand's second largest primary sector contributing more than NZ$7 billion (23%) to New Zealand's annual export revenue [325]. For the beef industry, North America represents New Zealand's major export market with a value of NZ$903 million *per annum* (year ending June 2011) including a market value of NZ$26 million for veal products alone [326]). The US Food Safety and Inspection Service has a zero tolerance policy for STEC O157 and six non-O157 STEC serogroups including O26, O45, O103, O111, O121, and O145 in all imported meat [30, 327], adding extra costs to the production of red meat in New Zealand to fulfil the overseas market access requirements.

Furthermore, the detection of STEC O157 or any of the six non-O157 STECs in imported veal or beef from New Zealand would result in the loss of both markets, as New Zealand's major export markets do not clearly distinguish between beef and veal. The economic consequences for New Zealand's trade would be significant, as overseas market access could be denied for prolonged periods until the issue is resolved.

To avoid possible economic losses to the red meat industry and New Zealand's trade due to denied access to overseas markets, a better understanding of the epidemiology of STEC O157 and non-O157 STECs in New Zealand's slaughter cattle population is required, with the aim to reduce the levels of STEC entering the food chain.

2.10.2 Public health

The incidence of STEC infections in New Zealand, compared to other foodborne enteric diseases, i.e. campylobacteriosis (152.9 per 100,000 in 2013; 6,837 cases) [31], is low, however the economic costs due to the rare but severe complications and premature death associated with the disease, are high. It was estimated that STEC infections accounted for NZ$14.6 million (11%) of economic costs caused by foodborne diseases in 2009 [328]; for the minority of STEC cases requiring lifetime treatment, the total costs were estimated to be NZ$1.51 million. Although the incidence of STEC infections in Australia is relatively low (0.4 per 100,000 in 2011 [89]), McPherson *et al.* [329] estimated that this foodborne illness costs the Australian economy approximately AU$2.6 million each year. In comparison, Frenzen *et al.* [330] reported that O157 STEC infections in the US account for an estimated annual cost of US$405 million (in 2003 dollars), with the average cost per case ranging from US$26 (case without medical care) to US$6.2 million (case with fatal HUS) depending on the severity of disease. A very recent Canadian study [331] estimated that the annual cost for primary and long-term illness due to *E. coli* O157 infection is CA$403.9 million. The conclusion for all these studies is that the major cost of illness is related to the severity of disease. Hence, efforts should be made to minimise the risks of STEC infections and reduce the burden of disease in New Zealand.

2.11 Conclusions

STEC is a zoonotic pathogen of significant importance to public health and the food producing industry worldwide, which can cause life-threatening illnesses such as haemorrhagic colitis and haemolytic uraemic syndrome in humans, and death in extreme cases. Ruminants, particularly cattle, are considered a reservoir of STEC and are recognised as important sources of STEC infection in humans. Humans acquire STEC infections via direct and indirect contact with STEC-shedding animals and the consumption of contaminated food and water. Although an enormous amount of research has been conducted to gain knowledge on the epidemiology of STEC O157 and non-O157 in cattle worldwide, there is very little information available on the prevalence and distribution of STEC in New Zealand's slaughter cattle population. Since the US declared STEC O157 and six non-O157 STECs (O26, O45, O103, O111, O121, and O145) as adulterants in beef, the extended testing requirements of beef and veal have become a costly aspect of international trade access conditions for New Zealand. Hence, basic epidemiological data on STEC O157 and non-

O157 STEC in slaughter cattle (bobby calves and adult cattle) and the levels of STEC entering the food chain are required to enable the implementation of appropriate and cost-effective controls that reduce the risk of contaminated red meat produced in New Zealand. The production of adulterant-free meat is important for New Zealand's economy to maintain overseas market access. Even more importantly, additional epidemiological data are required regarding the source and exposure pathways of STEC infections in humans to help inform useful control strategies to reduce New Zealand's annual STEC incidence rate, which is among the highest in the world.

Chapter 3

A repeated cross-sectional study investigating the prevalence of faecal shedding of Shiga toxin-producing *Escherichia coli* O157 and O26 in very young calves and adult cattle at slaughter in New Zealand

3.1 Abstract

Shiga toxin-producing *Escherichia coli* (STEC) are zoonotic pathogens of great concern to the food producing industry worldwide. Cattle have been identified as an important host of STEC and have been linked repeatedly to human infections, causing haemorrhagic diarrhoea and haemolytic uraemic syndrome.

A repeated cross-sectional study was conducted to investigate the prevalence (and concentration), spatial distribution, and seasonal patterns of faecal shedding of STEC O157 and STEC O26 in New Zealand slaughter cattle. Four selected New Zealand beef slaughter plants licensed for the beef and veal export market were visited repeatedly from July 2009 until June 2011 to collect recto-anal mucosal swab samples from very young calves (bobby calves) and adult cattle post-slaughter.

Enriched faecal samples from 695 calves and 895 adult cattle were screened for the presence of *E. coli* O157 and O26 (STEC and non-STEC) using real-time PCR and confirmed by isolation using serogroup-specific culture media. The prevalence of both *E. coli* O157 and O26 by real-time PCR was higher in calves (O157: 23.5% (163/695); 95% CI 20.4%–26.8%; O26: 33.4% (232/695); 95% CI 29.9%–37.0%) compared to adult cattle (O157: 7.0% (63/895); 95% CI 5.5%–9.0%; O26: 7.6% (68/895); 95% CI 6.0%–9.6%). A total of 44 O157 and 92 O26 *E. coli* isolates were collected and screened for the presence of virulence genes characteristic of STEC (*ehx*A, *eae*, *stx*1 and *stx*2) using multiplex PCR. The prevalence of STEC O157 and STEC O26 in calves was 2.3% (16/695; 95% CI 1.4%–3.8%) and 3.9% (27/695; 95% CI 2.6%–5.7%), respectively, and 1.6% (14/895; 95% CI 0.9%–2.7%) and 0.4% (4/895; 95% CI 0.1%–1.2%) in adult cattle, respectively.

Levels of faecal shedding of *E. coli* and STEC in calves and adult cattle were estimated based on counts of colony forming units (CFU) on selective culture media. Concentrations of all *E. coli* O157 and O26 (including STEC and non-STEC) ranged from 3.3 to 7.9 $\log_{10}$ CFU/g faeces (SD 1.32 $\log_{10}$ CFU/g) in calves, and from 3.8 to 6.9 $\log_{10}$ CFU/g faeces (SD 1.11 $\log_{10}$ CFU/g) in adult cattle. Similarly, STEC concentrations (O157 and O26) in calves ranged from 3.6 to 7.9 $\log_{10}$ CFU/g faeces (SD 1.39 $\log_{10}$ CFU/g), and in adult cattle from 4.2 to 6.6 $\log_{10}$ CFU/g faeces (SD 0.96 $\log_{10}$ CFU/g).

As confirmed by culture, 3.4% (34/1,009; 95% CI 2.4%–4.7%) and 8.0% (81/1,009; 95% CI 6.5%–9.9%) of farms were positive for *E. coli* O157 and O26 (STEC and non-STEC), respectively, while only 4.9% (49/1,009; 95% CI 3.6%–6.4%) of all farms were positive for STEC (O157 and/or O26). The spatial distributions of animals real-time PCR-positive for *E. coli* O157 and O26 (STEC and non-STEC) showed increased relative risks for *E. coli* O26 in calves in the central region (Waikato) of the North Island, and for *E. coli* O157 in adult cattle in the central region (Canterbury) of the South Island. STEC O157 and O26 isolates were recovered from calves and adult cattle in both islands of New Zealand, except for STEC O26, which was not detected in adult cattle sampled in the South Island.

No obvious seasonal pattern in prevalence of *E. coli* and STEC was observed in calves in any calving season (July/August to September/October). In adult cattle, however, an increased prevalence of faecal shedding of STEC O157 was apparent in spring (September/October) and summer/autumn (January to May), while STEC O26 was only prevalent in summer (December/February) ($p = 0.118$).

This nationwide study on the prevalence of STEC O157 and STEC O26 faecal carriage in slaughter cattle was the first of its kind in New Zealand, providing valuable new information on the epidemiology of these two important zoonotic pathogens. The findings on the levels of *E. coli* and STEC entering the food chain via slaughtered animals are of high importance for the New Zealand red meat industry for maintaining international market access and minimising the risks to domestic and overseas consumers of red meat produced in New Zealand.

3.2 Introduction

Shiga toxin-producing *Escherichia coli* (STEC) strains possessing the Shiga toxin-producing genes (*stx*1 and/or *stx*2) and the locus of enterocyte effacement (LEE) can cause serious illnesses in humans such as haemorrhagic colitis and haemolytic uraemic syndrome (HUS). Young children and the elderly in particular are at higher risk of developing HUS, a leading cause of acute renal failure, which is potentially life-threatening [332].

Epidemiological studies have identified ruminants as an important host of STEC, excreting the pathogen in their faeces. Cattle in particular have been linked repeatedly to human infections. Humans can acquire STEC infection via ingestion of faecally-contaminated food [8, 10], direct or indirect contact with STEC-infected animals or humans [76, 333, 334], or exposure to environmental sources of infection such as faecally-contaminated recreational water [335, 336] and drinking water [103, 337]. Meat products of bovine origin, contaminated with faecal material at slaughter, and undercooked meat are also possible sources of STEC infections [22, 24, 25].

E. coli O157:H7 (STEC O157) has become the most recognised STEC serotype since it was associated for the first time with two foodborne outbreaks in the United States (US) in 1982 [26]. Over the past three decades, this emerging pathogen has caused numerous large outbreaks of gastrointestinal illness worldwide. Although STEC O157 is still the most commonly identified serotype of STEC in clinical cases overall, non-O157 STEC serogroups with similar pathogenic characteristics have emerged more frequently in Europe and the US over the past ten years [33, 69]. The increased reports of non-O157 STEC cases could be associated with additional laboratory testing of specimens for non-O157 STEC as a result of the availability of improved isolation methods for non-O157 serogroups.

In New Zealand, STEC infections appear as sporadic cases and small clusters but have been reported with increasing frequency since the first case was observed in 1993 [314]. In 2013, 207 STEC cases (4.6 per 100,000 population) were reported to the Public Health Units across the country [31]. The Enteric Reference Laboratory at ESR reported 217 STEC cases, of which 194 (89.4%) and 22 (10.1%) were confirmed by culture isolation and identified as STEC O157 and non-O157 STEC, respectively (in one case *stx* was detected by PCR but no STEC could be isolated) [316]. High rates of STEC infections were observed in children of

<5 years of age and in residents of regions with intensive dairy farming, indicating animal/environmental-associated exposures play an important role in the epidemiology of STEC in New Zealand.

In 2012, 2.3 million adult cattle (beef and dairy animals) and up to 1.7 million bobby calves were slaughtered in New Zealand [338]. Testing requirements and the certification of export meat to be free from contamination of STEC O157 and other non-O157 STEC pathogens are an increasing and costly aspect of international trade access conditions for New Zealand's red meat industry. Hence, detailed knowledge on the levels of STEC O157 and related non-O157 STEC pathogens entering the food chain via slaughtered livestock in New Zealand is important to minimise public health risks and retain international market access.

A few studies have investigated the prevalence of *E. coli* in New Zealand's livestock and have reported STEC in cattle and sheep in the North Island of New Zealand [321, 322, 324]. However, only limited data are available on the epidemiology of carriage and seasonal shedding of STEC in New Zealand's slaughter cattle population, inevitably hampering meat industry efforts to eliminate or restrict contamination of STEC in fresh meat to extremely low levels. This study was designed with the primary objective to gain detailed epidemiological knowledge of prevalence (and concentration), spatial distribution, and seasonal patterns of faecal shedding of STEC O157 and STEC O26 in slaughtered cattle nationwide.

3.3 Materials and methods

3.3.1 Sample size calculations

Sample sizes for calves and adult cattle were calculated based on prevalence estimates derived from published research on the faecal shedding of *E. coli* O157 and non-O157 in New Zealand calves and adult cattle. Prevalence estimates with 99% binomical exact confidence intervals (± 5% precision limits) and calculated sample sizes are presented in Table 3.1.

Table 3.1: Statistical sample size estimations for New Zealand calves and adult cattle considering variable prevalence estimates (± 5% precision limits) and 99% binomial exact confidence intervals (CI).

Animal group	*E. coli* serogroup	Reference	Prevalence estimates (%) (precision limits)	99% binomial exact CI	Sample size
Calves	O157	[339]	18.0 (13.0–23.0)	13.39–23.63	392
	Non-O157	[322]	34.0 (29.0–39.0)	29.12–39.26	596
Adult cattle	O157	[321]	<1.0 (0.0–2.0)[a]	0.31–2.59	657
	Non-O157	[322]	20.0 (15.0–25.0)	15.25–25.44	425

[a] Precision limit of ± 1%.

To ensure sufficient numbers of samples were collected to detect *E. coli* serogroup O157 or O26 (non-O157) in faeces from calves and adult cattle, the larger sample size of either serogroup was chosen. Hence, it was estimated to collect faecal samples from at least 596 calves and 657 adult cattle nationwide, resulting in a minimum total sample size of 1,253 faecal samples for this study.

3.3.2 Sample collection

From a list of operating slaughter plants in New Zealand licensed to process veal and beef for export, four large slaughter plants were included in the study, with two plants in each of the North Island (A and B) and the South Island (C and D) of New Zealand. The slaughter plants were chosen based on the annual number of slaughtered/processed animals in 2007 and their location, to achieve a good geographical coverage in this nationwide repeated cross-sectional study. From July 2009 until June 2011, each plant was visited (i) fortnightly between July/August and September/October (calving season) to collect post-slaughter samples from bobby calves, and (ii) monthly between August and July to collect post-slaughter samples from adult cattle (beef and dairy animals) on the processing chain. Calves of different dairy farms in a catchment area of a slaughter plant were transported collectively and slaughtered as a mob (i.e. not grouped by farm), whereas adult cattle were transported from farms as separate mobs and slaughtered as separate 'lines'.

Faecal samples were collected systematically from carcasses of calves and adult cattle on the slaughter chain including every 20–25th calf carcass that had a numbered ear tag and did not

have a blocked anus, and 1–3 carcasses per line of adult cattle depending on the size of the line. The aim was to sample multiple lines of adult cattle to cover a large number of farms within the catchment area of a plant, thus increasing geographical coverage. The number of required samples per plant visit varied between the plants as it was calculated based on the plant's annual killing numbers from 2007 but also depended on the number of calves and adult cattle available on each sampling day. Hence, on average 27 calves (range 6–110) and 11 adult cattle (range 4–17) were sampled on each plant visit. Recto-anal mucosal swab samples were collected using sterile cotton-tipped swabs (Transystem®, Copan, Brescia, Italy), placed in the Amies transport medium provided with the swabs and kept at 0–5°C until processed at *m*EpiLab within 24–48 h of collection.

Details of the sampled animal such as breed, sex, tag number, carcass weight, and address of the farm were recorded. Cross-bred animals were allocated to the visually more dominant breed. The age of adult cattle was estimated based on the number of permanent incisors [340]. Dentition was also used for grading of beef carcasses at the slaughter plants, classifying female cattle with ≤4 and >4 permanent incisors as heifers and cows, respectively [2]. Data on the time spans of transportation (from farms to slaughter plants) and lairage (at slaughter plants) were not available.

3.3.3 Laboratory methods

Enrichments and direct culture plating

Before processing, faecal material from each recto-anal mucosal swab sample was weighed (difference between weight of swab with provided Amies transport medium before and after sampling) and recorded to enable enumeration of the concentration of *E. coli* O157 and O26 in each sample (as described later under *Concentration of* E. coli *in faecal samples*). Swab samples were transferred into 20 ml of buffered peptone water (BPW) (Difco™, Becton, Dickinson and Co, USA) for enrichment, vortexed for 10 s and an aliquot of 50 µl each was plated onto selective culture media for *E. coli* O157 and O26 using cefixime-tellurite sorbitol MacConkey agar (CT-SMAC, Fort Richard Laboratories, Auckland, New Zealand) and cefixime-tellurite rhamnose MacConkey agar (CT-RMAC, Fort Richard Laboratories, Auckland, New Zealand), respectively, and an automated spiral plater (Don Whitley, UK; model: WASP2). Culture plates and broths were incubated for 18–24 h at 37°C. Aliquots of

each pre-enriched and post-enriched broth sample were preserved in glycerol (20% (vol/vol) glycerol) and stored at –80°C.

Real-time PCR for detection of *E. coli* serogroups (STEC and non-STEC)

To improve the efficiency of culture isolation, enriched broth samples were initially screened by real-time PCR for the presence of *E. coli* O157 and O26 (see further below on testing of sensitivity and specificity of these assays). DNA was extracted from a 1 ml aliquot of enriched broth using 2% Chelex beads solution (Chelex® 100 Resin, Bio-Rad). Previously published primer sequences were used to detect genes encoding for serogroup-specific O-antigens of *E. coli* (*rfb*E_{O157} and *wzx*$_{O26}$) (Table 3.2). All genomic DNA extractions were analysed immediately and then stored at –20ºC.

Table 3.2: Nucleotide sequences of forward and reverse primers used for the detection of specific target genes of *E. coli* in faecal samples collected from New Zealand slaughter cattle.

Primer set	Primer	Primer sequence (5' – 3')	Target gene	Amplicon size (bp)	Reference
	rfbE-F	TTTCACACTTATTGGATGGTCTCAA	*rfb*E_{O157}	88	[341]
	rfbE-R	CGATGAGTTTATCTGCAAGGTGAT			
	wzx-F	CGCGACGGCAGAGAAAATT	*wzx*$_{O26}$	135	[341]
	wzx-R	AGCAGGCTTTTATATTCTCCAACTTT			
A	stx1-F	GACTGCAAAGACGTATGTAGATTCG	*stx*1	150	[342]
	stx1-R	ATCTATCCCTCTGACATCAACTGC			
A	stx2-F	ATTAACCACACCCCACCG	*stx*2	200	[342]
	stx2-R	GTCATGGAAACCGTTGTCAC			
A	eae-F	GACCCGGCACAAGCATAAGC	*eae*	384	[245]
	eae-R	CCACCTGCAGCAACAAGAGG			
A	ehxA-F	GCATCATCAAGCGTACGTTCC	*ehx*A	534	[245]
	ehxA-R	AATGAGCCAAGCTGGTTAAGCT			
B	stx2c-F	CGACAGGCCCGTTATAAAAA	*stx*2c	243	[269]
	stx2c-R	GGCCACTTTTACTGTGAATGTATC			[267]
C	fliC-F	TACCATCGCAAAAGCAACTCC	*fli*C_{H7}	247	[343]
	fliC-R	GTCGGCAACGTTAGTGATACC			
C	E16S-F	AAACGATGTCGACTTGGAGGT	16S rRNA	100	In house[a]
	E16S-R	TGAGTTTTAACCTTGCGGCCG			

[a] Enteric Reference Laboratory, Upper Hutt, New Zealand.

Real-time PCR DNA amplifications were performed using an automated real-time thermocycler (Rotor Gene 6200HRM, Corbett Research). The final 20 µl PCR reaction

mixture for O157 contained 2x PCR buffer (conc. Light Cycler 480 Probes Master, Roche), 50 μM SYTO® 9 dye (green fluorescent nucleic acid stain, Invitrogen), 2 μM of each primer, and 5.4 μl DNA. The PCR included an initial enzyme-activation step at 96°C for 5 min, followed by 45 cycles of denaturation at 96°C for 15 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s; the PCR product was detected by thermal melt from 73°C to 82°C at a rate of 0.1°C per 2 s. Similarly, the final 20 μl PCR reaction mixture for O26 contained 2x PCR buffer (Express qPCR SuperMix, Invitrogen), 50 μM SYTO® 9 dye, 10 μM of each primer, 2.0 μl DNA, and 5.4 μl sterile water. The PCR included an initial enzyme-activation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 10 s; the PCR product was detected by thermal melt from 72°C to 78°C at a rate of 0.1°C per 2 s. Positive and negative template controls were included in each PCR assay.

Testing sensitivity and specificity of real-time PCR assays

The sensitivity and specificity of the real-time PCR assays used for detection of *E. coli* O157 and O26 respectively in enriched broth samples were assessed. The sensitivity was determined using serial dilutions of enriched faecal broth samples spiked with *E. coli* O157 and O26. Aliquots of each dilution were taken for DNA extraction and direct plating on CT-SMAC/CT-RMAC culture media to enumerate colony forming units (CFU) at each dilution. The highest dilution detectable by real-time PCR was concluded to be the detection limit of CFU/ml of enriched broth sample. The detection limits for *E. coli* O157 and O26 were 260 CFU/ml and 198 CFU/ml, respectively.

The specificity of both real-time PCR assays was determined using negative enriched faecal broth samples spiked with *E. coli* O157 and O26 culture isolates. DNA was extracted from each spiked sample and tested with *rfb*E_{O157} and *wzx*$_{O26}$ primers. Real-time PCR amplicons using *rfb*E_{O157} and *wzx*$_{O26}$ primers were only obtained from samples spiked with *E. coli* O157 and O26, respectively, while no PCR products were detected in samples spiked with *E. coli* O157 using *wzx*$_{O26}$ primers, and *vice versa*. The real-time PCR amplicons of *E. coli* O157 and O26 were sequenced and showed identical gene sequences for *rfb*E_{O157} and *wzx*$_{O26}$, respectively, using BLAST [344].

Culture isolation from direct culture plates

Serogroup specific latex agglutination kits (E. coli O157 Latex, Oxoid, UK; Serocheck O26, Oxoid, UK) were used on real-time PCR-positive samples to identify suspected *E. coli* O157 and O26 colonies from direct culture on selective culture media. Up to 10 non-sorbitol-fermenting and 10 sorbitol-fermenting suspected *E. coli* O157 colonies per CT-SMAC plate were tested with latex agglutination and, similarly up to 10 non-rhamnose-fermenting and 10 rhamnose-fermenting suspected *E. coli* O26 colonies per CT-RMAC plate were tested with latex agglutination. Agglutination-positive *E. coli* O157 and O26 isolates were confirmed with real-time PCR for the presence of *rfb*E_{O157} and *wzx*$_{O26}$ genes, respectively, to exclude false-positive latex agglutination results. Confirmed isolates were preserved in glycerol broth (nutrient broth with 15% glycerol) and stored at –80°C for further molecular analysis.

In addition, suspected *E. coli* O157 colonies from a subset of real-time PCR-negative samples were also tested with latex agglutination to check for false-negative samples. This additional testing to detect false-negative samples was not implemented on suspected *E. coli* O26 colonies because of the limited availability of the serogroup specific agglutination kit.

Culture isolation using immunomagnetic separation (IMS)

If culture isolation from direct culture plates of real-time PCR-positive samples was unsuccessful, further tests were undertaken. Serogroup specific immunomagnetic beads coated with antibodies against surface antigens of *E. coli* O157 and O26 (Dynabeads® anti-E. coli O157 and Dynabeads® EPEC/VTEC O26, Invitrogen Dynal AS, Oslo, Norway) were used for isolation of *E. coli* O157 and O26 from enriched broths, following the manufacturer's instructions. A magnetic pen (PickPen® 1-M, BioNobile, Finland) was used for bead extraction. The beads-broth sample suspensions were inoculated onto serogroup specific culture media (CT-SMAC for O157, CT-RMAC for O26) and incubated for 18–24 h at 37°C. Again, up to 10 non-fermenting and 10 fermenting colonies per culture plate were tested with serogroup specific latex agglutination kits. Agglutination-positive *E. coli* O157 and O26 isolates were confirmed with real-time PCR for the presence of *rfb*E_{O157} and *wzx*$_{O26}$ genes, respectively, to exclude false-positive latex agglutination results. Confirmed isolates were preserved in glycerol broth and stored at –80°C for further molecular analysis.

Molecular analysis of isolates

Stored *E. coli* O157 and O26 isolates were re-grown on Columbia horse blood agar (Fort Richard Laboratories, Auckland, New Zealand). Bacterial DNA was extracted from five colonies using 2% Chelex beads solution and was analysed in the following series of PCR assays. All genomic DNA extractions were analysed immediately and then stored at –20ºC.

Multiplex PCR for detection of virulence genes

Confirmed *E. coli* O157 and O26 isolates were screened by multiplex PCR for the presence of Shiga toxin 1 (*stx*1) and Shiga toxin 2 (*stx*2) genes, and other virulence genes including enterohaemolysin (*ehx*A) and intimin (*eae*), characteristic of STEC. The multiplex PCR assay was performed on the automated real-time thermocycler, using previously published primer sequences (primer set A, Table 3.2). The final 25 µl PCR reaction mixture contained 2x PCR buffer (Express qPCR SuperMix, Invitrogen), 2 µM of each primer, 2.0 µl DNA, and 2.5 µl sterile water. The PCR included an initial enzyme-activation step at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 64°C for 20 s, and extension at 72°C for 20 s; the terminal extension was at 72°C for 5 min. Amplified PCR products were detected by electrophoresis using a 2% (wt/vol) agarose gel (Agarose low EEO, AppliChem, Germany), stained in ethidium bromide (0.5 µg/ml in TBE buffer) for 10 min, and visualised under ultraviolet light on a transilluminator (GelDoc™ XR, BIO-RAD Laboratories, Segrate (Milan), Italy).

PCR for detection of virulence gene subtype stx*2c*

E. coli isolates positive for *stx*2 were tested for the presence of the genetic subtype *stx*2c. This gene was detected in a separate PCR assay using primer sequences published by Besser *et al.* [267] and Shringi *et al.* [269] (primer set B, Table 3.2). DNA amplification was performed on the automated real-time thermocycler with a final 20 µl PCR reaction mixture containing 2x PCR buffer (Express qPCR SuperMix, Invitrogen), 2 µM of each primer, 2.0 µl DNA, and 6.0 µl sterile water. The PCR included an initial enzyme-activation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 20 s and annealing at 55°C for 20 s; no extensions were used. The amplified PCR product was detected by electrophoresis using a 2% (wt/vol) agarose gel, stained in ethidium bromide for 10 min, and visualised under UV light on the transilluminator.

PCR for detection of fli*C gene*

E. coli O157 isolates were tested also for the presence of the *fli*C gene encoding the H7 bacterial flagellum antigen, using previously published primer sequences [343] (primer set C, Table 3.2). In addition, to ensure the DNA template used in this series of PCR assays was of bacterial origin, an internal control (E16S) was included (primer set C, Table 3.2). DNA amplification of the *fli*C_{H7} and bacterial 16S rRNA gene sequences was performed using the automated real-time thermocycler. The final 20 µl PCR reaction mixture contained 2x PCR buffer (Taq PCR Mastermix, Qiagen), 5 µM of each primer for *fli*C_{H7}, 10 µM of each primer for bacterial 16S rRNA, 2.0 µl DNA, and 5.0 µl sterile water. The PCR included an initial enzyme-activation step at 95°C for 8 min, followed by 25 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 10 s, and extension at 72°C for 10 s; the terminal extension was at 72°C for 7 min. Amplified PCR products were detected by electrophoresis using a 2% (wt/vol) agarose gel, stained in ethidium bromide for 10 min, and visualised under UV light on the transilluminator.

Concentration of E. coli *in faecal samples*

Pre-enriched broth samples were inoculated directly on selective culture media as described earlier under *Enrichments and direct culture plating*. Putative non-fermenting *E. coli* O157 and O26 colonies were counted on each culture plate to enumerate the concentration of viable *E. coli* in faecal samples prior to enrichment. If agglutination-positive *E. coli* isolates were identified in pre-enriched samples, the concentration of *E. coli* was calculated as $\log_{10}$ CFU/g faecal material. If no *E. coli* isolates were identified due to concentrations below the detection limits of the selective culture media, the IMS method was used on the enriched samples as described above. If agglutination-positive *E. coli* isolates were identified in enriched samples by IMS, the concentration of *E. coli* was presented as a value below the detection limits of selective culture media of pre-enriched samples.

Of note, CT-RMAC culture media was not available at the start of the study and the direct inoculation of fresh faecal samples from 281 calves and 18 adult cattle could not be applied to enumerate the concentrations of presumptive *E. coli* O26 and STEC O26. However, 23 O26 isolates were retrieved with IMS from stored enrichments at a later stage in the study.

3.3.4 Data management and statistical analysis

R software (version 2.15.2) [345] was used for all statistical analysis, with significance set at $p < 0.05$. Descriptive statistics were calculated for each animal group to assess completeness and validity of data. Pearson χ^2 test was used to compare prevalence of all *E. coli* O157 with all O26, and STEC O157 with STEC O26 in samples from calves and adult cattle; and among beef and dairy farms.

Latent class analysis

A latent class analysis was applied to estimate the prevalence of *E. coli* O157 and O26 (STEC and non-STEC) in enriched faecal samples based on real-time PCR and culture methods (direct culture plating and IMS) used. Both methods are designed to detect *E. coli* serogroups O157 and O26 but do not distinguish between STEC and non-STEC. A 'two test – two population method' was applied as first described by Hui and Walter [346], assuming that (i) performances of both test methods were constant across both populations (two sets of samples from calves and adult cattle), (ii) prevalence of *E. coli* O157 (or O26) was different between the two populations, and (iii) the tests were independent.

The Bayesian inference approach was chosen to estimate the model parameters. Prior information on sensitivity and specificity of both tests and prevalence of *E. coli*, combined with data of test results were used to derive the posterior probability for each parameter. BetaBuster (version 1.0) [347] was used to estimate the prior probability distributions of each parameter, which were then included in Monte Carlo simulation modelling to derive the posterior probabilities with 95% credible intervals, using WinBUGS software (version 1.4.3) [348]. The computed prevalence estimates for *E. coli* O157 and O26 in calves and adult cattle were presented as medians with 95% credible intervals.

In addition, estimates of test performance of real-time PCR and direct culture plating (without IMS) were repeated on a subset of calf and adult cattle samples, which were real-time PCR-negative and test-negative with latex agglutination. This representative subset of samples enabled the comparison of the test performances of real-time PCR and direct culture plating more accurately, as not all real-time PCR-negative samples of calves and adult cattle were tested with latex agglutination.

Spatial and temporal analysis

To illustrate the spatial distribution of farm origins of calves and adult cattle sampled at slaughter plants, New Zealand Transverse Mercator coordinates (NZTM2000) of farms were plotted on the New Zealand map, using R packages 'maptools' [349] and 'spatstat' [350]. The same methods were applied to map the spatial distribution of STEC isolates (O157 and O26) retrieved from samples of calves and adult cattle.

Based on the spatial distribution of calves and adult cattle and animals' real-time PCR results (test-positive or test-negative for *E. coli* O157 or O26), relative risk surfaces were computed for New Zealand, using R package 'sparr' [351]. An adaptive estimate was utilised for densities of real-time PCR-positive and negative animals with an average smoothing bandwidth of 50km to account for spatial heterogeneity. Areas with values >0.0 indicate increased relative risks of animals being tested real-time PCR-positive for the specified serogroup of *E. coli*. Real-time PCR results were used for this analysis because of higher prevalence and statistical power, compared to culture results.

The monthly prevalence of *E. coli* O157 and O26 in calves and adult cattle was computed for each test method (real-time PCR, culture isolation, and multiplex PCR for STEC) as the number of test-positive animals divided by the total number of animals tested per calendar month.

Multivariate logistic regression analysis

Multivariate logistic regression analysis was applied to identify risk factors associated with a calf or adult cattle being test-positive for either *E. coli* O157 or O26 (STEC and non-STEC). Real-time PCR results were used for this analysis because of their higher prevalence and statistical power, compared to culture results. Explanatory variables were analysed using univariate and multivariate logistic regression. Exposure variables with Wald test or Likelihood ratio tests p-values <0.20 in univariate analysis were tested for correlation, and included in an initial multivariate model if their correlation values were < |0.30|.

Stepwise backward- and forward-elimination of least significant variables and those with correlation values of ≥ |0.30|, respectively, was applied, to generate a preliminary multivariate model. Eliminated variables were assessed for confounding, determined by a change of

>30% in a variable coefficient of the model after another variable was dropped from or added to the model. Even if non-significant, variables with confounding effect were retained in the model. Biologically plausible interactions between variables were assessed before generating the final multivariate model.

To evaluate the model's significance and goodness-of-fit, Likelihood ratio tests and the le Cessie-van Houwelingen normal test statistics [352] were applied, respectively, using R package 'rms' [353]. Models were compared using the Akaike information criterion (AIC), a measure of the relative goodness of fit. Odds ratios of model variables including 95% confidence intervals were calculated in Microsoft Excel 2010.

To account for clustering of animals originating from the same farm, a multivariate model with 'Farm' as a random effect variable was built as described above, using R package 'lme4' [354]. The model's significance was evaluated applying the Likelihood ratio tests.

3.4 Results

3.4.1 Animal data

Recto-anal mucosal swab samples of 695 calves and 895 adult cattle were collected at the selected slaughter plants. Numbers of collected faecal samples per animal group and year are summarised in Table 3.3.

Table 3.3: Number of faecal swab samples collected post-slaughter from calves and adult cattle at four large cattle slaughter plants in New Zealand from July 2009 to June 2011, stratified by island of New Zealand, slaughter plant, and type of missing data. [Total number of calves and adult cattle killed on each island of New Zealand [355]]

	Calves		Adult cattle		
Variable	2009	2010	2009	2010	2011
Island of New Zealand					
North (NI)	278 [1,012,812]	217 [1,063,820]	52 [1,804,671]	231 [1,757,569]	163 [1,758,873]
South (SI)	76 [451,365]	124 [486,246]	88 [632,764]	226 [618,408]	135 [622,294]
Plant					
A (NI)	205	80	22	111	79
B (NI)	73	137	30	120	84
C (SI)	36	64	53	124	72
D (SI)	40	60	35	102	63
Type of missing data					
Carcass weight[a]	10	15	0	1	0
Faecal sample weight[b]	0	0	0	11	0
Counts and concentration[c]	281	0	18	0	0

[a] Weights missing due to condemnation of carcasses at meat inspection.
[b] Weights missing as swabs with provided Amies transport medium were not weighed before sampling.
[c] Counts and concentration of presumptive *E. coli* O26 in faecal samples missing as selective culture media for *E. coli* O26 (CT-RMAC) was not available at the start of the study.

The geographical distribution of the farms of origin of the sampled calves and adult cattle is depicted in Figure 3.1.

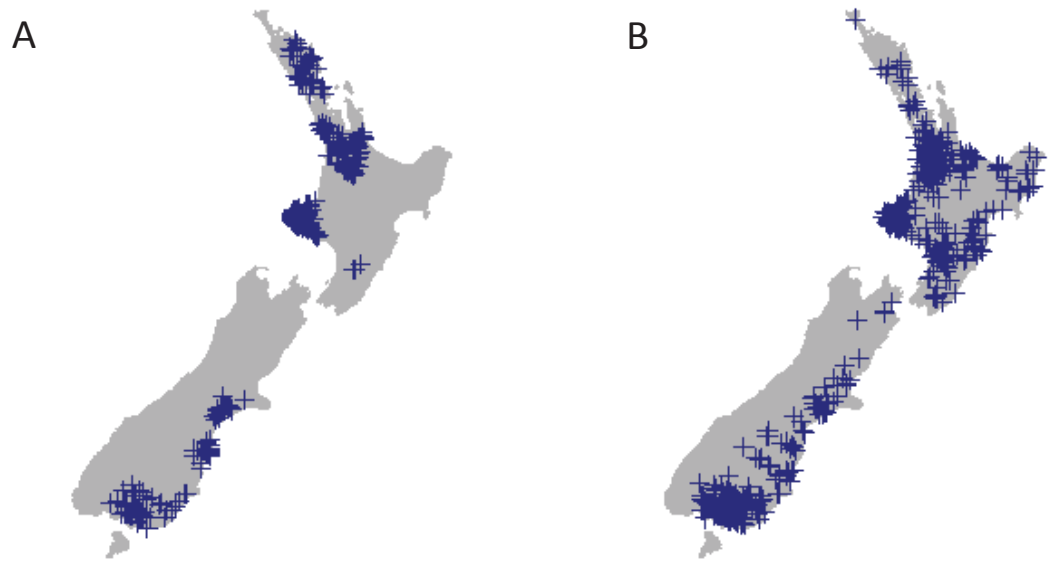

Figure 3.1: Map of New Zealand showing distribution of farms of origin of (A) calves (n = 695) and (B) adult cattle (n = 895) of which recto-anal faecal swab samples were collected post-slaughter at four large cattle slaughter plants from July 2009 to June 2011.

Calves and adult cattle originated from 496 and 513 different farms, respectively, of which 655 were dairy farms and 354 were beef farms. The group of calves comprised 512 bull calves and 183 heifer calves; they were 4–7 days-old and mainly of dairy breeds: Friesian (n = 469), Jersey (n = 220), and other breeds (n = 6). Almost all calves (99.1%; 689/695) originated from dairy farms (75.0%; 491/655).

The group of adult cattle comprised 173 bulls, 355 steers, 148 heifers, and 219 cows. The estimated age among adult cattle, stratified by sex, is depicted in Figure 3.2.

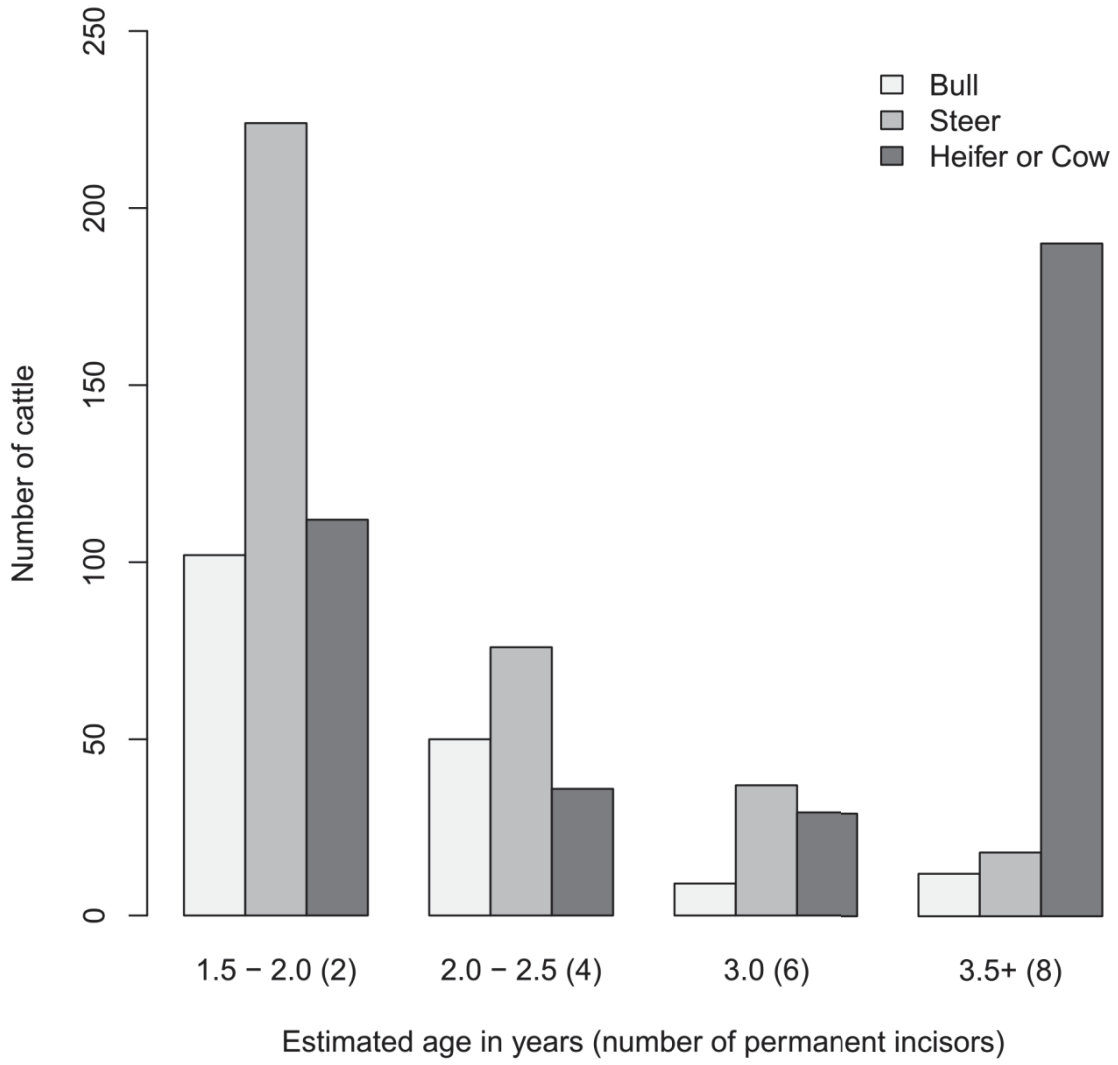

Figure 3.2: Age distribution of adult cattle (n = 895), stratified by sex. Age was estimated based on dentition (number of permanent incisors). Female cattle with ≤4 and >4 incisors were classified as heifers and cows, respectively.

The bimodal age distribution represents the two main groups of adult cattle killed at slaughter plants: the younger animals, which are fattened for the beef market and the older cattle, which are most likely to be cull animals.

Of the 895 adult cattle, 542 (60.6%) animals were of beef breeds: Angus (n = 314), Hereford (n = 159), Charolais (n = 25), Limousin (n = 15), Simmental (n = 15), and other beef breeds (n = 14); and 353 (39.4%) animals were of dairy breeds: Friesian (n = 276), Jersey (n = 75), and Ayrshire (n = 2). Angus and Hereford breeds were represented mainly by young steers

and heifers, and Friesian and Jersey breeds mostly by cows. The majority of adult cattle (74.3%; 665/895) originated from beef farms with 25.7% (230/895) from dairy farms.

The mean carcass weight of calves and adult cattle was 15.7 kg (95% confidence interval (CI) 9.7 kg–21.7 kg) and 271.2 kg (95% CI 133.9 kg–408.4 kg), respectively. Carcass weights were missing for 25 calves and one adult cattle due to condemnation at meat inspection.

3.4.2 Prevalence of *E. coli* (STEC and non-STEC) in animals

Prevalence estimates of *E. coli* O157 and O26 (including STEC and non-STEC) in faecal samples from calves and adult cattle as tested by real-time PCR and by culture isolation (direct culture plating and IMS combined), are summarised in Table 3.4. A proportion of samples from calves and adult cattle were test-positive for both serogroups.

Table 3.4: Prevalences (%) of *E. coli* O157 and O26 (including STEC and non-STEC) in recto-anal faecal swab samples collected from calves (n = 695) and adult cattle (n = 895) at slaughter. Prevalence is shown as the number of test-positive samples by real-time PCR and by culture isolation (direct culture plating and immunomagnetic separation combined)*.

		Real-time PCR		Culture isolation	
Animal group	Serogroup	Prevalence % (positive/tested)	95% CI[a]	Prevalence % (positive/tested)	95% CI[a]
Calves	O157	10.8 (75/695)	8.6–13.4	3.0 (21/695)	1.9–4.7
	O26	20.7 (144/695)	17.8–24.0	8.2 (57/695)	6.3–10.6
	O157 + O26	12.7 (88/695)	10.3–15.4	0.1 (1/695)	0.0–0.9
Adult cattle	O157	5.4 (48/895)	4.0–7.1	2.2 (20/895)	1.4–3.5
	O26	5.9 (53/895)	4.5–7.7	3.0 (27/895)	2.0–4.4
	O157 + O26	1.7 (15/895)	1.0–2.8	0.2 (2/895)	0.0–0.9

[a] CI = 95% confidence interval.

* A proportion of animals were test-positive for both serogroups (O157+O26).

In calves, the real-time PCR prevalence of *E. coli* O26 (STEC and non-STEC) (33.4% (232/695); 95% CI 29.9%–37.0%) was significantly higher than for *E. coli* O157 (STEC and non-STEC) (23.5% (163/695); 95% CI 20.4%–26.8%) ($p < 0.001$), but no statistical difference was observed between *E. coli* serogroups in adult cattle (O157: 7.0% (63/895); 95% CI 5.5%–9.0%; O26: 7.6% (68/895); 95% CI 6.0%–9.6%) ($p = 0.717$). The culture prevalence of *E.*

coli O26 (STEC and non-STEC) in calves (8.3% (58/695); 95% CI 6.4%–10.7%) was also significantly higher compared to *E. coli* O157 (STEC and non-STEC) (3.2% (22/695); 95% CI 2.0%–4.8%) (p <0.001), but no statistically significant difference was apparent in adult cattle (O157: 2.5% (22/895); 95% CI 1.6%–3.8%; O26: 3.2% (29/895); 95% CI 2.2%–4.7%) (p = 0.394).

Recovery rates of isolates from real-time PCR-positive samples and prevalence estimates of all *E. coli* O157 and O26 (STEC and non-STEC) in calves and adult cattle, adjusted for sensitivity and specificity of test methods applied, are shown in Table 3.5. Estimate calculations of test performances are presented in the following section (3.4.3 *Test performance of real-time PCR and culture isolation*).

Table 3.5: Prevalences (%) of *E. coli* O157 and O26 (including STEC and non-STEC) in recto-anal faecal swab samples from calves (n = 695) and adult cattle (n = 895) as tested by real-time PCR; the recovery rates (%) of isolates from real-time PCR-positive samples by culture isolation methods (direct culture plating and immunomagnetic separation combined); and prevalence estimates (%) of *E. coli* O157 and O26 (STEC and non-STEC) adjusted for test sensitivity (Se) and specificity (Sp) of real-time PCR and culture methods used.

Animal group	Serogroup	Prevalence by real-time PCR (95% CI)[a] [positive/tested]	Recovery rate (95% CI)[a] [culture positive/real-time PCR positive]	Prevalence estimates adjusted for Se and Sp (95% CrI)[b]
Calves	O157	23.5 (20.4–26.8) [163/695]	13.5 (8.8–19.9) [22/163]	23.1 (18.2–28.4)
	O26	33.4 (29.9–37.0) [232/695]	25.0 (19.7–31.2) [58/232]	32.8 (28.3–37.5)
Adult cattle	O157	7.0 (5.5–9.0) [63/895]	34.9 (23.6–48.1) [22/63]	6.7 (4.8–9.0)
	O26	7.6 (6.0–9.6) [68/895]	42.6 (30.9–55.2) [29/68]	6.0 (4.2–8.2)

[a] CI = 95% confidence interval.
[b] Prevalence estimates and 95% credible intervals (CrI) were computed based on 100,000 iterations.

The recovery rate of *E. coli* O26 (STEC and non-STEC) in samples from calves was significantly higher than for *E. coli* O157 (p = 0.007). Although there was no statistically significant difference in recovery rates between *E. coli* serogroups from samples of adult

cattle (p = 0.467), the percentages of recovery for both *E. coli* serogroups were higher than from samples of calves.

3.4.3 Test performance of real-time PCR and culture isolation

Estimates of test performances of real-time PCR and culture isolation (direct culture plating and IMS combined), including prior and posterior probabilities with 95% credible intervals, are summarised in Table 3.6.

Table 3.6: Prior and posterior probability estimates of sensitivity (Se) and specificity (Sp) of real-time PCR (PCR) and culture methods (CM: direct culture plating and immunomagnetic separation combined) used to test recto-anal faecal swabs from calves (n = 695) and adult cattle (n = 895) for the prevalence of all *E. coli* O157 and O26 (including STEC and non-STEC).

		Prior estimates (%)		Posterior estimates (%)		
Parameter	Serogroup	Mode value[a]	95% certainty value[b]	Standard deviation	Median	95% credible interval[c]
	O157					
Se PCR		55.0	>40.0	4.60	80.89	70.85–88.80
Se CM		90.0	>60.0	3.52	23.49	17.31–31.08
Sp PCR		55.0	>40.0	0.95	95.68	93.62–97.34
Sp CM		85.0	>60.0	0.13	99.82	99.48–99.96
	O26					
Se PCR		55.0	>40.0	2.93	88.48	81.95–93.36
Se CM		90.0	>60.0	3.21	32.80	26.89–39.47
Sp PCR		55.0	>40.0	0.91	95.79	93.83–97.39
Sp CM		70.0	>40.0	0.15	99.76	99.38–99.94

[a] Prior information on prevalence of all *E. coli* O157 or O26, e.g. 55.0%.
[b] Being 95% certain that sensitivity or specificity is greater or less than e.g. 40.0%.
[c] 95% credible intervals were computed based on 100,000 iterations.

Although the specificity of real-time PCR for both *E. coli* O157 and *E. coli* O26 (STEC and non-STEC) was estimated as >95%, the test sensitivity was better for *E. coli* O26 than for *E. coli* O157. The culture methods had also very high estimated specificities for both *E. coli* serogroups (>99%), but performed poorly in test sensitivity (<32%).

Estimates of test performances of real-time PCR and direct culture plating (without IMS) for *E. coli* O157 (STEC and non-STEC) were repeated including a subset of negative samples (Table 3.7). This subset comprised a percentage of direct culture plates of real-time PCR-negative samples for *E. coli* O157 from calves (40.3%, 211/532) and adult cattle (37.4%, 311/832), which had tested negative with latex agglutination for suspect *E. coli* O157 colonies.

Table 3.7: Prior and posterior probability estimates of sensitivity (Se) and specificity (Sp) of real-time PCR (PCR) and direct culture plating (DCP) methods calculated on a subset of recto-anal faecal samples from calves (n = 286) and adult cattle (n = 359) tested for the prevalence of *E. coli* O157 (including STEC and non-STEC).

	Prior estimates (%)		Posterior estimates (%)		
Parameter	Mode value[a]	95% certainty value[b]	Standard deviation	Median	95% credible interval[c]
Se PCR	80.0	>50.0	6.06	90.53	74.97–98.00
Se DCP	50.0	<80.0	7.19	22.82	12.88–40.76
Sp PCR	55.0	<80.0	2.69	90.70	85.44–95.92
Sp DCP	75.0	>50.0	0.38	99.32	98.36–99.80

[a] Prior information on prevalence of all *E. coli* O157, e.g. 55.0%.
[b] Being 95% certain that sensitivity or specificity is greater or less than e.g. 70%.
[c] 95% credible intervals were computed based on 100,000 iterations.

While the test performance of direct culture plating for *E. coli* O157 remained unchanged, the test sensitivity of real-time PCR improved to 90%, but its specificity decreased slightly to 90%.

3.4.4 Characterisation of confirmed isolates

A total of 44 O157 and 92 O26 isolates were collected and analysed by multiplex PCR for the presence of virulence genes. Five samples contained two characteristically different virulence types of *E. coli* O26. Figure 3.3 depicts PCR amplicons of bacterial DNA from *E. coli* isolates and the identification of STEC isolates (primer set A), the detection of the gene subtype *stx*2c (primer set B), the presence of the *fli*C_{H7} gene in STEC O157 and the gene for bacterial 16S rRNA (internal control) (primer set C).

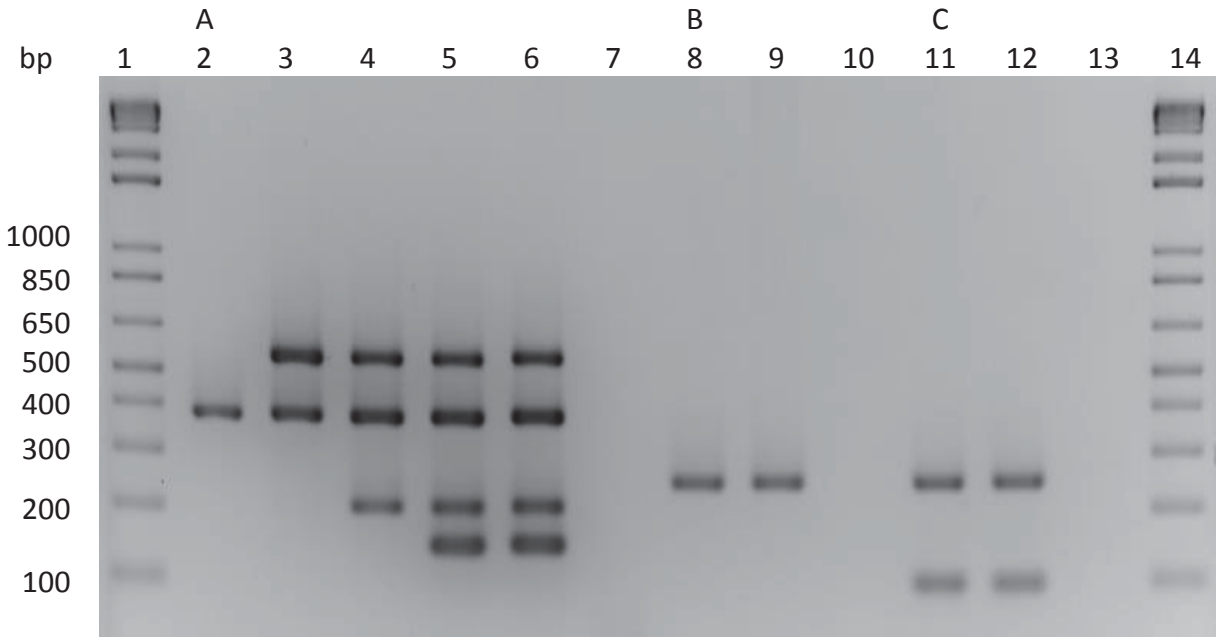

Figure 3.3: Agarose (2% wt/vol) gel electrophoresis showing PCR amplicons of DNA from *E. coli* isolates using primer sets A (lanes 2–7), B (lanes 8–10), and C (lanes 11–13) for the detection of different virulence genes. Lanes 1 and 14: 1kb Plus DNA ladder (Invitrogen); 2, *eae*; 3, *ehx*A and *eae*; 4, *ehx*A, *eae*, and *stx*2; 5, *ehx*A, *eae*, *stx*2, and *stx*1; 6, positive control; 7, negative control; 8, stx2c; 9, positive control; 10, negative control; 11, *fli*C_{H7} and bacterial 16S rRNA (internal control); 12, positive control; 13, negative control.

Characteristics of all *E. coli* isolates collected, stratified by animal group and serogroup, are presented in Table 3.8.

Table 3.8: Characteristics of *E. coli* O157 (n = 44) and O26 (n = 92) isolates retrieved from faecal samples collected from New Zealand slaughter cattle, stratified by sampled animal group and serogroups. PCR methods were used to test for the presence (+) of virulence genes encoding for enterohaemolysin (*ehx*A), intimin (*eae*), Shiga toxins (*stx*2, genetic subtype *stx*2c, and *stx*1), and the *fliC*$_{H7}$ gene encoding the H7 flagellum antigen from STEC O157.

					Virulence genes of STEC					
Animal group	Serogroup	Number of isolates	Sorbitol[a]	Rhamnose[b]	*ehx*A	*eae*	*stx*2	*stx*2c	*stx*1	H7
Calves	O157	2	–		+	+	+	–	+	+
(n = 695)		2	–		+	+	+	+	–	+
		12	–		+	+	+	–	–	+
		6	+		–	–	–	–	–	–
	O26	27		–	+	+	–	–	+	ne
		15		–	+	+	–	–	–	ne
		3		+	+	+	–	–	–	ne
		17		+	–	+	–	–	–	ne
		1		+	–	–	–	–	–	ne
Adult cattle	O157	1	–		+	+	+	–	+	+
(n = 895)		8	–		+	+	+	+	–	+
		5	–		+	+	+	–	–	+
		8	+		–	–	–	–	–	–
	O26	4		–	+	+	–	–	+	ne
		7		–	+	+	–	–	–	ne
		5		+	+	+	–	–	–	ne
		13		+	–	+	–	–	–	ne

[a] Sorbitol-fermenting (+) or non-sorbitol-fermenting (–) on cefixime-tellurite sorbitol MacConkey culture plate.

[b] Rhamnose-fermenting (+) or non-rhamnose-fermenting (–) on cefixime-tellurite rhamnose MacConkey culture plate.

ne = not examined.

3.4.5 Concentrations of *E. coli* and STEC in faecal samples

The median weight of faecal material collected from calves and adult cattle was 0.07g (interquartile range (IQR) 0.05g–0.10g) and 0.09g (IQR 0.08g–0.12g; 11 missing values), respectively.

The counts of fermenting and non-fermenting colonies on direct culture plates inoculated with recto-anal faecal samples from calves and adult cattle are summarised in Table 3.9. Both selective culture media (CT-SMAC and CT-RMAC) showed a higher aerobic growth in faecal samples from calves compared to adult cattle.

Table 3.9: Minimum, maximum, and quartiles for counts of fermenting and non-fermenting colonies on direct culture plates inoculated with recto-anal faecal samples (n) collected from calves and adult cattle at slaughter. Only colony counts on CT-SMAC[a] and CT-RMAC[b] plates of samples real-time PCR-positive for *E. coli* O157 and O26, respectively, are presented. Counts in faecal samples are shown as CFU/ml of pre-enriched broth sample.

		CT-SMAC[a] plates						CT-RMAC[b] plates					
				Percentile						Percentile			
Age group[c]	Colony type	n	Minimum	25th	50th	75th	Maximum	n	Minimum	25th	50th	75th	Maximum
Calves	Fermenting	163	0	1695	11360	54000	200000	241	0	210	2080	8480	200000
	Non-fermenting		0	0	20	760	200000		0	10	100	3040	200000
Younger cattle	Fermenting	41	0	0	20	200	200000	41	0	0	0	30	1360
	Non-fermenting		0	0	0	10	200000		0	0	0	20	19200
Older cattle	Fermenting	22	0	10	40	165	1160	27	0	0	0	5	780
	Non-fermenting		0	0	0	10	43200		0	0	0	0	310

[a] CT-SMAC = cefixime-tellurite sorbitol MacConkey culture plate.

[b] CT-RMAC = cefixime-tellurite rhamnose MacConkey culture plate.

[c] Age groups: calves 4–7 days-old; younger cattle (male and female of beef or dairy breed) with ≤4 permanent incisors; older cattle (male and female of beef or dairy breed) with >4 permanent incisors.

The distributions of estimated concentrations of all *E. coli* (O157 and O26, including STEC and non-STEC) as derived from direct culture plating and IMS of samples from calves and adult cattle, are presented in Table 3.10. Higher concentrations of *E. coli* were observed in faecal samples from calves compared to adult cattle.

Table 3.10: Minimum, maximum and quartiles for estimated concentrations of all *E. coli* O157 and O26 (including STEC and non-STEC) in recto-anal swab samples (n) collected from calves and adult cattle at slaughter, stratified by detection method. Concentrations are shown as $\log_{10}$ CFU/g faeces.

				Percentile				
Age group[a]	Detection method	n	Minimum	25th	50th	75th	Maximum	Standard deviation
Calves	DC[b]	37	3.3	4.6	5.9	6.7	7.9	1.32
	IMS[c]	20	<3.2	<3.7	<4.8	<5.9	<7.9	1.25
Younger cattle	DC	9	3.8	4.2	4.6	5.6	6.9	1.11
	IMS	29	<2.7	<3.3	<3.4	<4.0	<6.6	0.79
Older cattle	DC	1	-	-	6.3	-	-	-
	IMS	12	<3.2	<3.2	<3.4	<3.7	<4.8	0.56

[a] Age groups = calves 4–7 days-old; younger cattle (male and female of beef or dairy breed) with ≤4 permanent incisors; older cattle (male and female of beef or dairy breed) with >4 permanent incisors.
[b] DC = Direct culture.
[c] IMS = Immunomagnetic separation. Serogroup specific beads for *E. coli* O157 and O26 were used when *E. coli* concentrations in samples were very low and no colonies were identified with direct culture. As IMS method provides no information on pathogen concentrations, *E. coli* concentrations in these samples were below the presented values, which represent enumerated concentrations from direct culture but without having detected *E. coli*.

The distributions of estimated concentrations of STEC as detected by direct culture plating and IMS, are summarised in Table 3.11. Higher concentrations of all STEC (O157 and O26) were observed in samples from calves compared to adult cattle.

Table 3.11: Minimum, maximum and quartiles for estimated concentrations of all STEC (O157 and O26) in recto-anal swab samples (n) collected from calves and adult cattle at slaughter, stratified by detection method. Concentrations are shown as $\log_{10}$ CFU/g faeces.

Age group[a]	Detection method	n	Minimum	Percentile 25th	Percentile 50th	Percentile 75th	Maximum	Standard deviation
Calves	DC[b]	23	3.6	4.6	6.1	7.0	7.9	1.39
	IMS[c]	7	<3.5	<3.7	<4.9	<6.2	<7.9	1.68
Younger cattle	DC	5	4.2	4.4	4.6	4.9	6.6	0.96
	IMS	11	<2.7	<3.3	<3.5	<4.3	<6.6	1.13
Older cattle	DC	1	-	-	6.3	-	-	-
	IMS	1	-	-	<3.2	-	-	-

[a] Age groups = calves 4–7 days-old; younger cattle (male and female of beef or dairy breed) with ≤4 permanent incisors; older cattle (male and female of beef or dairy breed) with >4 permanent incisors.
[b] DC = Direct culture.
[c] IMS = Immunomagnetic separation. Serogroup specific beads for *E. coli* O157 and O26 were used when *E. coli* concentrations were below the detection limits of direct culture plating. Hence, *E. coli* concentrations were below the presented values.

3.4.6 Prevalence of STEC in animals

Prevalence estimates of STEC O157 and STEC O26 in calves and adult cattle are summarised in Table 3.12; one calf and two adult cattle shed both STEC O157 and STEC O26.

Table 3.12: Prevalence estimates (%) of STEC O157 and STEC O26, and overall STEC prevalence (STEC O157 and/or STEC O26 combined) in recto-anal faecal swab samples from calves (n = 695) and adult cattle (n = 895) collected at slaughter. STEC prevalence is shown as the number of samples with *E. coli* isolates containing *stx* virulence genes. One calf and two adult cattle shed both STEC O157 and STEC O26.

Animal group	Serogroup	*stx* positive/tested	STEC prevalence (95% CI)[a]
Calves	O157	16/695	2.3 (1.4–3.8)
	O26	27/695	3.9 (2.6–5.7)
	Combined	42/695	6.0 (4.4–8.1)
Adult cattle	O157	14/895	1.6 (0.9–2.7)
	O26	4/895	0.4 (0.1–1.2)
	Combined	16/895	1.8 (1.1–3.0)

[a] CI = 95% confidence interval.

No significant differences were apparent between the prevalence of STEC O157 and STEC O26 in either of the animal groups. The overall STEC prevalence (STEC O157 and/or O26 combined) was significantly higher in calves than in adult cattle ($p < 0.001$).

3.4.7 Prevalence of *E. coli* and STEC between farm types

Prevalence estimates of all *E. coli* O157 and O26 (including STEC and non-STEC), and of STEC O157 and STEC O26 in beef and dairy farms were calculated based on farm of origin of sampled animals (calves and adult cattle), test results of real-time PCR, and culture isolation from recto-anal faecal samples (Table 3.13). A proportion of farms were positive for both serogroups.

The following farm-level prevalence values are likely to be grossly underestimated as only a small number of animals were sampled from each farm (range 1–11), reducing the sensitivity of the assays to detect positive farms. Hence, the prevalence estimates are only of value when comparing farm types (assuming a similar bias in each farm type).

Table 3.13: Prevalence (%) of all *E. coli* O157 and O26 (including STEC and non-STEC) and all STEC O157 and STEC O26 among beef and dairy farms of which animals (calves and adult cattle) were faecal sampled at slaughter plants. Prevalence is shown as the number of test-positive samples by real-time PCR, culture isolation (direct culture plating and immunomagnetic separation combined), and the number of samples with *E. coli* isolates containing *stx* virulence genes (STEC)*.

		Prevalence (%)		
Farm type	Serogroup	Real-time PCR (95% CI)[a] [positive/tested]	Culture (95% CI)[a] [positive/tested]	STEC (95% CI)[a] [positive/tested]
Beef	O157	6.2 (4.0–9.4)	2.3 (1.1–4.6)	2.0 (0.9–4.2)
(n = 354)		[22/354]	[8/354]	[7/354]
	O26	10.5 (7.6–14.2)	5.1 (3.1–8.1)	0.6 (0.1–2.2)
		[37/354]	[18/354]	[2/354]
	O157 + O26	2.0 (0.9–4.2)	0.6 (0.1–2.2)	0.3 (0.0–1.8)
		[7/354]	[2/354]	[1/354]
Dairy	O157	9.9 (7.8–12.5)	3.4 (2.2–5.1)	2.3 (1.3–3.8)
(n = 655)		[65/655]	[22/655]	[15/655]
	O26	17.3 (14.5–20.4)	9.0 (7.0–11.5)	3.4 (2.2–5.1)
		[113/655]	[59/655]	[22/655]
	O157 + O26	12.7 (10.3–15.5)	0.3 (0.1–1.2)	0.3 (0.1–1.2)
		[83/655]	[2/655]	[2/655]

[a] CI = 95% confidence interval.
* A proportion of animals were test-positive for both serogroups (O157+O26).

No significant differences in real-time PCR, culture or STEC prevalence were observed between the serogroups in beef farms. However, in dairy farms both real-time PCR and culture prevalence for *E. coli* O26 (STEC and non-STEC) were significantly higher compared to *E. coli* O157 (both $p < 0.001$), but no statistically significant difference was apparent between prevalence of STEC O26 and STEC O157 ($p = 0.317$). There was a significant difference in real-time PCR prevalence for *E. coli* O26 between beef and dairy farms ($p = 0.005$).

As confirmed by culture, 3.4% (34/1,009; 95% CI 2.4%–4.7%) and 8.0% (81/1,009; 95% CI 6.5%–9.9%) of all farms were positive for all *E. coli* O157 and O26 (including STEC and non-STEC), respectively. Overall, 4.9% (49/1,009; 95% CI 3.6%–6.4%) of all farms were positive for STEC (O157 and/or O26), with 2.8% (10/354; 95% CI 1.4%–5.3%) and 6.0%

(39/655; 95% CI 4.3%–8.1%) of beef farms and dairy farms being STEC-positive, respectively.

3.4.8 Spatial distribution of *E. coli* and STEC

Distributions of real-time PCR prevalence of all *E. coli* O157 and O26 (including STEC and non-STEC) and prevalence of all STEC O157 and STEC O26 in faecal samples collected in the North Island and the South Island of New Zealand, are presented in Table 3.14. Prevalences in calves and adult cattle, stratified by island, are also shown.

Table 3.14: Real-time PCR prevalence (%) of all *E. coli* O157 and O26 (including STEC and non-STEC) and prevalence of STEC O157 and STEC O26 in faecal samples collected from slaughter cattle (calf and adult cattle) in the North Island and the South Island of New Zealand. Prevalence is also shown for each animal group, stratified by island.

		Real-time PCR			STEC		
Island of New Zealand	Serogroup	Prevalence % (positive/tested)	95% CI[a]	Within island difference p-value[b]	Prevalence % (positive/tested)	95% CI[a]	Within island difference p-value[b]
North Island (n = 941)	O157	15.8 (149/941)	13.6–18.4	0.001	2.3 (22/941)	1.5–3.6	0.752
	O26	21.8 (205/941)	19.2–24.6		2.0 (19/941)	1.3–3.2	
South Island (n = 649)	O157	11.9 (77/649)	9.5–14.7	0.164	1.2 (8/649)	0.6–2.5	0.499
	O26	14.6 (95/649)	12.1–17.6		1.8 (12/649)	1.0–3.3	
Calves							
North Island (n = 495)	O157	24.2 (120/495)	20.6–28.3	0.001	2.8 (14/495)	1.6–4.8	1.000
	O26	33.7 (167/495)	29.6–38.1		3.0 (15/495)	1.8–5.1	
South Island (n = 200)	O157	21.5 (43/200)	16.1–28.0	0.018	1.0 (2/200)	0.2–3.9	0.011*
	O26	32.5 (65/200)	26.2–39.5		6.0 (12/200)	3.3–10.5	
Adult cattle							
North Island (n = 446)	O157	6.5 (29/446)	4.5–9.3	0.310	1.8 (8/446)	0.8–3.6	0.384
	O26	8.5 (38/446)	6.2–11.6		0.9 (4/446)	0.3–2.4	
South Island (n = 449)	O157	7.6 (34/449)	5.4–10.5	0.697	1.3 (6/449)	0.5–3.0	-
	O26	6.7 (30/449)	4.6–9.5		nd	nd	

[a] CI = 95% confidence interval.

[b] Statistical difference between serogroups on each island.

*Calculated with Fisher's exact test.

nd = not detected.

Considering all animals (calves and adult cattle), real-time PCR prevalence of both *E. coli* O157 and O26 (STEC and non-STEC) were significantly higher in the North Island compared to the South Island ($p = 0.031$ and $p < 0.001$, respectively); no significant differences were observed at the STEC level (note, the potential clustering of animals within farms was not considered in this analysis; see section 3.4.10 for random effects modelling). Except for significant differences in prevalence between *E. coli* serogroups within islands in calves, no further within- or between-island differences were observed in calves or adult cattle.

Based on the spatial distribution of calves and adult cattle with faecal samples tested real-time PCR-positive for all *E. coli* O157 and O26 (including STEC and non-STEC), estimates of relative risks for each animal group and serogroup of *E. coli* were computed for New Zealand and mapped as depicted in Figure 3.4. Increased relative risks for *E. coli* O26 were observed in calves from the central region (Waikato) of the North Island, and for *E. coli* O157 in adult cattle from the central region (Canterbury) of the South Island.

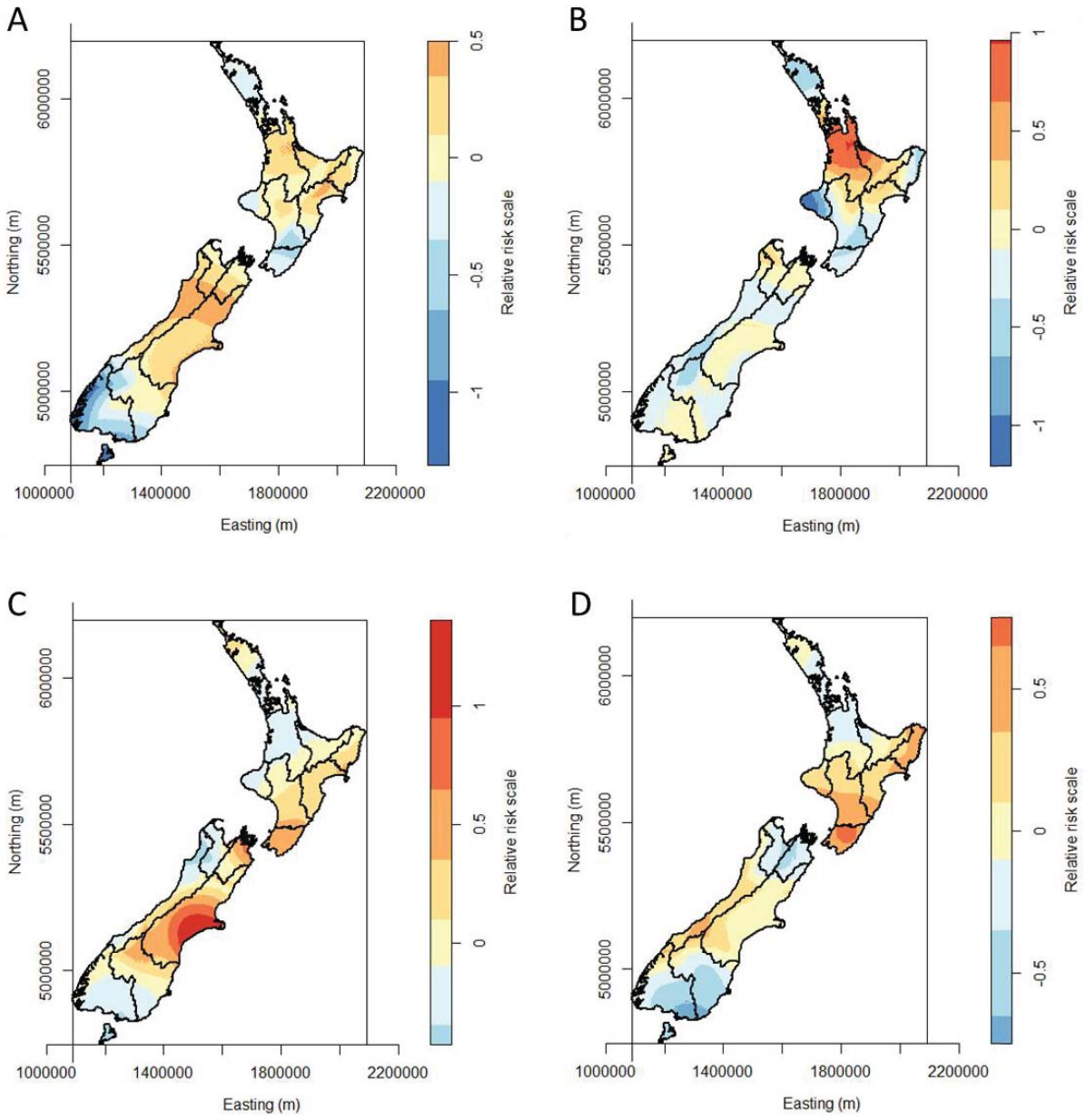

Figure 3.4: Map of New Zealand with bivariate kernel density plots showing estimates of relative risks of calves (n = 695) and adult cattle (n = 895) being tested real-time PCR-positive for all *E. coli* O157 and O26 (STEC and non-STEC). The risk estimates are shown for calves being test-positive for *E. coli* (A) O157 and (B) O26; and for adult cattle being test-positive for *E. coli* (C) O157 and (D) O26. Values >0.0 on the relative risk scale indicate increased risk of being tested real-time PCR-positive for *E. coli*.

The spatial distribution of STEC isolates (O157 and O26) retrieved from sampled calves and adult cattle is illustrated in Figure 3.5.

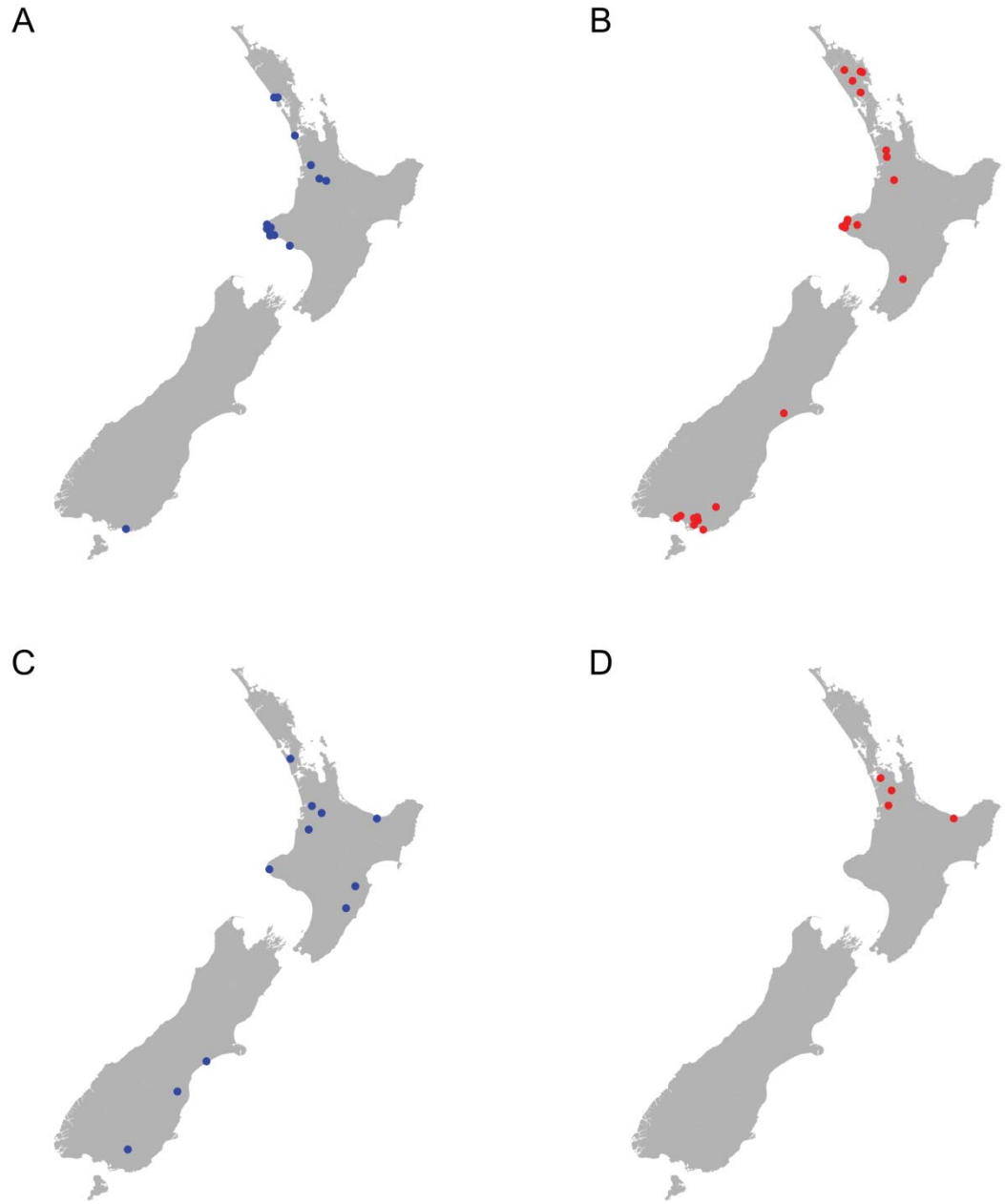

Figure 3.5: Map of New Zealand showing distribution of animals from which STEC isolates were recovered: (A) calves with STEC O157, (B) calves with STEC O26, (C) adult cattle with STEC O157, and (D) adult cattle with STEC O26.

STEC O157 and STEC O26 isolates were recovered from calves and adult cattle in both islands of New Zealand, except for STEC O26, which was not detected in adult cattle in the South Island.

3.4.9 Seasonality of *E. coli* and STEC

Calves

Slaughter plants started their calf processing season with the beginning of the calving season in July/August and production continued for approximately two months. The calf processing seasons varied by two to three weeks between slaughter plants and seasons, influenced by the seasonal farming conditions and marketing decisions of the slaughter plant's co-operatives. No obvious seasonal pattern in prevalence of *E. coli* and STEC was observed in calves as depicted in Figure 3.6

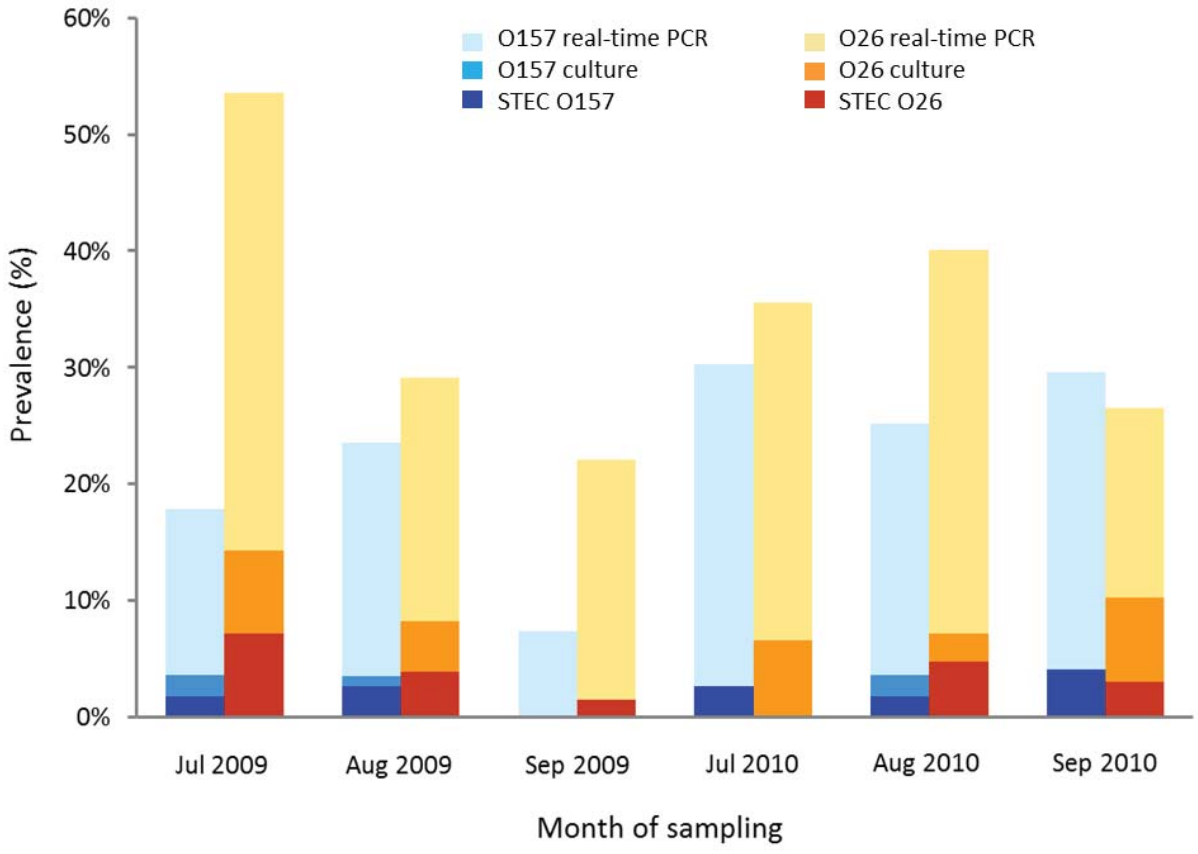

Figure 3.6: Seasonal prevalence of *E. coli* and STEC in calves (n = 695) at slaughter, stratified by serogroups O157 and O26. Prevalences are shown as the percentage of faecal samples test-positive for *E. coli* (STEC and non-STEC) by real-time PCR (light coloured bars) and by culture isolation (medium coloured bars). STEC prevalences are shown as the percentage of samples with *E. coli* isolates containing *stx* virulence genes (dark coloured bars).

Adult cattle

A seasonal pattern in faecal shedding of all *E. coli* O157 and O26 (including STEC and non-STEC) was observed in adult cattle. An increased prevalence of STEC O157 was apparent in spring (September/October) and summer/autumn (January to May), while STEC O26 was only prevalent in summer (December/February) (Figure 3.7). There was no significant difference between season and the prevalence of STEC serogroups (p = 0.118).

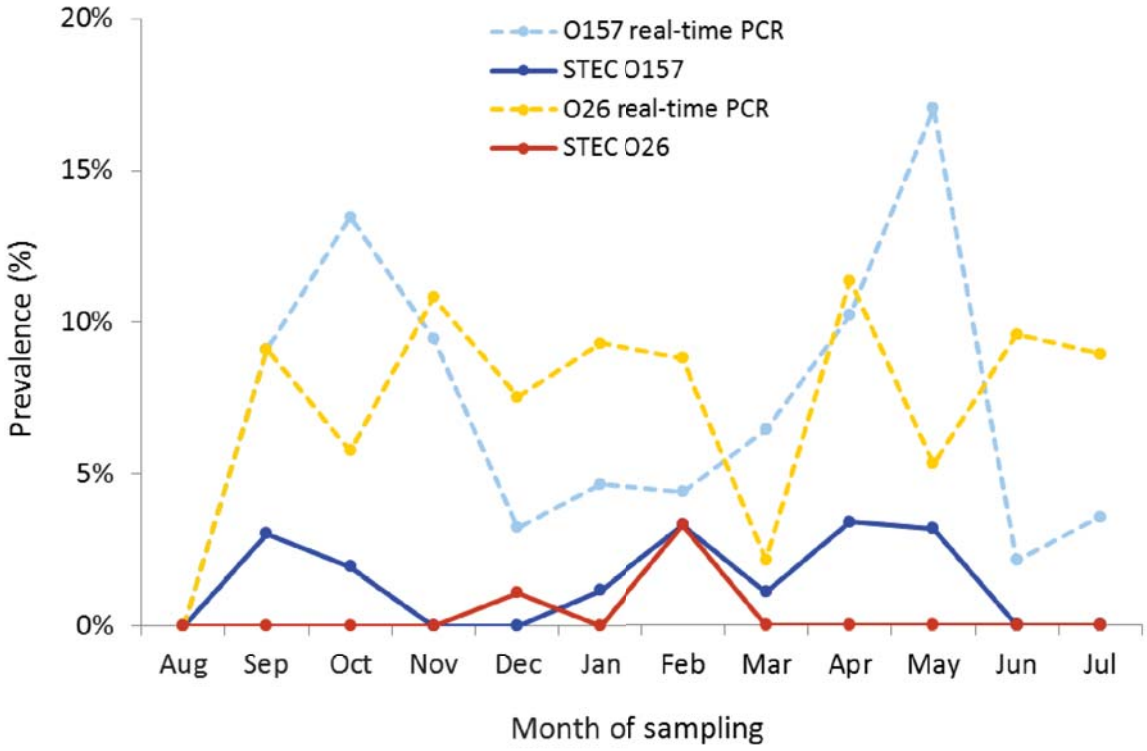

Figure 3.7: Seasonal prevalence of *E. coli* and STEC in adult cattle (n = 895) at slaughter, stratified by serogroups O157 and O26. Prevalences are shown as the percentage of faecal samples test-positive for *E. coli* (STEC and non-STEC) by real-time PCR (dashed lines); STEC prevalences are shown as the percentage of samples with *E. coli* isolates containing *stx* virulence genes (solid lines).

3.4.10 Risk factors for faecal shedding of *E. coli* (STEC and non-STEC) at slaughter

Univariate logistic regression results for calves and adult cattle, stratified by serogroup of *E. coli* (STEC and non-STEC), are summarised in Appendix 1 and Appendix 2, respectively.

Statistically significant risk factors and confounding variables of the final multivariate logistic regression models for calves being real-time PCR-positive for *E. coli* O157 and O26 (STEC and non-STEC) are presented in Table 3.15 and Table 3.16, respectively; and correspondingly for adult cattle in Table 3.17 and Table 3.18.

Table 3.15: Results of multivariate logistic regression analysis for a calf being tested real-time PCR-positive for *E. coli* O157 (STEC and non-STEC). In Model B 'Farm' was considered as a random effect. From 695 observations, 25 were deleted due to missing carcass weights.

	Model A			Model B		
Variable	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Intercept	–1.50 (0.63)			–1.95 (0.76)		
Slaughter plant			<0.001*			<0.001*
A (North Island)	Ref	-	-	Ref	-	-
B (North Island)	–0.01 (0.24)	0.99 (0.62–1.58)	0.965	–0.05 (0.31)	0.95 (0.52–1.73)	0.871
C (South Island)	0.50 (0.28)	1.65 (0.96–2.83)	0.070	0.56 (0.36)	1.75 (0.86–3.57)	0.126
D (South Island)	–1.33 (0.40)	0.26 (0.12–0.58)	0.001	–1.61 (0.53)	0.20 (0.07–0.57)	0.002
Presence of *E. coli* O26						
No	Ref	-	-	Ref	-	-
Yes	1.22 (0.20)	3.39 (2.28–5.02)	<0.001	1.54 (0.25)	4.65 (2.86–7.55)	<0.001
Sex						
Male	Ref	-	-	Ref	-	-
Female	–0.40 (0.23)	0.67 (0.42–1.06)	0.089	–0.48 (0.29)	0.62 (0.35–1.09)	0.096
Carcass weight	0.02 (0.03)	1.02 (0.96–1.09)	0.464	0.03 (0.04)	1.03 (0.95–1.11)	0.476

Likelihood ratio test (df = 6, p <0.001) was 63.97 for Model A, and 90.016 for Model B.

[a] SE = Standard error.

[b] CI = 95% confidence interval.

* p-value of variable as whole using Likelihood ratio test.

Ref = reference level for comparison.

Table 3.16: Results of multivariate logistic regression analysis for a calf being tested real-time PCR-positive for *E. coli* O26 (STEC and non-STEC). In Model B 'Farm' was considered as a random effect. From 695 observations, 25 were deleted due to missing carcass weights.

	Model A			Model B		
Variable	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Intercept	–1.37 (1.03)			–1.83 (1.24)		
Slaughter plant			<0.001*			<0.001*
A (North Island)	Ref	-	-	Ref	-	-
B (North Island)	–1.06 (0.24)	0.35 (0.22–0.56)	<0.001	–1.29 (0.30)	0.28 (0.15–0.50)	<0.001
C (South Island)	–0.53 (0.29)	0.59 (0.34–1.02)	0.061	–0.59 (0.36)	0.56 (0.27–1.13)	0.105
D (South Island)	0.13 (0.31)	1.13 (0.62–2.07)	0.684	0.17 (0.39)	1.18 (0.56–2.52)	0.662
Month			0.004*			0.006*
July	Ref	-	-	Ref	-	-
August	–0.67 (0.23)	0.51 (0.33–0.79)	0.003	–0.82 (0.27)	0.44 (0.26–0.75)	0.003
September	–0.88 (0.31)	0.41 (0.22–0.76)	0.005	–1.06 (0.38)	0.35 (0.16–0.73)	0.005
Presence of *E. coli* O157						
No	Ref	-	-	Ref	-	-
Yes	1.26 (0.20)	3.54 (2.38–5.26)	<0.001	1.57 (0.24)	4.82 (3.01–7.72)	<0.001
Breed			0.366*			0.400*
Friesian	Ref	-	-	Ref	-	-
Jersey	–0.27 (0.20)	0.76 (0.51–1.13)	0.179	–0.32 (0.24)	0.73 (0.45–1.17)	0.185
Other breed	0.36 (1.04)	1.44 (0.19–10.97)	0.725	0.42 (1.23)	1.52 (0.14–17.08)	0.735
Farm type						
Dairy	Ref	-	-	Ref	-	-
Beef	1.25 (0.89)	3.48 (0.61–19.90)	0.162	1.58 (1.08)	4.87 (0.59–40.46)	0.143
Carcass weight	0.01 (0.03)	1.01 (0.95–1.07)	0.862	0.01 (0.04)	1.01 (0.94–1.08)	0.820

Likelihood ratio test (df = 10, p <0.001) was 85.40 for Model A, and 111.79 for Model B.

[a] SE = Standard error. [b] CI = 95% confidence interval. * p-value of variable as whole using Likelihood ratio test. Ref = reference level for comparison.

'Slaughter plant' and 'Presence of *E. coli* O26' were identified as significant risk factors for a calf being tested real-time PCR-positive for *E. coli* O157 (STEC and non-STEC), with 'Sex' and 'Carcass weight' as confounding variables. Similarly, risk factors for a calf being test-positive for *E. coli* O26 by real-time PCR were 'Slaughter plant' and 'Presence of *E. coli* O157', but also 'Month', including confounding variables 'Breed', 'Farm type', and 'Carcass weight'.

A multivariate logistic regression model with 'Farm' as a random effect could not be fitted on data of adult cattle tested real-time PCR-positive for *E. coli* O157 and *E. coli* O26 due to non-convergence of data. Hence, only one model is shown in Table 3.17 and Table 3.18.

Table 3.17: Results of multivariate logistic regression analysis for an adult cattle being tested real-time PCR-positive for *E. coli* O157 (STEC and non-STEC). From 895 observations, one was deleted due to missing carcass weight.

Variable	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Intercept	–3.92 (1.01)		
Season			<0.001*
Spring (Sep–Nov)	Ref	-	-
Summer (Dec–Feb)	–0.86 (0.42)	0.42 (0.19–0.97)	0.043
Autumn (Mar–May)	0.48 (0.36)	1.62 (0.79–3.30)	0.187
Winter (Jun–Aug)	–1.57 (0.58)	0.21 (0.07–0.65)	0.007
Presence of *E. coli* O26			
No	Ref	-	-
Yes	1.72 (0.35)	5.58 (2.80–11.13)	<0.001
Sex			0.060*
Male	Ref	-	-
Male castrated	1.10 (0.51)	3.00 (1.10–8.21)	0.032
Female	0.84 (0.55)	2.32 (0.79–6.85)	0.126
Breed			0.202*
Friesian	Ref	-	-
Jersey	–0.78 (0.78)	0.46 (0.10–2.12)	0.319
Angus	0.24 (0.39)	1.27 (0.59–2.75)	0.545
Hereford	–0.65 (0.52)	0.52 (0.19–1.46)	0.216
Other breeds	0.49 (0.53)	1.64 (0.58–4.62)	0.352
Carcass weight	<0.01 (<0.01)	1.00 (1.00–1.01)	0.541

Likelihood ratio test = 58.73 (df = 11, p <0.001).
[a] SE = Standard error.
[b] CI = 95% confidence interval.
* p-value of variable as whole using Likelihood ratio test.
Ref = reference level for comparison.

Table 3.18: Results of multivariate logistic regression analysis for an adult cattle being tested real-time PCR-positive for *E. coli* O26 (STEC and non-STEC). From 895 observations, one was deleted due to missing carcass weight.

Variable	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Intercept	–2.98 (0.22)		
Presence of *E. coli* O157			
No	Ref	-	-
Yes	1.55 (0.33)	4.69 (2.45–8.99)	<0.001
Age (in years)			0.135*
≤2.25	Ref	-	-
2.5–3.0	0.56 (0.34)	1.75 (0.89–3.44)	0.102
3.25–3.75	–0.06 (0.56)	0.95 (0.32–2.82)	0.920
≥4.0	0.62 (0.30)	1.86 (1.02–3.37)	0.042

Likelihood ratio test = 23.18 (df = 4, p <0.001).
[a] SE = Standard error.
[b] CI = 95% confidence interval.
* p-value of variable as whole using Likelihood ratio test.
Ref = reference level for comparison.

Significant risk factors for an adult animal being tested real-time PCR-positive for *E. coli* O157 (STEC and non-STEC) were 'Season' and 'Presence of *E. coli* O26', with 'Sex', 'Breed', and 'Carcass weight' as confounders. In comparison, 'Presence of *E. coli* O157' was the only risk factor for adult cattle being real-time PCR-positive for *E. coli* O26, with 'Age' as the only confounding variable.

3.5 Discussion

This two-year nationwide study was the first of its kind in New Zealand to provide data on the prevalence of shedding STEC O157 and STEC O26 in very young calves and adult cattle at slaughter, and was undertaken to gain detailed knowledge on the epidemiology of these two important STEC strains entering the food chain via slaughtered animals in New Zealand. The selection of two large slaughter plants in each main island of New Zealand and the systematic sampling of animals on the slaughter chain resulted in a good nationwide coverage of farms within the catchment areas of the participating plants. Animals sampled in this study are therefore considered to be broadly representative of New Zealand's slaughter cattle population.

3.5.1 Prevalence of STEC O157 and STEC O26 in animals and farms

To understand the epidemiology of STEC O157 and STEC O26 and their importance to public health, this prevalence study was conducted first to assess the extent of presence/absence of these pathogens in New Zealand's slaughter cattle population and generate basic but essential data for the design of further epidemiological studies. This study identified a significantly higher STEC prevalence (STEC O157 and/or STEC O26 combined) in calves compared to adult cattle, especially for STEC O26. When comparing prevalence of STEC in faecal samples from animals of previous studies, it is essential to recognise variations in study design and methods of isolation, in addition to animal management practices and environmental factors.

Calves

Overseas, it is not general practice to slaughter calves at <7 days of age, hence, most previous studies have investigated the prevalence of faecal carriage of STEC in calves on-farm, and in animals of different age categories, ranging from <24 hours and <1 month to <1–2 months of age. Fernández *et al.* [356] reported a 0.8% (2/252) prevalence of STEC O157 in milk-fed calves <2 months of age on five dairy farms in Argentina, while Rugbjerg *et al.* [357] observed a faecal carriage of STEC O157 of 2.2% (6/278) in 1–2 month-old calves on eight dairy farms in Denmark. A prevalence of 1.3% (1/79) was reported for STEC O157 in calves during a longitudinal cohort study on three dairy farms in south-east Queensland, Australia [165]. In contrast, a survey at two slaughter plants in the lower North Island of New Zealand [324] showed an STEC O157 prevalence of 3.2% (10/309) in calves at slaughter, consistent with our findings (2.3%, 16/695). It can be hypothesised that the faecal shedding rates of STEC in calves is higher at slaughter compared to on-farm due to effects of transportation to and lairage at slaughter plants. The impacts of transportation and lairage on faecal carriage have been studied in adult cattle [358] [359] [298] but there are no data available for calves of this very young age.

Compared to STEC O157, the prevalence of STEC O26 in calves was higher (3.9%, 27/695), which is consistent with findings reported in the Australian study (3.8% (3/79)) [165] but is not directly comparable due to differences in study design. A longitudinal study in northern Scotland [262], assessing the shedding patterns of STECs O26, O103, O111, O145, and O157 in a cohort of beef calves from birth over a five-month period, described STEC O26 as the

most prevalent STEC serogroup in calves followed in decreasing order by STECs O103, O145, and O157. STEC O26 was detected in 93.9% (46/49) of calves studied, with first shedding at mean age of 16.5 days (standard deviation 12.8 days). It was noted that a higher proportion of dams shed STEC O26 at time of calving than at study end, suggesting dams as a possible source of infection for calves. Cobbold and Desmarchelier [165] reported in their Australian study that calves were twice as likely to shed STEC when born to STEC-positive dams compared to STEC-negative dams. Although vertical transmission of STEC in cattle is a possible pathway of infection on-farm, other factors such as animal housing and management, and the environment could have an impact on the prevalence of STEC observed in calves on-farm.

Adult cattle

A number of previous studies have investigated the faecal carriage of STEC O157 and other non-O157 STEC in adult cattle at slaughter. The faecal carriage of STEC O157 in adult cattle in this study was 1.6% (14/895) and within the range of prevalence estimates detected in adult slaughter cattle in Ireland (2.3%, 7/301) [360], Italy (3.3%, 6/182) [190], Japan (1.6%, 5/306) [361], and Australia (1.7%, 5/300) [362]. In contrast, a New Zealand study investigating the prevalence of STEC O157 in 371 healthy dairy cows at a slaughter plant in the North Island of New Zealand detected STEC O157 in faecal samples from only two animals (0.5%, 2/371) [321]. The difference in STEC O157 prevalence to our study could be due to differences in the detection and isolation methods used, season and the distribution of animals sampled (local *versus* nationwide).

Compared to STEC O157, the faecal carriage of STEC O26 in adult cattle was only 0.4% (4/895) and was consistent with low STEC O26 prevalence reported for slaughter cattle in Italy (0.5%, 1/182) [190], Switzerland (1.6%, 9/573) [363], Ireland (1.5%, 6/402) [360], Belgium (1.5%, 6/399) [364], Japan (1.3%, 4/306) [361], and Australia (0.3%, 1/300) [362]. Although no previous study has investigated the faecal shedding of STEC O26 in adult cattle at slaughter in New Zealand, Cookson *et al.* [323] reported the isolation of STEC O26 from healthy cattle (weaned calves, heifers, and dairy cattle) on-farm in the lower North Island (1.1%, 2/187), providing evidence of STEC O26 being prevalent in New Zealand cattle.

In general, the prevalence of STEC in adult cattle was lower compared to calves and might be associated with the fully developed gastrointestinal tract of the adult cattle. Microbial fermentation of feed in the developed rumen of adult cattle produces high concentrations of volatile fatty acids, which lower the pH conditions, and are thought to inhibit the growth of STEC O157 and other pathogenic strains of *E. coli* [365]. These conditions and a naïve gut could therefore account for a higher susceptibility for STEC colonisation and thereby the higher prevalence of STEC observed in calves, in addition to other external factors such as husbandry systems and animal management.

Beef and dairy farms

Considering the origins of all animals tested, 6.0% (39/655) of dairy farms were positive for STEC (O157 and/or O26) compared to 2.8% (10/354) of beef farms. It is noteworthy that the sensitivity of the test applied at farm-level was low as only a very small number of animals were tested per farm at slaughter; hence, these values represent estimates of the minimum farm level prevalence. Two New Zealand studies have investigated the prevalence of STEC in dairy cattle on-farm. Cookson *et al.* [322, 323] reported in their survey on four separate research farm sites in the lower North Island the isolation of various STEC serogroups (including STEC O26) from recto-anal faecal samples of 187 cattle (weaned calves, heifers, and dairy cattle), but did not detect STEC O157. In a one-year study, Moriarty *et al.* [366] collected freshly deposited faecal samples on four dairy farms (two farms on each island of New Zealand) in each season, but did not isolate STEC O157 or STEC O26 in any of the 155 samples collected. Although neither studies showed the presence of STEC O157 in dairy cattle on-farm, this STEC serogroup was detected in dairy animals at slaughter [321], providing additional evidence of STEC O157-positive dairy farms in New Zealand.

The difference in STEC prevalence between beef and dairy farms presented might be influenced by husbandry and environmental factors. Beef farms are located mainly in the hill and high country areas of both islands of New Zealand, while dairy farming is practiced principally in the flatter and/or wetter areas in the upper, central, and lower North Island; and from the central east coast to the south of the South Island. Geographical differences could have an effect on STEC prevalence at the farm level but no epidemiological study has been conducted to investigate risk factors associated with on-farm prevalence of STEC between these two farming systems in New Zealand.

3.5.2 Diagnostic methods

The prevalence of faecal shedding of *E. coli* O157 and O26, and STEC O157 and STEC O26, in calves and adult cattle at slaughter was determined by the number of positive samples detected using real-time PCR and culture isolation. However, some care is needed in interpreting prevalence based on real-time PCR results or culture isolation, as other factors might influence the results of these methods.

Enrichment

The combination of enrichment and immunomagnetic separation with inoculation on CT-SMAC culture media has been proven to be a sensitive method to isolate STEC O157 from bovine faeces [218, 224]. However, different enrichments and incubation periods have a significant effect on the recovery of STEC O157 from naturally infected bovine faeces, as demonstrated by Tutenel *et al.* [367]. They reported that a 24-h enrichment in BPW followed by IMS and plating on CT-SMAC culture media (as used in the present study) was a significantly more sensitive isolation method, compared to a 6-h enrichment in BPW or enrichment in modified tryptone soya broth supplemented with novobiocin. A study by Foster *et al.* [225] showed that BPW without additives was a superior enrichment to isolate STEC O157 from bovine faecal samples, compared to BPW with added antibiotics vancomycin, cefixime and cefsulodin.

Other common enrichment media used for enhancing growth rate of STEC are tryptic soy broth (TSB), *E. coli* broth, EHEC broth, and brain heart infusion broth. TSB is a highly nutritious and nonselective medium commonly used for the enrichment of different foodborne pathogens, such as *E. coli*, *Listeria* spp., and *Salmonella* spp.. It has been demonstrated that TSB is a robust medium for the detection of STEC O157 in bovine faecal, hide, and meat samples [368, 369]. To suppress the growth of competitive natural bacteria in samples, modified TSB enrichments with antibacterial additives such as novobiocine, vancomycin, or rifampicine have been developed for the detection of STEC from bovine faeces and food samples [238, 370].

Real-time PCR

According to our latent class analysis, the specificity of our real-time PCR method was estimated to be >90%, meaning that some false-positive results were likely to occur, resulting in an overestimate of the prevalence of the PCR target genes for *E. coli* O157 and O26 in our samples. The specificity of the assay to detect the presence of *E. coli* O157 and O26 might be further reduced by primers cross-reacting with other *Escherichia* species harbouring the same target genes. Despite using previously published primers to detect genes encoding for serogroup specific O-antigens of *E. coli* O157 (*rfb*E) and O26 (*wzx*), false-positive results could have been caused by this phenomenon. Fegan *et al.* [371] reported a strain of *Escherichia fergusonii* isolated during routine screening of beef meat for *E. coli* O157, which cross-reacted with O157-specific antibody-based tests. The isolate grew on SMAC culture media (but not CT-SMAC) and carried the O-antigen gene of *E. coli* O157 (*rfb*E) as confirmed by sequencing, suggesting horizontal gene transfer between *E. coli* O157 and *E. fergusonii*. Further, despite best laboratory practice and care, false-positive real-time PCR results could also occur via cross-contamination of samples during processing and testing.

As real-time PCR is unable to differentiate DNA from viable and dead *E. coli*/STEC cells, this method might identify *E. coli* O157/O26-positive samples containing only dead cells that could not be confirmed by culture. Quantitative PCR-methods have been developed to detect and differentiate viable from dead *E. coli* bacteria in biosolids (spiked dewatered cake from wastewater treatment plant) [372] and STEC O157 in food (spiked beef) [373], using selective DNA-binding dyes like ethidium monoazide and propidium monoazide. These dyes penetrate into dead cells, which have compromised membrane integrity, intercalate with bacterial DNA and inhibit the amplification by PCR. To establish a similar method for the detection of viable *E. coli* cells in faecal samples would be of great value to assess more accurately the prevalence of faecal shedding of *E. coli*/STEC in ruminants.

Culture isolation

As shown by latent class analysis, the specificities of the culture methods used were almost 100% but showed very low sensitivities (<33%), resulting in a high proportion of false-negative samples. Culture isolation is crucial to confirm real-time PCR-positive samples but the sensitivity of these assays is influenced by other factors such as the enrichment, culturability of *E. coli*, the specificity of immunomagnetic beads, and the selectivity of culture

media. The use of 18-h to 24-h enrichment in BPW without additives supported the growth of *E. coli* cells in faecal samples, but inevitably enhanced the growth of background bacteria, which probably overgrew some of the *E. coli* cells. This might have occurred in samples from calves as they showed a considerably higher aerobic growth compared to samples from adult cattle, explaining some of the lower recovery rates of *E. coli* isolates from calves.

Bacterial cells are able to enter a physiological stage of being viable but non-culturable when exposed to natural stress factors such as incubation temperature outside the range required for growth, starvation, or white light [237]. Research has demonstrated that a large number of bacterial pathogens can enter this stage, including *Campylobacter* spp., *E. coli* as well as STEC, *Helicobacter pylori*, *Legionella pneumophila, Listeria monocytogenes, Mycobacterium tuberculosis, Pseudomonas aeruginosa, Salmonella* and *Shigella* spp., and *Vibrio cholera* [237]. Viable but non-culturable cells show very low levels of metabolic activity and do not multiply to form colonies on culture media. It is possible that some real-time PCR-positive/culture-negative samples contained cells in the viable but non-culturable stage, contributing to some of the false-negative culture results and the lower recovery rate of *E. coli*/STEC isolates observed in our study.

Immunomagnetic separation was applied to increase the isolation of *E. coli* O157/O26 from real-time PCR-positive samples. The immunomagnetic beads are coated with polyclonal antibodies against surface antigens of *E. coli* O157/O26, but could cross-react with antigenically similar organisms, such as *Escherichia hermannii*, *Salmonella O group N*, or *Proteus* spp. [374]. According to the beads manufacturer, a false-negative rate of 2–10% might be expected depending on the inoculum level, sample matrix, and background microbiota [374]. A rather unlikely (but still possible) hypothesis could be that the polyclonal antibodies on these commercial beads are not specific enough to detect New Zealand strains, hence, affecting the recovery of *E. coli* isolates. An increased size and magnetite content of beads would improve the recovery rate of *E. coli* isolates as was demonstrated in a study by Parham *et al.* [375] when isolating *E. coli* directly from bovine faecal samples (without enrichment).

CT-SMAC and CT-RMAC are recognised as selective culture media for the isolation of *E. coli* O157 [228, 376] and O26 [233], respectively, and have been used in many studies to isolate *E. coli* from different types of samples (food, faeces, environment). However, these

culture media are not 100% selective as was demonstrated in a longitudinal study by Evans *et al.* [235], comparing the selectivity of CT-RMAC and tryptone bile X-glucuronide (TBX) agar on the isolation of STEC O26 from faeces of Scottish cattle and sheep. Although the authors observed a significant association between STEC O26 and the inability to ferment rhamnose, two STEC O26 isolates fermented rhamnose on CT-RMAC plates, and 21.3% (10/47) of non-rhamnose-fermenting STEC O26 isolates did not grow on CT-RMAC plates. These results are somewhat inconsistent with previous findings reported by Hiramatsu *et al.* [233], showing that all their STEC O26 isolates (31/31), selected from clinical cases in Japan, grew on CT-RMAC plates as non-rhamnose-fermenting colonies. It is not unexpected to observe some differences in selectivity of culture media, considering the genetic variations between strains of *E. coli*.

Latent class analysis

In the absence of a 'gold standard' or a single reference diagnostic test that is both 100% sensitive and 100% specific, the latent class analysis assessed the test performances of both the real-time PCR and culture methods used in this study, and computed adjusted prevalence estimates for *E. coli* O157 and O26 in calves and adult cattle. Findings of the analysis showed that the real-time PCR method performed better compared to the culture methods, but also highlighted the importance of using them in combination. For example, real-time PCR cannot differentiate DNA from viable and non-viable cells and is therefore likely to overestimate the prevalence of animals shedding infectious *E. coli*.

The parameter values obtained with the latent class analysis model were estimated using the Bayesian inference approach, including prior information and on the disease prevalence in both populations (calves and adult cattle) and probabilities of the diagnostic test outcomes. Although the assumption was that both tests were independent, it can be argued that both methods did not differ considerably in the actual mechanisms the organisms were detected in the faecal samples. Neither the model fit nor the stability of the estimates was evaluated.

3.5.3 *E. coli* concentrations

Counts and concentrations of *E. coli* and STEC in faeces from calves and adult cattle were determined to provide the New Zealand red meat industry with quantitative data on the levels

of *E. coli* and STEC entering the food chain via slaughtered cattle. This information will assist in quantitative risk assessments and developments of intervention strategies associated with food safety management.

In our study, based on direct culture, the median concentration of *E. coli* was higher in calves (5.9 $\log_{10}$ CFU/g) than (young) adult cattle (4.6 $\log_{10}$ CFU/g faeces). A survey of North American dairy farms detected *E. coli* O157:H7 in dairy calves in concentrations from $<10^2$ to 10^5 CFU/g faeces [187]. Using the most probable number (MPN) technique for enumeration, Moriarty *et al.* [366] reported an overall median count of 8.2×10^4 MPN/g faeces (equals 4.9 $\log_{10}$) for *E. coli* in cow pats collected on four New Zealand dairy farms during a one-year study, consistent with our findings in adult cattle. Fegan *et al.* [240] determined *E. coli* concentrations in 22 *E. coli* O157-positive faecal samples from 10 pasture-fed and 12 grain-fed cattle in Australia. Their numbers ranged from undetectable (<3 MPN/g faeces) to 2.4×10^4 MPN/g faeces (equals 4.4 $\log_{10}$), and only a marginal significance in MPN was observed between grain-fed and pasture-fed cattle faeces ($p = 0.06$). Although both studies used MPN, their findings were consistent with our results when presented as $\log_{10}$ results (as indicated in brackets).

It is important to note that *E. coli*/STEC counts in faecal samples can be affected by intermittent shedding of *E. coli*/STEC, which occurs in colonised cattle [161, 163]. Hence, it is possible that some samples were collected in the non-shedding phase of colonised animals, or when *E. coli*/STEC were shed at undetectable levels. In addition, the occurrence of 'supershedder', animals, which shed *E. coli*/STEC in higher concentrations ($>10^3$ to $\geq 10^4$ CFU/g faeces) [168-171], could have increased the median counts in this study.

3.5.4 Spatial and temporal findings

The selection of two large slaughter plants on each island of New Zealand resulted in good nationwide coverage of farms and sampled animals within the catchment areas of the participating plants, providing data on the spatial distribution of *E. coli* O157 and O26 across New Zealand.

Overall, real-time PCR prevalence for both *E. coli* O157 and O26 was higher in the North Island compared to the South Island. It is possible the higher prevalence is due to increased

beef and dairy cattle densities in the North Island or could be affected by a difference in climate. Increased relative risk for *E. coli* O26 PCR-positive animals was observed in calves in the central region (Waikato) of the North Island, suggesting a region-associated prevalence in calves. Waikato is a high density dairy farming region and it can be hypothesised that the local farming conditions have an impact on the increased risk for *E. coli* O26 in this region. Similarly, this could apply to the central region (Canterbury) of the South Island, where increased relative risk for *E. coli* O157 was found in adult cattle.

STEC O157 and STEC O26 were detected in slaughter cattle on both islands of New Zealand, except for STEC O26, which was not isolated from adult cattle in the South Island although *E. coli* O26 real-time PCR-positive faecal samples were identified. Having observed PCR-positive samples, it can be assumed that *E. coli* O26, and very likely STEC O26, is prevalent in adult cattle in the South Island.

No obvious seasonal patterns in prevalence of *E. coli* O157/O26 or STEC O157/O26 was found in calves, which was probably due to the relative short sampling period of only a few weeks. In adult cattle, however, clear patterns of seasonal shedding were observed with a bimodal prevalence for STEC O157 (spring and summer/autumn) and a peak in summer for STEC O26. Seasonal variations in the prevalence of faecal shedding of STEC in cattle were described in previous studies in the Netherlands [377] and Great Britain [176, 177], consistent with peaks of STEC prevalence in the summer months.

3.5.5 Risk factors for faecal shedding of *E. coli* (STEC and non-STEC)

Calves

Plant and co-infections with either serogroup of *E. coli* (O157 or O26) were identified as risk factors for faecal shedding of *E. coli* in calves at slaughter, suggesting common sources of infection for both serogroups on the farm of origin and/or during transport to and lairage at the slaughter plant. Plant as a risk factor could indicate other risks associated with the catchment area of the plant, but also plant-associated factors, such as animal handling or lairage management, which could affect the prevalence of faecal shedding of *E. coli* in animals at slaughter.

Risk factors for STEC O157 infections in dairy calves were assessed in previous studies [166, 378], indicating that increased prevalence of STEC O157 on-farm is associated with age of calves and housing/farm management factors. It is general practice on New Zealand dairy farms to keep calves in groups in barns for the first week after birth, before selecting heifer calves for stock replacement and bull calves for the dairy beef market, and sending the remaining animals as bobby calves for slaughter. Grouping calves before weaning was identified as an increased risk factor for American dairy farms to be positive for STEC O157, compared to farms where grouping of calves was practiced only after weaning [166, 263]. The use of open pails compared to nipples to feed calves was also associated with increased infection of STEC O157 in calves on Canadian dairy farms [379, 380]. Given the lack of risk factor studies of STEC infections on New Zealand dairy farms, it can only be assumed that similar housing and feeding practices contribute to the level of STEC infection in calves on-farm, and consequently would have an impact on the prevalence of STEC detected in calves at slaughter.

Adult cattle

Similar to observations in calves, co-infection with either serogroup of *E. coli* (O157 or O26) was identified as the main risk factor for faecal shedding of *E. coli* in adult cattle, suggesting a common source of infection either on-farm or on-plant. Interestingly, season was also associated with *E. coli* O157 infection in adult cattle, showing a significantly lower risk in winter. It can be speculated whether seasonal changes in weather or feed could have an effect on the seasonality of *E. coli* O157 infection in adult cattle; the majority of New Zealand's beef and dairy animals are grazed on pasture year-round and are supplemented with hay, baleage, or maize silage during winter.

Feed compositions have been studied as possible risk factors for STEC infections in cattle but have shown controversial findings. Hovde *et al.* [381] demonstrated that steers experimentally inoculated with STEC O157 shed the organism for longer periods when on a diet of hay compared to grain-fed animals. Similar findings were observed in hay-fed sheep with shedding of STEC O157 for twice as long compared to animals fed a mixture of corn and pelleted alfalfa [382]. In contrast, Berry *et al.* [383] reported no significant difference in faecal shedding of STEC O157 in growing beef cattle that were fed chopped bromegrass hay or corn silage, while other studies showed increased concentrations of generic *E. coli* in

rumen and faeces of grain-fed feedlot cattle [384, 385]. Therefore, it is uncertain whether feed compositions could have an impact on the seasonality of *E. coli*/STEC infections in adult cattle in New Zealand.

3.6 Conclusions

The findings of this study contributed to our understanding that STEC O157 and STEC O26 are prevalent in slaughter cattle populations on both islands of New Zealand, which could represent an important source of STEC infection in humans via animal contact, environmental contamination, or contaminated food. Calves were identified as more prevalent carriers of STEC than adult cattle at slaughter, and might therefore be considered as an important source of STEC likely to enter the food chain. Epidemiological data collected in this study will be used for the development of a risk management strategy for STEC and inform decision making in the meat industry to reduce the public health risks associated with the consumption of red meat produced in New Zealand.

3.7 Acknowledgements

We thank all four beef slaughter plants and their managers for participation in this study, and their staff for providing important carcass data; Angie Reynolds from ^{m}EpiLab for great assistance with processing and laboratory testing of samples; and other laboratory staff from ^{m}EpiLab for their technical support.

Having identified calves compared to adult cattle as more likely shedders of STEC at slaughter in the prevalence study, the following chapter describes a cohort study in calves, which was conducted to evaluate the impact of transportation and lairage on the rectal carriage of *E. coli* O157 and O26 (STEC and non-STEC) in calves at slaughter and the levels of carcass contamination through meat processing.

Chapter 4

Population dynamics of *Escherichia coli* O157 and O26 – the effect of transportation and lairage on faecal shedding and carcass contamination of very young calves in New Zealand

4.1 Abstract

In New Zealand slaughter cattle, the proportion of very young calves (4–7 days old) shedding Shiga toxin-producing *Escherichia coli* O157:H7 (STEC O157) and STEC O26 at slaughter is higher compared to adult cattle, however the reasons for this are not well understood. As a higher shedding prevalence at slaughter could increase the risks of hide and carcass contamination, this study investigated the effect of transportation and lairge on the faecal shedding and post-slaughter contamination of carcasses in calves.

A cohort study was designed for a large dairy farming region in the central part of the North Island of New Zealand. Calves from 'high' and 'low' risk farms (risk category determined by pre-testing on-farm *E. coli* prevalence among calves) were followed from farm to slaughter at a regional calf-processing plant. Recto-anal faecal swab samples were collected from each calf both prior to farm departure (on-farm sample) and at the end of lairage just prior to slaughter (on-plant sample). On the slaughter chain, three sponge swab samples were taken from each carcass at: (i) pre-skinning (hide sample), (ii) post-evisceration (pre-intervention sample), and (iii) pre-boning (post-intervention sample). A hot water wash (82ºC) was used as an antimicrobial intervention after carcass inspection and prior to hot boning.

Enrichment samples were analysed for *E. coli* O157 (*rfb*E_{O157}) and O26 (*wzx*$_{O26}$) using real-time PCR. Isolates recovered using immunomagnetic separation were screened with multiplex PCR for the presence of virulence genes *ehx*A, *eae*, *stx*2 and *stx*1, and were genotyped using pulsed-field gel electrophoresis (*Xba*I-PFGE).

A total of 60 calves were followed from farm to slaughter from six dairy farms (three high-risk and three low-risk farms), with 60 on-farm; 60 on-plant; 59 hide; 58 pre-intervention and 47 post-intervention samples collected. In total, 56 *E. coli* O157 and 115 *E. coli* O26 isolates (including STEC and non-STEC) were retrieved (15 and 19 on-farm, 10 and 17 on-plant, 27 and 62 hide, 3 and 12 pre-, and 1 and 5 post-intervention, respectively). PFGE analysis of *E. coli* O157 and O26 faecal isolates revealed little evidence that transmission of *E. coli* infection had occurred during transportation and lairage, i.e. faecal-oral transmission resulting in new animals shedding *E. coli*. However, there was evidence of cross-contamination of hides and carcasses with *E. coli* (STEC and non-STEC) between high- and low-risk groups. Despite this, there was no evidence of a difference in residual contamination on the carcasses of high- and low-risk calves at the pre-boning stage.

Multivariate logistic regression analysis identified risk factors such as 'being positive on-farm' and originating from a high-risk farm (for *E. coli* O157) or a farm with high prevalence of *E. coli* O26 as risk factors for calves being real-time PCR-positive for *E. coli* O157 or O26 in faeces on-plant after transportation and lairage.

This was the first study to investigate the effect of transportation and lairage on faecal shedding and carcass contamination with *E. coli* O157 and O26 (STEC and non-STEC) in very young calves. These findings provide evidence that transportation and lairage under New Zealand conditions does result in an increased cross-contamination of hides and carcasses at pre-intervention with *E. coli* O157 and O26 (STEC and non-STEC) from high-risk to low-risk calves. However, the study was unable to demonstrate an increased prevalence of contaminated carcasses through to the boning room, indicating that good hygiene practices and routine industry intervention strategies effectively control carcass contamination.

4.2 Introduction

Ruminants, in particular cattle, have been identified as an important reservoir of Shiga toxin-producing *Escherichia coli* O157:H7 (STEC O157) and related non-O157 STEC serogroups (e.g. O26, O45, O103, O111, O121, and O145). STEC are zoonotic pathogens and can cause serious illnesses such as diarrhoea, haemorrhagic colitis, and haemolytic uraemic syndrome (HUS), if ingested by humans [26, 59]. HUS is characterised by haemolytic anaemia,

thrombocytopaenia, and acute renal failure, leading to kidney dysfunction, seizures, coma, and death [106]. Young children (<4 years) and the elderly (>65 years) infected with STEC are at higher risk of developing HUS [4], with mortalities of 3% to 5% (cited in [37]).

The most frequent sources of STEC infections in humans are STEC-shedding animals or humans [76, 333, 334], faecally-contaminated drinking water [103, 337] and recreational water [335, 336], or faecally-contaminated food [8, 10]. Undercooked meat and meat products of bovine origin, contaminated with faecal material at slaughter, have been identified frequently as sources of foodborne STEC outbreaks overseas [22, 24, 25].

Although good hygiene practices on meat processing plants limit the faecal contamination of carcasses at slaughter, hides have been identified as the main source of carcass contamination during processing of cattle [285, 288, 306], highlighting the importance of minimising the level of faecal contamination of hides prior to slaughter. STEC-infected cattle shed the pathogen within their feaces and contaminate the environment and other animals during transportation and lairage. For example, *E. coli* have been found on walls of transport trucks [358], lairage pen floors [386, 387], and on hides of animals after transportation and lairage [283, 386, 388].

Slaughter animals are exposed to changing stress levels during transportation and lairage, which might lead to increased faecal shedding of STEC and contamination of hides and consequently an increased risk of carcass contamination. In addition, transport and lairage times can vary considerably, which could increase the likelihood of possible transmission and infection between slaughter cattle during transportation. A Canadian study investigating the effect of long- and short-haul transportation of slaughter heifers on hide contamination with *E. coli* O157 concluded that the *E. coli* prevalence in feedlots had a higher impact on hide contamination at the slaughter plant than factors associated with transportation [359]. Similarly, a cohort study in Irish feedlot cattle was not able to find an increased prevalence in faecal shedding of *E. coli* O157 after transportation and lairage [298]. However, no data are currently available on the impact of transportation and lairage on the hide and carcass contamination in very young calves.

STEC pose a risk to domestic and overseas consumers of red meat produced in New Zealand, but little is known about the levels of STEC entering the food chain via slaughtered livestock

in New Zealand. Findings of the nationwide cross-sectional study investigating the epidemiology and prevalence of STEC in New Zealand slaughter cattle (Chapter 3) indicated that a higher proportion of very young calves shed STEC O157 and STEC O26 compared to adult cattle. Based on these observations, it can be hypothesised that conditions associated with transportation and lairage could have an effect on the faecal prevalence of STEC in calves at time of slaughter, which could increase the post-slaughter contamination of carcasses from infected to non-infected animals.

To determine the impact of transportation and lairage on calf rectal carriage and carcass contamination with specific *E. coli* serogroups, a cohort study of calves from 'high' and 'low' risk farms was designed with the following two objectives: (i) to determine the prevalence and level of faecal shedding of *E. coli* O157 and O26 in calves at time of slaughter, and (ii) study the effect on the levels of carcass contamination through meat processing.

4.3 Materials and methods

4.3.1 Pre-selection, pre-testing and classification of farms

A cohort study was designed for the Waikato region, a large dairy farming area in the central part of the North Island of New Zealand, to follow calves from farms to a regional meat processing plant licenced for veal and beef export market. Dairy farms eligible for this study were those, from which calves have been sampled randomly for the nationwide cross-sectional study (Chapter 3) at the same slaughter plant in the preceding year, and had tested positive for *E. coli* O157 and/or O26 in faeces. Eight dairy farms from three locations (A, B, and C; three farms in A (A1, A2, A3), two farms in B (B1, B2), three farms in C (C1, C2, C3); 52–71 km distance between each location) in the Waikato region were pre-selected for this study but had to be pre-tested and classified, before being included in the study. The study scheme with pre-testing is outlined in Figure 4.1.

			1. Run	2. Run	3. Run
	Week 1	*Week 2*	*Week 3*	*Week 4*	*Week 5*
Location A	1. pre-test	2. pre-test	Study		
Location B		1. pre-test	2. pre-test	Study	
Location C			1. pre-test	2. pre-test	Study

Figure 4.1: Study scheme with pre-testing of dairy farms in three locations in the Waikato region in the North Island of New Zealand to investigate the effect of transportation and lairage on faecal carriage and carcass contamination with *E. coli* O157 and O26 in very young calves.

This study was conducted during the 2010 calving season (July to September). The pre-testing of farms was completed within four weeks and overlapped with the three-week study period (Figure 4.1), enabling the study to be completed before the end of the calf processing season at the slaughter plant.

For pre-testing, all pre-selected farms from the same location were visited twice within a period of two weeks at the beginning of calving to (i) confirm *E. coli* O157 and O26 were present, and (ii) to classify two farms as a 'high' risk and a 'low' risk farm based on *E. coli* O157 prevalence for the study (see paragraph below). On each visit, rectal-anal mucosal swab samples from up to 10 calves per farm were collected as described under *Sample collection*. If more than 10 animals were available on a visit, samples were collected from the first 10 calves in the order they could be approached/handled. Each faecal sample was screened for the presence of *E. coli* O157 and O26 by real-time PCR as described under *Laboratory methods for faecal samples*, but no further laboratory methods were applied to these samples. It was estimated that sampling a total of 20 calves on a farm with 50 calves would detect a low prevalence of 1% with 95% confidence interval (CI) of 0%–4.4%, and a high prevalence of 35% with 95% CI of 19%–51%.

The classification of 'high' and 'low' risk farms was based on the pooled real-time PCR results for *E. coli* O157 from both visits, using Chi-square test. The pair of farms in each location that were most significantly different from each other in terms of prevalence (i.e. they had the lowest p-value in all pairwise comparisons of prevalence) was included in the study; the farm with the higher number of positive samples was classified as the 'high' risk and the other as the 'low' risk farm, recruiting three high-risk and three low-risk farms for the

study. Collected farm/animal data were analysed based on the initial classification of farms (high or low risk) using the same principal of 'intention-to-treat' analysis commonly applied in randomised clinical trials.

4.3.2 Sample size calculations

Preliminary data from the nationwide cross-sectional study (Chapter 3) were used for sample size calculations in this study. The study size for the applied cluster sampling was determined by calculating the design effect (D) from the intra-cluster correlation (ρ) for a range of cluster sizes (b) using the following formula [389]:

$$D = 1 + (b - 1) \times \rho$$

ρ was calculated by estimating the cluster and global variance of the proportion of positive animals infected on each farm in the cross-sectional study (Chapter 3), using the 'aod' package in R.

Practical issues concerning the number of calves to be sampled per farm were taken into consideration and example estimates of power for a sample size of 10 calves per farm (considering D and ρ) were then calculated with Epi Info software (version 6.04b) [390]; the results are presented in Table 4.1.

Table 4.1: Example estimates of power for cluster sampling on selected farms, considering design effect (D) and intra-cluster correlation (ρ) for a sample size of 10 animals per farm.

Study size per farm	D	ρ	Expected frequency of disease in unexposed group	Expected frequency of disease in exposed group	Confidence level (1-α)	Power (1-β)
10	3.6	0.29	20%	80%	95%	80%
10	3.6	0.29	10%	90%	95%	99%

Based on the above calculations, there was sufficient statistical power (at least 80%) at a confidence level of 95% to detect *E. coli* in 10 animals, using 20% or 10% as the expected frequencies of exposure among unexposed animals.

4.3.3 Animal selection and description of transportation and lairage

In the morning of the study day on the first, second and third run (Figure 4.1), calves on a high- and a low-risk farm were selected and tagged for slaughter by the farmer, and provided with a final feed before transportation. If more than 10 tagged calves were available, the first 10 animals were included in the study in the order they could be approached/handled to collect recto-anal faecal samples; otherwise all available calves were used. The study animals were identified with livestock spray marker before being collected by a regional livestock transporter, who picked-up calves from local dairy farms on a pre-determined route and transported them collectively to the described meat processing plant. Where possible, calves from a high- and a low-risk farm were transported in the same pen in the crate but were separated from other calves on the transporter. The duration of livestock transportation (from pick-up on the farm to drop-off at the slaughter plant) was less than two hours. On arrival at the slaughter plant, the study animals were kept in the same holding pen until being slaughtered in the early hours of the following day. During lairage, the study animals were not mixed with calves from other farms but were able to have direct contact with neighbouring calves through the rails of the pen's walls and shared the same trough for *ad libitum* water access. The animals were not fed during lairage. The pen size was large enough for all calves to lie down on the meshed flooring. All holding pens were cleaned with a high-pressure water hose after every use.

4.3.4 Sample collection

Multiple samples were collected from each calf as the cohort of study animals was followed from their farm of origin to the slaughter plant and along the processing chain. Two recto-anal mucosal swab samples were taken from each calf at pre-slaughter, the first on farm before the animal was loaded on the transport truck (on-farm), and the second at the end of lairage immediately before the animal was slaughtered (on-plant). Faecal samples were collected by rotating a sterile plastic cotton-tipped swab (Transystem®, Copan, Brescia, Italy) inside the first 5–7 cm of the rectal mucosa of the animal before being placed into the provided Amies transport medium. The samples were kept at 0–5°C and processed at ^{m}EpiLab within 24–48 h of collection.

The study animals were slaughtered with mobs of calves from other farms from different regions in the upper half of the North Island. To facilitate the sample collection on the

processing chain (processing speed of 6.8 calves per min), the study animals were slaughtered as approximately every 10th calf. Three sponge swab samples were aseptically collected from each carcass post-slaughter, using sponges pre-moistened with 10 ml of maximum recovery diluent (MRD, Fort Richard Laboratories, Auckland, New Zealand), which were sealed in Whirlpak™ bags (Nasco, Fort Atkinson, WI, USA).

Swab samples were taken from (i) the hide of the opening Y-cut area (sampling area of 282 ± 21 cm^2) before skinning (hide sample), (ii) opening Y-cut area and anal cavity (289 ± 30 cm^2) of the left half of the carcass at post-evisceration but pre-intervention (pre-intervention sample), and (iii) opening Y-cut area and anal cavity (289 ± 30 cm^2) of the right half of the carcass at post-intervention (post-intervention sample) when entering the cold room. A hot water wash (82ºC) was used as an intervention on the study plant. As areas of the opening cut and anal cavity had to be sampled twice, a left and right half of carcass sampling was applied to obviate the first sampling procedure modifying or removing the microbial load for the second sampling of the same site. It was assumed that (i) both halves of the carcass were likely to be equally exposed to any possible contamination, and (ii) variability of individual sampling between the left and right half of the carcass was negligible.

Immediately after collection, all hide and carcass swab samples were kept at 0–5°C and processed in the laboratory within 24 h of collection. Post-slaughter collection of samples and initial processing of carcass samples were conducted by staff of the Food Microbiology and Safety Team from AgResearch in Ruakura, Hamilton, New Zealand.

4.3.5 Financial restrictions

Financial restrictions limited the type of samples collected and processed in this study. The initial study design had included the additional collection of hide samples on-farm, and faeces and hides after transportation to the slaughter plant to (i) assess the level of hide contamination before and after transportation and (ii) to observe the single effect of transportation on the faecal shedding of *E. coli* O157 and O26 in calves. Furthermore, laboratory methods such as direct culture plating and most probable number (MPN) analysis for *E. coli* O26 were not applied on samples collected from low-risk animals as specified under *Laboratory methods*.

4.3.6 Data recording

Animal data including age, sex, breed, ear tag number, and carcass weight were recorded. Farm data were collected on geographical location, farm size, housing system of calves, and cleanliness of calves' bedding on the study day. The faecal contamination/hygiene status of calves was scored at the time of sampling on farm and at the end of lairage. Where possible, time spans of transport and lairage were recorded. On plant, lairage conditions such as size and cleanliness of pen, and possible contacts with animals from neighbouring pens were documented.

4.3.7 Laboratory methods

Faecal samples

Enrichments and direct culture plating

Before processing, collected faecal material of each recto-anal mucosal swab sample was weighed (difference between weight of swab with provided Amies transport medium before and after sampling) and recorded to enable enumeration of the concentration of *E. coli* O157 and O26 in each sample, as described later under *Concentration of* E. coli *in faecal samples*. Swab samples were transferred into 20 ml of 100% tryptic soy broth (TSB, Bacto™, Becton, Dickinson and Co, USA) for enrichment, vortexed for 10 s and an aliquot of 50 µl each was plated onto selective culture media for *E. coli* O157 and O26 using cefixime-tellurite sorbitol MacConkey agar (CT-SMAC, Fort Richard Laboratories, Auckland, New Zealand) and cefixime-tellurite rhamnose MacConkey agar (CT-RMAC, Fort Richard Laboratories, Auckland, New Zealand), respectively, and an automatic spiral plater (Don Whitley, UK; model: WASP2). Because of financial restrictions, direct culture plating on CT-SMAC plates was completed on samples from all high- and low-risk animals, but CT-RMAC plates were used for samples from high-risk animals only. Culture plates were incubated for 18–24 h at 37°C, while broth samples were incubated first for 2 h at 25°C, and then 6 h at 42°C. Aliquots of each pre-enriched and post-enriched broth sample were preserved in glycerol (20% (vol/vol) glycerol) and stored at –80°C.

Real-time PCR for detection of E. coli *serogroups*

After enrichment, each broth sample was screened initially by real-time PCR for the presence of *E. coli* O157 and O26. DNA was extracted from an aliquot of 1 ml of enriched broth using

2% Chelex beads solution (Chelex® 100 Resin, Bio-Rad). Previously published primer sequences were used to detect genes encoding for serogroup specific O-antigens of *E. coli* (*rfb*E_{O157} and *wzx*$_{O26}$) (Table 4.2). All DNA extractions were stored at –20°C.

Table 4.2: Nucleotide sequences of forward and reverse primers used for the detection of specific target genes of *E. coli* to investigate the effect of transportation and lairage on the faecal carriage and carcass contamination with *E. coli* O157 and O26 in very young calves.

Primer set	Primer	Primer sequence (5' – 3')	Target gene	Amplicon size (bp)	Reference
	rfbE-F	TTTCACACTTATTGGATGGTCTCAA	*rfb*E_{O157}	88	[341]
	rfbE-R	CGATGAGTTTATCTGCAAGGTGAT			
	wzx-F	CGCGACGGCAGAGAAAATT	*wzx*$_{O26}$	135	[341]
	wzx-R	AGCAGGCTTTTATATTCTCCAACTTT			
A	stx1-F	GACTGCAAAGACGTATGTAGATTCG	*stx*1	150	[342]
	stx1-R	ATCTATCCCTCTGACATCAACTGC			
A	stx2-F	ATTAACCACACCCCACCG	*stx*2	200	[342]
	stx2-R	GTCATGGAAACCGTTGTCAC			
A	eae-F	GACCCGGCACAAGCATAAGC	*eae*	384	[245]
	eae-R	CCACCTGCAGCAACAAGAGG			
A	ehxA-F	GCATCATCAAGCGTACGTTCC	*ehx*A	534	[245]
	ehxA-R	AATGAGCCAAGCTGGTTAAGCT			
B	stx2c-F	CGACAGGCCCGTTATAAAAA	*stx*2c	243	[269]
	stx2c-R	GGCCACTTTTACTGTGAATGTATC			[267]
C	fliC-F	TACCATCGCAAAAGCAACTCC	*fli*C_{H7}	247	[343]
	fliC-R	GTCGGCAACGTTAGTGATACC			
C	E16S-F	AAACGATGTCGACTTGGAGGT	16S rRNA	100	(In house[a])
	E16S-R	TGAGTTTTAACCTTGCGGCCG			

[a] Enteric Reference Laboratory, Upper Hutt, New Zealand.

Real-time PCR assays for DNA amplifications were performed using an automated real-time thermocycler (Rotor Gene 6200HRM, Corbett Research). The final 20 µl PCR reaction mixture for O157 contained 2x PCR buffer (conc. Light Cycler 480 Probes Master, Roche), 50 µM SYTO® 9 dye (green fluorescent nucleic acid stain, Invitrogen), 2 µM of each primer, and 5.4 µl DNA. The PCR included an initial enzyme-activation step at 96°C for 5 min, followed by 45 cycles of denaturation at 96°C for 15 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s; the PCR product was detected by thermal melt from 73°C to 82°C at a rate of 0.1°C per 2 s. Similarly, the final 20 µl PCR reaction mixture for O26 contained 2x PCR buffer (Express qPCR SuperMix, Invitrogen), 50 µM SYTO® 9 dye, 10 µM of each

primer, 2.0 µl DNA, and 5.4 µl sterile water. The PCR included an initial enzyme-activation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 10 s; the PCR product was detected by thermal melt from 72°C to 78°C at a rate of 0.1°C per 2 s. Positive and negative template controls were included in each PCR assay.

Culture isolation from direct culture plates

Serogroup specific latex agglutination kits (E. coli O157 Latex, Oxoid, UK; Serocheck O26, Oxoid, UK) were used on real-time PCR-positive samples to identify suspected *E. coli* O157 and O26 colonies from direct culture on selective culture media. Up to 10 non-fermenting and 10 fermenting colonies per culture plate were tested with latex agglutination. Agglutination-positive *E. coli* O157 and O26 isolates were confirmed with real-time PCR for the presence of *rfb*E_{O157} and *wzx*$_{O26}$ genes, respectively, to exclude false-positive latex agglutination results. Confirmed isolates were preserved in glycerol broth (nutrient broth with 15% glycerol) and stored at –80°C for further molecular analysis.

In addition, suspected *E. coli* O157 colonies from real-time PCR-negative samples were tested also with latex agglutination to check for false-negative samples. This additional testing to detect false-negative samples was not implemented on suspected *E. coli* O26 colonies because of limited availability of the serogroup specific agglutination kit.

Culture isolation using immunomagnetic separation (IMS)

Where culture isolation from direct culture plates of real-time PCR-positive samples was unsuccessful, the samples were subjected to culture confirmation using IMS. Serogroup specific immunomagnetic beads coated with antibodies against surface antigens of *E. coli* O157 and O26 (Dynabeads® anti-E. coli O157 and Dynabeads® EPEC/VTEC O26, Invitrogen Dynal AS, Oslo, Norway) were used for isolation of *E. coli* O157 and O26 from enriched broths, following the manufacturer's instructions. A magnetic pen (PickPen® 1-M, BioNobile, Finland) was used for beads extraction. The beads-broth sample suspensions were inoculated onto serogroup specific culture media (CT-SMAC for O157, CT-RMAC for O26) and incubated for 18–24 h at 37°C.

Up to 10 non-fermenting and 10 fermenting colonies per culture plate were tested with serogroup specific latex agglutination kits. Agglutination-positive *E. coli* O157 and O26

isolates were confirmed with real-time PCR for the presence of *rfb*E_{O157} and *wzx*$_{O26}$ genes, respectively, to exclude false-positive latex agglutination results. Confirmed isolates were preserved in glycerol broth and stored at -80°C for further molecular analysis.

Concentration of E. coli *in faecal samples*

Pre-enriched broth samples were inoculated directly on selective culture media as described above under *Enrichments and direct culture plating*. To enumerate the concentration of viable *E. coli* O157 and O26 in faecal samples prior to enrichment, putative sorbitol/rhamnose non-fermenting *E. coli* O157 and O26 colonies were counted on each culture plate of real-time PCR-positive samples. If agglutination-positive isolates were identified in pre-enriched samples, the concentration of non-fermenting suspect *E. coli* O157 and O26 was calculated as colony forming units (CFU) per gram of faecal material and presented as $\log_{10}$ CFU/g faeces. If no *E. coli* isolates were identified due to concentrations below the detection limits of the selective culture media, the IMS method was used on the enriched samples as described above. If agglutination-positive *E. coli* isolates were identified in enriched samples by IMS, then the concentration of *E. coli* was presented as a value below the detection limits of selective culture media of pre-enriched samples.

Carcass samples

Enrichments and direct culture plating

After sample collection, 90 ml of MRD was added to each sponge swab sample and mixed in a stomacher for 2 min. From each sample, an aliquot of 1 ml was taken for a 10-fold serial dilution of each sample. Diluted samples were inoculated onto CT-SMAC and CT-RMAC selective media for direct culture plating to enumerate concentrations of *E. coli* O157 in carcass samples from high- and low-risk animals, but *E. coli* O26 from high-risk animals only (financial restrictions). A further aliquot of 35 ml was stored at 4ºC for later analysis using the most probable number method as described below, and the remaining volume (approximately 55 ml) was used for enrichment by adding 220 ml of 118% TSB. Culture plates were incubated for 24 h at 35ºC, while enrichment broths were incubated for 2 h at 25ºC followed by a 6-hour incubation at 42ºC. Aliquots of each original sample and enriched broth sample were preserved in glycerol (25% (vol/vol) glycerol) and stored at –80ºC.

TaqMan/Real-time PCR for detection of E. coli *serogroups*

After enrichment, genomic DNA was extracted from 1 ml of enriched broth, using PrepMan™ Ultra (Applied Biosystems, USA) according to the manufacturer's instructions. Each DNA sample was initially screened by TaqMan using probes to detect genes encoding for serogroup specific O-antigens of *E. coli* O157 and O26 (*rfb*E_{O157} and *wzx*$_{O26}$) [341, 391]. Cycle threshold (Ct)-values of less than 35 were considered positive, while Ct-values between 35 and 40 or no response were considered negative. All assays included internal reaction controls for PCR proficiency, and negative and positive culture controls were included on all 96-well plates.

All genomic DNA extractions from enrichments were analysed immediately and then stored at –20ºC for later re-testing by the same real-time PCR as described for faecal samples to ensure concordance between the methods used at each stage.

Culture isolation from direct culture plates

TaqMan-positive samples plus 10% of randomly selected TaqMan-negative samples were subjected to culture confirmation. Where possible, up to three non-fermenting and three fermenting colonies per CT-SMAC and CT-RMAC plates from direct culture plating were transferred to tryptic soy agar plates (TSA, Fort Richard Laboratories, Auckland, New Zealand) for further analysis. Presumptive *E. coli* O157 and O26 isolates were identified using the same serogroup specific latex agglutination kits as applied on faecal samples. Presumptive isolates were stored in glycerol broth (tryptic soy broth with 15% glycerol) at –80°C and later transferred to ^{m}EpiLab's laboratory for further molecular analysis including confirmation as *E. coli* O157 and O26 by real-time PCR (*rfb*E_{O157} and *wzx*$_{O26}$).

Culture isolation using immunomagnetic separation (IMS)

If culture isolation from direct culture plates of TaqMan-positive samples was unsuccessful, enriched samples were subjected to culture confirmation using IMS as described for faecal samples, but with external magnets. Where possible, up to three non-fermenting and three fermenting colonies per CT-SMAC and CT-RMAC plates were transferred to TSA plates for further analysis. Presumptive *E. coli* O157 and O26 isolates were identified using latex agglutination and stored in glycerol broth at –80°C for confirmation and further molecular analysis at ^{m}EpiLab's laboratory.

After re-testing genomic DNA extractions from enrichments by real-time PCR for the presence of *E. coli* O157 and O26, real-time PCR-positive samples without isolates on initial screening were subjected to culture confirmation using IMS on stored enrichments.

Concentration of E. coli *in carcass samples*

Direct plating and most probable number (MPN) analysis were used to enumerate the concentration of viable *E. coli* O157 and O26 in carcass samples prior to enrichment. Equivalent to direct culture plating of faecal samples, putative non-fermenting *E. coli* O157 and O26 colonies were counted on each culture plate of TaqMan-positive samples and identified using latex agglutination. If agglutination-positive *E. coli* isolates were identified, the concentration of *E. coli* was calculated as colony forming units (CFU)/cm^2 swabbed carcass area. If agglutination-positive *E. coli* isolates were not identified on direct culture plates with growth of non-fermenting and fermenting colonies, but with IMS in enriched samples, then the concentration of *E. coli* was presented as a value below the calculated concentration of non-fermenting/fermenting colonies on direct culture plates of pre-enriched samples.

Multiple-tube MPN analysis was applied on O157 TaqMan-positive samples of high- and low-risk animals but only on O26 TaqMan-positive samples of high-risk animals (financial restrictions). This method was used to estimate the concentration of *E. coli* O157 and O26 in hide and carcass samples where non-fermenting colonies did not grow by direct plating of the original samples because the concentrations of microbes were below the detection limits of culture media. A standard 3 x 3 row MPN protocol (10 ml in 40 ml; 1 ml in 4 ml; and 0.1 ml in 4 ml) was used on the original samples with 118% TSB [392]. After incubation for 2 h at 25ºC followed by a 6-hour incubation at 42ºC, genomic DNA was extracted from all tubes with visible growth using PrepMan™ Ultra. TaqMan assays were performed to confirm the presence of *E. coli* O157 and O26 (*rfb*E_{O157} and *wzx*$_{O26}$). The observed pattern of tubes with visible growth was recorded and concentrations as MPNs were read off published standard tables [392].

Molecular analysis of isolates

Stored *E. coli* O157 and O26 isolates were re-grown on Columbia horse blood agar (Fort Richard Laboratories, Auckland, New Zealand). Bacterial DNA was extracted from five

colonies using 2% Chelex beads solution and was analysed in the following series of PCR assays. All genomic DNA extractions were analysed immediately and then stored at –20ºC.

Multiplex PCR for detection of virulence genes

Confirmed *E. coli* O157 and O26 isolates were screened by multiplex PCR for the presence of virulence genes encoding for enterohaemolysin (*ehx*A), intimin (*eae*), and Shiga toxin 1 (*stx*1) and Shiga toxin 2 (*stx*2), characteristic of STEC. The multiplex PCR assay was performed on the automated real-time thermocycler using previously published primer sequences (primer set A, Table 4.2). The final 25 µl PCR reaction mixture contained 2x PCR buffer (Express qPCR SuperMix, Invitrogen), 2 µM of each primer, 2.0 µl DNA, and 2.5 µl sterile water. The PCR included an initial enzyme-activation step at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 64°C for 20 s, and extension at 72°C for 20 s; the terminal extension was at 72°C for 5 min. Amplified PCR products were detected by electrophoresis using a 2% (wt/vol) agarose gel (Agarose low EEO, AppliChem, Germany), stained in ethidium bromide (0.5 µg/ml in TBE buffer) for 10 min, and visualised under ultraviolet light on a transilluminator (GelDoc™ XR, BIO-RAD Laboratories, Segrate (Milan), Italy).

*PCR for detection of virulence gene subtype stx*2c

E. coli isolates positive for *stx*2 were tested for the presence of the genetic subtype *stx*2c. This gene was detected in a separate PCR assay using primer sequences published by Besser *et al.* [267] and Shringi *et al.* [269] (primer set B, Table 4.2). DNA amplification was performed on the automated real-time thermocycler with a final 20 µl PCR reaction mixture containing 2x PCR buffer (Express qPCR SuperMix, universal, Invitrogen), 2 µM of each primer, 2.0 µl DNA, and 6.0 µl sterile water. The PCR included an initial enzyme-activation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 20 s and annealing at 55°C for 20 s; no extensions were used. The amplified PCR product was detected by electrophoresis using a 2% (wt/vol) agarose gel, stained in ethidium bromide for 10 min, and visualised under ultraviolet light on the transilluminator.

PCR for detection of fli*C gene*

E. coli O157 isolates were tested also for the presence of the *fli*C gene encoding for the H7 bacterial flagellum antigen, using previously published primer sequences [343] (primer set C, Table 4.2). In addition, to ensure the DNA template used in this series of PCR assays was of

bacterial origin, an internal control (E16S) was included (primer set C, Table 4.2). DNA amplification of the *fli*C_{H7} and bacterial 16S rRNA gene sequences was performed using the automated real-time thermocycler. The final 20 µl PCR reaction mixture contained 2x PCR buffer (Taq PCR Mastermix, Qiagen), 5 µM of each primer for *fli*C_{H7}, 10 µM of each primer for bacterial 16S rRNA, 2.0 µl DNA, and 5.0 µl sterile water. The PCR included an initial enzyme-activation step at 95°C for 8 min, followed by 25 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 10 s, and extension at 72°C for 10 s; the terminal extension was at 72°C for 7 min. Amplified PCR products were detected by electrophoresis using a 2% (wt/vol) agarose gel, stained in ethidium bromide for 10 min, and visualised under ultraviolet light on the transilluminator.

Pulsed-field gel electrophoresis (PFGE)

Each confirmed *E. coli* O157 and O26 isolate was subtyped using PFGE (restriction enzyme *Xba*I) following the standardised laboratory protocol published by PulseNet International [393].

4.3.8 Ethical approval

This study was approved by the Animal Ethics Committee of Massey University, Palmerston North, New Zealand, on 21 May 2010, protocol number 10/44.

4.3.9 Data management and statistical analysis

R software (version 2.15.2) [345] was used for all statistical analysis, with significance set at $p < 0.05$.

Multivariate logistic regression analysis

Multivariate logistic regression analysis was applied to identify risk factors associated with a calf being positive for either serogroup of *E. coli* on plant after transportation and lairage. Real-time PCR results were used for this analysis because of their higher prevalence and statistical power compared to culture results. Only explanatory variables such as the risk type of farm, being real-time PCR-positive on-farm, and the prevalence of *E. coli* (O157 or O26) on-farm were included in the initial multivariate model. The on-farm prevalence for a

low/high-risk animal was calculated as the percentage of the number of real-time PCR-positive animals divided by the total number of animals selected on the low/high-risk farm.

Stepwise backward-elimination of the least significant variables was applied to generate a final multivariate model. Eliminated variables were assessed for confounding, determined by a change of >30% in a variable coefficient of the model after another variable was dropped from the model. Even if non-significant, variables with confounding effect were retained in the model.

To evaluate the model's significance and goodness-of-fit, Likelihood ratio tests and the le Cessie-van Houwelingen normal test statistics [352] were applied, respectively, using R package 'rms' [353]. The models were compared using the Akaike information criterion (AIC), a measure of the relative goodness of fit. Odds ratios of model variables including 95% confidence intervals were calculated in Microsoft Excel 2010.

Molecular analysis of isolates

BioNumerics software (version 6.6) [394] was used to analyse and compare PFGE profiles of *E. coli* O157 and O26 isolates, and to create a dendrogram applying UPGMA (unweighted pair group method with arithmetic mean) cluster analysis using the Dice similarity coefficient, with a band matching tolerance of 1%. Clusters of PFGE profiles were established with cut-off levels of 97.3% and 97.7% similarity for *E. coli* O157 and O26, respectively, and cluster numbers were assigned. The cluster numbers were used to analyse the diversity of PFGE profiles by calculating the Simpson's diversity index (1 – D) [395] including 95% confidence intervals, using PAST software [396]. The Simpson's index ranges from 0 to 1, with values closer to 1 indicating a higher diversity of PFGE profiles. The assigned cluster numbers were used also to differentiate groups of *E. coli* O157 and O26 originating from the same high/low-risk farm from farms not included in the study, using a colour scheme.

Counts of aerobic growth and concentrations of *E. coli* in samples

To assess the distribution of aerobic growth of pre-enriched faecal and carcass samples real-time PCR-positive for *E. coli* O157 and O26, sorbitol/rhamnose-fermenting and non-fermenting colonies of samples were counted on direct culture plates and presented as

minimum, maximum and quartiles of counts, stratified by sample type. Similarly, distributions of concentrations of *E. coli* O157 and O26 (STEC and non-STEC) in faecal and carcass samples were presented also as minimum, maximum and quartiles per sample type, but stratified by detection methods applied. Where the MPN method was applied, the concentrations were estimated using published standard tables [392] and calculated per 100 cm^2 of swabbed carcass area.

4.4 Results

4.4.1 Data from pre-testing

Recto-anal faecal samples from a total of 144 calves from eight pre-selected dairy farms were collected for pre-testing; the results of real-time PCR, stratified by farm, are summarised in Table 4.3.

Table 4.3: Recto-anal faecal swab samples collected from calves on eight pre-selected dairy farms in three locations in the Waikato region and screened for the presence of *E. coli* O157 and O26 (STEC and non-STEC) by real-time PCR (real-time PCR positive/samples (%)).

			O157		O26		
Location	Farm	Total calves tested	1. pre-testing	2. pre-testing	1. pre-testing	2. pre-testing	Classified risk type of farm
A	A1	18	0/9 (0)	0/9 (0)	6/9 (67)	4/9 (44)	-
	A2	20	3/10 (30)	3/10 (30)	0/10 (0)	0/10 (0)	Low
	A3	10	- [a]	8/10 (80)	- [a]	8/10 (80)	High
B	B1	20	1/10 (10)	4/10 (40)	0/10 (0)	2/10 (20)	Low
	B2	20	5/10 (50)	10/10 (100)	8/10 (80)	7/10 (70)	High
C	C1	20	8/10 (80)	3/10 (30)	8/10 (80)	6/10 (60)	-
	C2	20	2/10 (20)	2/10 (20)	8/10 (80)	9/10 (90)	Low
	C3	16	8/10 (80)	3/6 (50)	7/10 (70)	3/6 (50)	High

[a] No calves available.

In general, the prevalence of *E. coli* O157 and O26 in calves on farms remained relatively unchanged or decreased, over the two weeks of pre-testing; however, a sharp increase in prevalence of *E. coli* O157 was observed on both farms in Location B, but not in *E. coli* O26.

4.4.2 Number of animals and samples collected

A total of 60 calves were followed from three high-risk and three low-risk dairy farms to the slaughter plant and multiple samples were collected from each calf at pre- and post-slaughter. A summary of collected samples per run, stratified by risk type of farm, is presented in Table 4.4.

Table 4.4: Total number of animals and samples collected at pre- and post-slaughter during three separate runs to investigate the effect of transportation and lairage on the faecal shedding and contamination of carcasses with *E. coli* O157 and O26 (STEC and non-STEC) in very young calves. Reasons for loss of carcasses at post-slaughter are explained in superscripts (c-e).

			Number of samples collected				
			Pre-slaughter (faeces)[a]		Post-slaughter (carcass)[b]		
Run	Risk type of farm	Number of animals	On-farm	On-plant	Hide	Pre-intervention	Post-intervention
1	Low	10	10	10	9[c]	8[c]	1[d]
	High	10	10	10	10	10	7[d]
2	Low	8	8	8	8	8	8
	High	10	10	10	10	10	9[e]
3	Low	11	11	11	11	11	11
	High	11	11	11	11	11	11

[a] Pre-slaughter: faecal sample on-farm before transportation, faecal sample on-plant at the end of lairage.
[b] Post-slaughter: hide swab at pre-skinning, carcass swab after evisceration but pre-intervention, and carcass swab post-intervention at pre-boning.
[c] Carcass missed on processing chain.
[d] Identification tags lost in hot water shower (intervention).
[e] Carcass dropped at pre-intervention sampling and was therefore condemned.

On the first run, 12 carcasses were lost to follow-up at post-slaughter as they were either missed on the processing chain, or the tags of marked study carcasses got lost at intervention in the hot water shower. On the second run, only eight calves were available at the low-risk farm and one carcass was condemned at pre-intervention. To adjust for some losses of animals and carcasses, which occurred on the first and second run, 11 high- and low-risk calves were selected on the third run.

4.4.3 Real-time PCR and culture isolation

All collected samples were tested by real-time PCR for the presence of *E. coli* O157 and O26 (STEC and non-STEC) and confirmed with culture isolation; the results are presented in Table 4.5.

Table 4.5: Results of real-time PCR and culture confirmed isolation of *E. coli* O157 and O26 (STEC and non-STEC) from pre- and post-slaughter samples collected from very young calves to investigate the effect of transportation and lairage on the faecal shedding and carcass contamination with *E. coli* O157 and O26.

		O157		O26	
Sample type	Number of samples	Real-time PCR positive (%)	Culture confirmed (%)	Real-time PCR positive (%)	Culture confirmed (%)
Pre-slaughter (faeces)[a]					
On-farm	60	38 (63.3)	15 (25.0)	37 (61.7)	19 (31.7)
On-plant	60	39 (65.0)	10 (16.7)	44 (73.3)	17 (28.3)
Post-slaughter (carcass)[b]					
Hide	59	56 (94.9)	24 (40.7)	59 (100.0)	48 (81.4)
Pre-intervention	58	8 (13.8)	3 (5.2)	20 (34.5)	10 (17.2)
Post-intervention	47	2 (4.3)	1 (2.1)	7 (14.9)	5 (10.6)

[a] Pre-slaughter: faecal sample on-farm before transportation, faecal sample on-plant at the end of lairage.
[b] Post-slaughter: hide swab at pre-skinning, carcass swab after evisceration but pre-intervention, and carcass swab post-intervention at pre-boning.

No significant change in real-time PCR-positive faecal samples was observed on-plant ($p = 1$ for *E. coli* O157 and $p = 0.242$ for *E. coli* O26), providing no evidence for transmission of *E. coli* infection, i.e., ingestion of *E. coli*, gastrointestinal passage and colonisation of the recto-anal junction during transportation and lairage. In contrast, however, 56 out of 59 calves were real-time PCR-positive for both serogroups at hide sampling ($p < 0.001$ for *E. coli* O157 and $p < 0.001$ for *E. coli* O26), showing a high cross-contamination of hides with *E. coli*. The prevalence of PCR-positive samples dropped considerably at both pre- and post-intervention.

Detailed results of real-time PCR and culture isolation of *E. coli* O157 and O26 (STEC and non-STEC), stratified by run and risk type of farm, are presented in Table 4.6.

Table 4.6: Results of real-time PCR (Real-time PCR positive/samples) and culture confirmed isolation (culture positive/samples) of *E. coli* O157 and O26 (STEC and non-STEC) from pre- and post-slaughter samples collected from very young calves during three separate runs to investigate the effect of transportation and lairage on the faecal shedding and carcass contamination with *E. coli* O157 and O26, stratified by risk type of farm.

			Pre-slaughter (faeces)[a]				Post-slaughter (carcass)[b]					
			On-farm		On-plant		Hide		Pre-intervention		Post-intervention	
Run	Risk type of farm	Sero-group	Real-time PCR	Culture	Real-time PCR	Culture	Real-time PCR	Culture	Real-time PCR	Culture	Real-time PCR	Culture
1	Low	O157	1/10	0/10	2/10	0/10	9/9[c]	1/9[c]	1/8[c]	0/8[c]	0/1[d]	0/1[d]
		O26	1/10	0/10	4/10	1/10	9/9	8/9	4/8	3/8	1/1	1/1
	High	O157	10/10	3/10	9/10	0/10	10/10	2/10	4/10	1/10	0/7[d]	0/7[d]
		O26	10/10	10/10	9/10	9/10	10/10	9/10	5/10	4/10	4/7	4/7
2	Low	O157	5/8	5/8	4/8	3/8	7/8	6/8	0/8	0/8	0/8	0/8
		O26	1/8	1/8	1/8	0/8	8/8	8/8	3/8	2/8	0/8	0/8
	High	O157	10/10	1/10	10/10	0/10	10/10	1/10	1/10	0/10	0/9[e]	0/9[e]
		O26	9/10	8/10	10/10	7/10	10/10	10/10	3/10	1/10	0/9	0/9
3	Low	O157	3/11	0/11	5/11	1/11	10/11	6/11	0/11	0/11	1/11	0/11
		O26	8/11	0/11	10/11	0/11	11/11	8/11	2/11	0/11	1/11	0/11
	High	O157	9/11	6/11	9/11	6/11	10/11	8/11	2/11	2/11	1/11	1/11
		O26	8/11	0/11	10/11	0/11	11/11	5/11	3/11	0/11	1/11	0/11

[a] Pre-slaughter: faecal sample on-farm before transportation, faecal sample on-plant at the end of lairage.

[b] Post-slaughter: hide swab at pre-skinning, carcass swab after evisceration but pre-intervention, and carcass swab post-intervention at pre-boning.

[c] Carcass missed on processing chain.

[d] Identification tags lost in hot water shower (intervention).

[e] Carcass dropped at pre-intervention sampling and was therefore condemned.

On each run, faecal samples (on-farm and on-plant) from calves of high-risk farms were generally real-time PCR-positive for *E. coli* O157 and O26 compared to samples from animals of low-risk farms, while almost all hide samples from calves of both risk types were real-time PCR-positive for *E. coli* O157 and O26. At pre-intervention, however, fewer samples from high-risk and low-risk animals were real-time PCR-positive for *E. coli* O157 and O26 compared to hide samples, and the number of positive samples declined even further to only two at post-intervention. No consistent patterns could be observed in culture isolation of *E. coli* O157 and O26 (STEC and non-STEC) among faecal and carcass samples of calves from high-risk and low-risk farms.

4.4.4 Characterisation of confirmed isolates

In total, 56 *E. coli* O157 and 115 *E. coli* O26 isolates were retrieved and analysed by multiplex PCR for the presence of various target genes. PCR amplicons of bacterial DNA were visualised as depicted in Figure 4.2, identifying the presence of virulence genes (primer set A), the gene subtype *stx*2c (primer set B), the *fli*C_{H7} gene in STEC O157 and the gene for bacterial 16S rRNA (internal control) (primer set C).

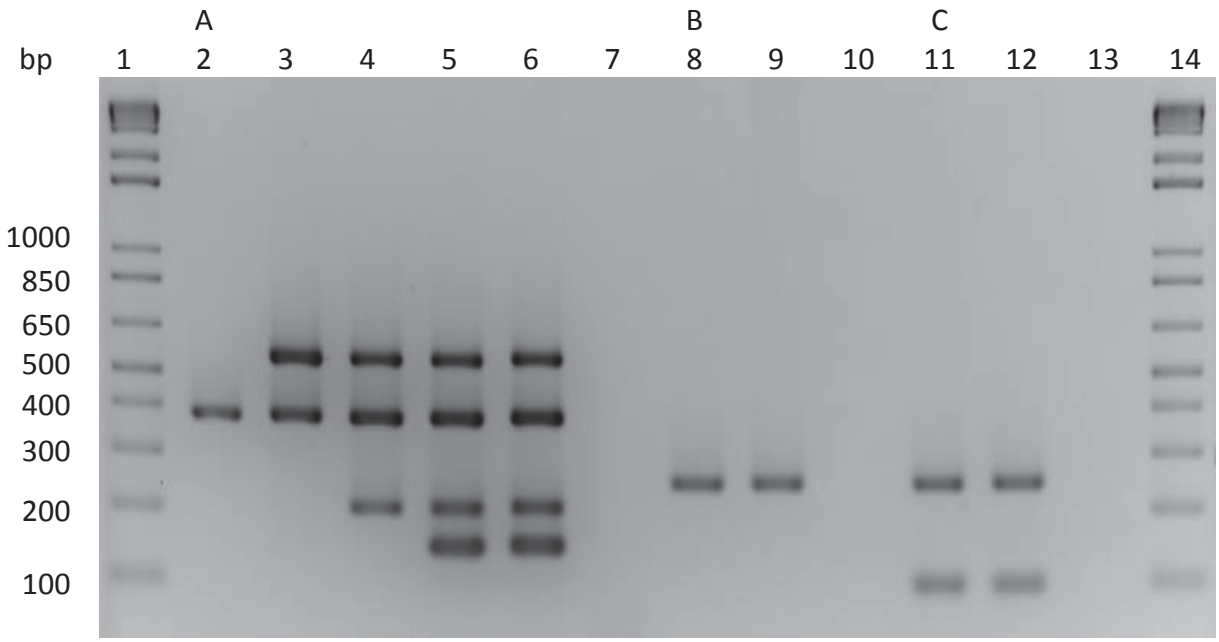

Figure 4.2: Agarose (2% wt/vol) gel electrophoresis showing PCR amplicons of DNA from *E. coli* isolates using primer sets A (lanes 2–7), B (lanes 8–10), and C (lanes 11–13) for the detection of different virulence genes. Lanes 1 and 14: 1Kb Plus DNA ladder (Invitrogen); 2, *eae*; 3, *ehx*A and *eae*; 4, *ehx*A, *eae*, and *stx*2; 5, *ehx*A, *eae*, *stx*2, and *stx*1; 6, positive control; 7, negative control; 8, *stx*2c; 9, positive control; 10, negative control; 11, *fliC*$_{H7}$ and bacterial 16S rRNA (internal control); 12, positive control; 13, negative control.

Seventeen samples contained two or more different strain types of *E. coli* O157 or O26. Characteristics of all confirmed *E. coli* isolates, stratified by sample type, serogroup and risk type of farm, are presented in Table 4.7.

Table 4.7: Characteristics of *E. coli* O157 (n = 56) and O26 (n = 115) isolates collected from multiple samples from very young calves at pre-slaughter (prior to transportation on-farm, after transportation to and lairage on-plant) and post-slaughter (hide, pre- and post-intervention on processing chain). Isolates are stratified by sample type and serogroups. PCR methods were used to test for the presence (+) of virulence genes encoding for enterohaemolysin (*ehx*A), intimin (*eae*), Shiga toxins (*stx*2, subtype *stx*2c, and *stx*1), and the *fli*C_{H7} gene encoding the H7 flagellum antigen from STEC O157.

		Number of isolates from animals				Virulence genes of STEC					
Sample type	Serogroup	High-risk	Low-risk	Sorbitol[a]	Rhamnose[b]	*ehx*A	*eae*	*stx*2	*stx*2c	*stx*1	H7
On-farm (faeces)	O157	6	5	–		+	+	+	–	+	+
		4	0	+		–	–	–	–	–	ne
	O26	0	1		–	+	+	–	–	–	ne
		8	0		+	–	+	–	–	–	ne
		10	0		+	–	–	–	–	–	ne
On-plant (faeces)	O157	0	3	–		+	+	+	–	+	+
		5	1	–		+	+	+	–	–	+
		1	0	+		–	–	–	–	–	ne
	O26	2	0		–	–	–	–	–	–	ne
		7	0		+	–	+	–	–	–	ne
		7	1		+	–	–	–	–	–	ne
Hide (carcass)	O157	0	1	–		+	+	+	+	+	+
		1	1	–		+	+	+	–	+	+
		4	7	–		+	+	+	+	–	+
		6	4	–		+	+	+	–	–	+
		2	1	+		–	–	–	–	–	ne
	O26	3	3		–	+	+	–	–	+	ne
		5	4		–	+	+	–	–	–	ne
		1	1		–	–	+	–	–	–	ne
		13	18		+	–	+	–	–	–	ne
		7	7		+	–	–	–	–	–	ne

Table 4.7 continued

Sample type	Serogroup	Number of isolates from animals		Sorbitol[a]	Rhamnose[b]	Virulence genes of STEC					H7
		High-risk	Low-risk			*ehx*A	*eae*	*stx*2	*stx*2c	*stx*1	
Pre-intervention	O157	2	0	–		+	+	+	–	–	+
(carcass)		1	0	+		–	–	–	–	–	ne
	O26	2	1		–	+	+	–	–	+	ne
		1	0		–	+	+	–	–	–	ne
		1	1		+	–	+	–	–	–	ne
		3	3		+	–	–	–	–	–	ne
Post-intervention	O157	1	0	–		+	+	+	–	–	+
(carcass)	O26	1	0		–	+	+	–	–	+	ne
		4	0		+	–	–	–	–	–	ne

[a] Sorbitol-fermenting (+) or non-sorbitol-fermenting (–) on CT-SMAC culture plate.

[b] Rhamnose-fermenting (+) or non-rhamnose-fermenting (–) on CT-RMAC culture plate.

ne = not examined.

Twice as many *E. coli* isolates of serogroup O26 compared to O157 were recovered, however, only 8.7% (10/115) of the *E. coli* O26 isolates carried an *stx* gene and were considered as STEC, while 83.9% (47/56) of the *E. coli* O157 isolates were STEC. A large variety of different strain types of *E. coli* O157 and O26 was observed, particularly in the hide samples. The lowest numbers of *E. coli* isolates (including STEC O157 and STEC O26) were detected in carcass samples post-intervention.

4.4.5 Risk factors

Statistically significant risk factors and confounding variables estimated from the multivariate logistic regression models for a calf being real-time PCR-positive for *E. coli* O157 and O26 on-plant, are presented in Table 4.8 and Table 4.9, respectively.

Table 4.8: Results of multivariate logistic regression analysis for a calf being real-time PCR-positive for *E. coli* O157 in faeces on-plant after transportation and lairage (60 observations).

Variable	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Intercept	–1.16 (0.50)		
Being positive on-farm	1.98 (0.78)	7.26 (1.57–33.61)	0.011
Risk type of farm			
Low	Ref		
High	1.66 (0.84)	5.27 (1.01–27.43)	0.049

Likelihood ratio test = 26.22 (df = 2, p <0.001).
[a] SE = Standard error.
[b] CI = 95% confidence interval.
Ref = reference level for comparison.

A calf being real-time PCR-positive for *E. coli* O157 in faeces on-plant was highly associated with being positive on-farm or if the animals originated from a high-risk farm.

Table 4.9: Results of multivariate logistic regression analysis for a calf being real-time PCR-positive for *E. coli* O26 in faeces on-plant after transportation and lairage (60 observations).

Variable	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Intercept	–1.51 (0.64)		
On-farm prevalence of *E. coli* O26	3.34 (1.34)	28.31 (2.03–394.39)[c]	0.013
Being positive on-farm[d]	1.93 (1.00)	6.89 (0.98–48.73)	0.053

Likelihood ratio test = 29.83 (df = 2, p <0.001).
[a] SE = Standard error.
[b] CI = 95% confidence interval.
[c] The odds ratio of this continuous variable refers to an increase in prevalence of *E. coli* O26 of 0.1 (10%) on the farm.
[d] Confounding variable.

Similarly to O157, a calf being real-time PCR-positive for *E. coli* O26 in faeces on-plant was also associated with being positive on-farm. The second variable was 'on-farm prevalence', which was closely related to the 'risk type of farm' variable, but for O26 this continuous variable provided the better fit.

4.4.6 Genotype diversity of isolates

Based on cluster analysis of PFGE profiles of *E. coli* (STEC and non-STEC) isolates recovered, the Simpson's index (1 – D) was calculated to demonstrate the diversity of PFGE profiles of isolates from specific sample types (Table 4.10).

Table 4.10: Simpson's index (1 – D) values with 95% confidence intervals (CI) showing the diversity of PFGE profiles of *E. coli* O157 (n = 56) and O26 isolates (n = 115) recovered from faeces from very young calves on-farm and after transportation to and lairage on-plant, and from carcasses (presenting hides only) on the processing chain at the slaughter plant.

	O157 isolates		O26 isolates	
Sample type	Number of isolates	Simpson index (95% CI)	Number of isolates	Simpson index (95% CI)
All included	56	0.911 (0.858–0.921)	115	0.899 (0.852–0.920)
On-farm + on-plant	25	0.816 (0.672–0.851)	36	0.676 (0.568–0.728)
Hide	27	0.867 (0.752–0.889)	62	0.955 (0.916–0.955)

A high diversity was observed for both serogroups when analysing all isolates collectively; however, after stratification by sample type, the highest diversity was apparent at the hide stage for either serogroup. For O26, the difference in diversity between pre-slaughter (on-farm and on-plant isolates combined) and hide samples was statistically significant.

To investigate the transmission of *E. coli* infection and/or cross-contamination of *E. coli* among calves, the diversity of PFGE profiles of *E. coli* O157 and O26 isolates at sampling sites was visualised as depicted in Figure 4.3 and Figure 4.4, respectively, based on cluster analysis of the isolates' PFGE profiles. Cluster analysis of PFGE profiles of *E. coli* O157 and O26 isolates including virulence profiles are presented in Appendix 3 and Appendix 4, respectively.

		Sampling sites				
Run	Type of risk farm	On-farm	On-plant	Hide	Pre-int.	Post-int.
1	low			6		
1	high			8		
1	high	7			2	
1	high			21*		
1	high	3				
1	high	7				
2	low	21*	21*	18*		
2	low	21*		18*		
2	low			15*		
2	low	21*		21* | 17*		
2	low	21*	21*	19*		
2	low	21*	21*	17*		
2	high	1				
2	high			17*		
3	low			9*		
3	low			9*		
3	low			20*		
3	low			9*		
3	low			20*		
3	low			20*		
3	low		11*			
3	high	13*	4	12*		
3	high	14*	14*	14*		
3	high			16*	12*	10*
3	high	12*		20*		
3	high	14*	10*	9* | 20*		
3	high	14*	10*	20*		
3	high		10*	9*		
3	high	14*	10*		12*	
3	high			9* | 5		

Figure 4.3: Diversity of PFGE profiles of *E. coli* O157 isolates (n = 56) recovered at multiple sampling sites from very young calves at pre-slaughter (prior to transportation on-farm, after transportation to and lairage on-plant) and post-slaughter (hide, pre-intervention, and post-intervention on processing chain). Only animals with one or more recovered isolates are listed, with split cells showing two characteristically different PFGE profiles of isolates, which were recovered from the same sample, coloured by farm of origin. Dark blue cells represent PFGE profiles prevalent on high-risk farms, light blue on low-risk farms, and red on neither low- or high-risk farms included in the study and therefore likely of different origin. Type of risk farm was determined by on-farm prevalence of *E. coli* O157 at pre-testing. Numbers in cells represent assigned PFGE cluster numbers and * identifies STEC isolates.

There was no evidence to support that transmission of *E. coli* infection (following ingestion, gut passage and colonisation) had occurred during transportation and lairage; on hides, however, a high level of cross-contamination with genotypes (red cells), which were not recovered from calves' faeces on-farm or at the end of transportation and lairage was observed.

		Sampling sites				
Run	Type of risk farm	On-farm	On-plant	Hide	Pre-int.	Post- int.
1	low			29 9		
1	low		12	15* 9		
1	low			35 12		
1	low			9		9
1	low			7 9	9	
1	low				9	
1	low			34 12		
1	low			13*	11	
1	low			9		
1	high	9	9	30 32		
1	high	9	9	9		
1	high	9	9	9	9	9
1	high	9	12	12		13*
1	high	9	12	9	12	
1	high	12	9	11		11
1	high	9	9	13*	13*	
1	high	9	9		9 14*	
1	high	10	12	11		
1	high	9		34		12
2	low			1 24		
2	low			23		
2	low			23		
2	low			40	18*	
2	low			23 4 18*		
2	low			38	41	
2	low			38		
2	low	2		39 1		
2	high	23	23	5 20 25		
2	high	3	23	8 27		
2	high	23	23	1		
2	high	23		6 16*		
2	high	23		36	23 1	
2	high	23	23	21		
2	high		23	1		
2	high	23	23	19		
2	high	23	23	37		
2	high			22		
3	low			28		
3	low			28		
3	low			28		
3	low			28		
3	low			42		
3	low			43		
3	low			33		
3	low			26		
3	high			31		
3	high			31		
3	high			31		
3	high			31		
3	high			17*		

Figure 4.4: Diversity of PFGE profiles of *E. coli* O26 isolates (n = 115) recovered at multiple sampling sites from very young calves at pre-slaughter (prior to transportation on-farm, after transportation to and lairage on-plant) and post-slaughter (hide, pre-intervention, and post-intervention on processing chain). Only animals with one or more recovered isolates are

listed, with split cells showing up to three characteristically different PFGE profiles of isolates, which were recovered from the same sample, coloured by farm of origin. Dark blue cells represent PFGE profiles prevalent on high-risk farms, light blue on low-risk farms, and red on neither low- or high-risk farms included in the study and therefore likely of different origin. Type of risk farm was determined by on-farm prevalence of *E. coli* O157 at pre-testing. Numbers in cells represent assigned PFGE cluster numbers and * identifies STEC isolates.

Similar to data for O157, no obvious transmission of *E. coli* O26 infection during transportation and lairage resulting in an increased faecal shedding of *E. coli* at the end of lairage had occurred (although one low-risk calf was positive with PFGE type 12), and also a high level of cross-contamination at hide level with genotypes of isolates from other farms of origin was observed. However, there was evidence of hide and carcass cross-contamination at pre-intervention from high- to low-risk animals. Despite this, there was no evidence of a difference in residual contamination on the carcasses of high- and low-risk calves at the pre-boning stage.

4.4.7 Counts and concentrations of *E. coli* in faecal and carcass samples

The aerobic growth of faecal (pre-slaughter) and carcass (post-slaughter) samples real-time PCR-positive for *E. coli* O157 and O26 (STEC and non-STEC) was assessed, counting fermenting and non-fermenting colonies on direct culture plates (Table 4.11). The lowest counts were observed in pre- and post-intervention samples.

In addition, concentrations of *E. coli* O157 and O26 (STEC and non-STEC) in faecal and carcass samples were enumerated/estimated based on detection methods applied (Table 4.12).

Table 4.11: Minimum, maximum, and quartiles for counts of fermenting and non-fermenting colonies in recto-anal faecal samples (n) at pre-slaughter (on-farm, on-plant) and carcass swab samples (n) at post-slaughter (hide, pre- and post-intervention) from very young calves, using direct culture plating. Only colony counts on CT-SMAC[a] and CT-RMAC[b] plates of samples real-time PCR-positive for *E. coli* O157 and O26 (STEC and non-STEC), respectively, are presented. Faecal sample counts are shown as CFU/ml of pre-enriched broth sample, and carcass samples counts as CFU/ml of original swab sample.

		CT-SMAC[a]						CT-RMAC[b]					
				Percentile						Percentile			
Sample type	Colony type	n	Minimum	25th	50th	75th	Maximum	n	Minimum	25th	50th	75th	Maximum
On-farm	Fermenting	38	0	2050	4480	9200	200000	27	20	1375	3320	6620	24480
(faeces)	Non-fermenting		0	13	75	340	200000		0	105	300	1710	60480
On-plant	Fermenting	39	130	2920	8720	15840	200000	29	20	900	3440	5760	17280
(faeces)	Non-fermenting		0	0	10	135	110400		60	420	1060	10240	200000
Hide[c]	Fermenting	56	100	500	1100	3225	23000	31	0	200	400	950	5900
(carcass)	Non-fermenting		0	0	100	350	4000		0	100	300	800	14000
Pre-intervention[d]	Fermenting	8	0	0	100	125	200	11	0	0	0	50	200
(carcass)	Non-fermenting		0	0	0	0	100		0	0	0	0	100
Post-intervention[e]	Fermenting	2	0	50	100	150	200	5	0	0	0	0	200
(carcass)	Non-fermenting		0	0	0	0	0		0	0	100	100	400

[a] CT-SMAC = cefixime-tellurite sorbitol MacConkey culture plate.
[b] CT-RMAC = cefixime-tellurite rhamnose MacConkey culture plate.
[c] Sampling of hide (approximately 282 cm^2) in Y-cut region at pre-skinning.
[d] Sampling of opening Y-cut area and anal cavity (approximately 289 cm^2) of left half of carcass at post-evisceration but pre-intervention.
[e] Sampling of opening Y-cut area and anal cavity (approximately 289 cm^2) of right half of carcass at pre-boning but post-intervention.

Table 4.12: Minimum, maximum and quartiles for estimated concentrations of *E. coli* O157 and O26 (STEC and non-STEC) in recto-anal faecal samples (n) collected from very young calves on-farm and on-plant at pre-slaughter, and in carcass swab samples (n) at post-slaughter, stratified by detection method (direct culture plating, immunomagnetic separation (IMS), and most probable number analysis (MPN)). Only concentrations of samples real-time PCR-positive for *E. coli* O157 and O26 (STEC and non-STEC) are presented. Concentrations in faecal samples are shown as $\log_{10}$ CFU/g faeces collected and in carcass samples as CFU/cm^2 swabbed carcass surface; MPN values are presented as CFU/100 cm^2.

		O157						O26					
				Percentile						Percentile			
Sample type	Detection method	n	Minimum	25th	50th	75th	Maximum	n	Minimum	25th	50th	75th	Maximum
On-farm	Direct culture	6	3.4	4.7	5.3	5.8	6.2	7	4.0	5.9	6.0	6.5	6.6
(faeces)	IMS[a]	9	<3.3	<3.8	<4.6	<6.7	<7.4	11	<4.7	<5.1	<5.8	<6.5	<6.8
On-plant	Direct culture	4	4.7	5.5	6.0	6.3	6.5	5	5.2	5.7	6.1	6.2	6.4
(faeces)	IMS	6	<3.4	<3.4	<3.5	<4.4	<6.9	11	<3.9	<5.3	<5.6	<6.0	<7.0
Hide[b]	Direct culture	4	35.5	35.5	407.8	957.5	1489.4	5	106.4	106.4	141.8	354.6	1276.6
(carcass)	IMS	29	<35.5	<70.9	<177.3	<354.6	<4964.5	16	<34.6	<70.5	<140.1	<320.1	<553.6
	MPN	28	1.1	3.3	3.3	18.1	390.1	14	1.1	1.1	2.0	13.2	390.1
Pre-intervention[c]	Direct culture	3	34.6	69.2	177.3	354.6	4964.5	0	-	-	-	-	-
(carcass)	IMS	1	-	-	<69.2	-	-	2	-	-	<34.6	-	-
	MPN	7	1.0	1.0	1.3	1.3	14.9	7	1.0	1.0	1.3	5.6	8.0
Post-intervention[d]	Direct culture	0	-	-	-	-	-	0	-	-	-	-	-
(carcass)	IMS	0	-	-	-	-	-	0	-	-	-	-	-
	MPN	2	-	-	1.0	-	-	0	-	-	-	-	-

[a] Serogroup specific beads for *E. coli* O157 and O26 were used when *E. coli* concentrations were below the detection limits of direct culture plating. Hence, *E. coli* concentrations were below the presented values.

[b] Sampling of hide (approximately 282 cm^2) in Y-cut region at pre-skinning.

[c] Sampling of opening Y-cut area and anal cavity (approximately 289 cm^2) of left half of carcass at post-evisceration but pre-intervention.

[d] Sampling of opening Y-cut area and anal cavity (approximately 289 cm^2) of right half of carcass at pre-boning but post-intervention.

A wide range of concentrations was observed among hide and pre-intervention samples, reflecting the different detection limits of methods used. Only very low *E. coli* O157 concentrations of 1 CFU/100 cm^2 were estimated in two post-intervention samples. No *E. coli* O26 MPN results were available from four post-intervention samples tested TaqMan-positive at initial screening.

4.5 Discussion

To the best of our knowledge, this is the first study to investigate the effect of transportation and lairage on faecal shedding and carcass contamination of *E. coli* O157 and O26 (STEC and non-STEC) in very young calves. Although some samples could not be collected from calves due to financial restrictions, this study provided sufficient epidemiological data to highlight critical control points in the contamination of veal carcasses with *E. coli* O157 and O26 after transportation and lairage under New Zealand conditions. Having collected faecal samples from the first 10 animals in the order in which they could be approached or handled might have introduced a selection bias; more animals of ill health may have been included in the study, as they were easier to approach.

4.5.1 Effect of transportation and lairage on faecal shedding

A slight increase in real-time PCR-prevalence of faecal shedding of *E. coli* O157 or O26 was observed in calves after transportation and lairage at the slaughter plant (<24 h); however this increase in prevalence could not be confirmed by culture isolation. It is possible that some calves had ingested *E. coli* during transportation and lairage but either the pathogen did not reach the recto-anal junction to be detected at time of sample collection on-plant, or *E. coli* was shed in concentrations below the detection limits of the culture methods used. Calves exposed to STEC O157 can shed the pathogen within one day/24 h as has been described in other studies with three month-old bull calves (natural transmission) [397], calves of body weight 97.8 ± 16.6 (S.D.) kg (natural transmission via exposure to inoculated house flies) [398], or 6–9 month-old steers (oral inoculation of *E. coli*) [399].

Transportation has been identified as a potential stressor to induce faecal shedding of *Salmonella* spp. in feeder calves [400] and adult feedlot cattle [401], but could not be confirmed for *E. coli* O157 in the study by Barham *et al*.. It appears that transportation of

only a few hours has no or very little impact on the prevalence of faecal shedding of *E. coli* O157 in cattle. Minihan *et al*. [298] did not observe any increased prevalence of *E. coli* O157 in faeces from adult feedlot cattle after 1.5 h and 6 h transportation to slaughter plants in Ireland. Similarly, Bach *et al.* [299] reported no increased faecal shedding of STEC O157 in steer calves (220 ± 37 kg) after relocation to a feedlot in 3 h (short-haul transportation, with or without preconditioning of animals), in contrast to a 15 h transportation (long-haul, without preconditioning). Although no faecal samples were collected from calves on arrival at the slaughter plant in our study, one would suggest that transportation of <2 h is less likely to induce or increase the faecal shedding of *E. coli* in very young calves, but it cannot be ruled out.

E. coli O157 have been isolated from samples of lairage pen floors and rails [386-388]. It is possible that *E. coli* was shed with faeces from infected calves and transmitted to non-infected animals at lairage on-plant via pen floors and rails, the shared water trough, or via direct contact with neighbouring calves through rails of the pen's walls. Water troughs have been identified as environmental sources/reservoirs of *E. coli* infections for calves and cattle on farms [216, 402] and slaughter plants [386, 387]. In addition, grooming [399] and social activities of calves are other possible means of ingesting *E. coli*. Although the calves were kept at lairage for 17–20 h, this was probably not long enough to transmit an *E. coli* infection (requiring ingestion, gut passage and colonisation of the recto-anal junction) to exposed calves.

'Being positive on-farm' and 'high-risk farm' (for serogroup O157), and 'on-farm prevalence of *E. coli* O26' (for serogroup O26) were identified as risk factors for calves being real-time PCR-positive in faeces after transportation and lairage on-plant. It is important to note that this analysis has some limitations as cluster effects between animals and between samples of the same animal were not taken into account, and only on-farm related risk factors were assessed. Including this source of variation may have resulted in wider confidence intervals and larger p-values. However, the findings indicated that the real-time PCR-status of an animal on-plant was strongly associated with *E. coli* prevalence at the animal/farm level. Housing/farm management factors and age of calves have been identified as risk factors for increased prevalence of STEC O157 on dairy farms in previous studies [166, 378]. Cobbold and Desmarchelier [403] reported that higher prevalence of STEC in dairy calves was associated with group penning of animals, with pen floors and calf hides as important means

of horizontal STEC transmission between calves. These observations support our findings as it is general practice on New Zealand dairy farms to keep very young calves in groups in barns, pointing to husbandry as a critical control point to reduce prevalence of STEC at the farm and animal level. Hence, faecal shedding of *E. coli* in very young calves on-plant is less likely to be affected by transportation and lairage under New Zealand conditions, but more likely to be associated with farm-related factors.

4.5.2 Effect of transportation and lairage on hide contamination

Almost every hide sample was positive by real-time PCR or culture. As no comparable hide samples were collected from calves before and after transportation, it cannot be concluded that the large proportion of hide contamination was due to transportation and/or lairage. However, there was an indication of on-plant contamination of hides as some PFGE profiles of *E. coli* not detected in the on-farm samples appeared in both high- and low-risk groups on the same run; e.g. PFGE type 17 on the second run, and PFGE types 9 and 20 on the third run for *E. coli* O157; and similarly for *E. coli* O26 with PFGE types 11, 13, and 34 on the first run, and PFGE type 1 on the second run. Previous studies have identified the use of commercial transportation as a (borderline) significant risk factor for increased *E. coli* O157 cross-contamination of cattle hides at slaughter [300], and holding animals in *E. coli* O157-positive or faecally contaminated lairage pens have also been described as higher risks of hide contamination in finished beef cattle [302]. Similarly, Arthur *et al.* [301] reported that the transfer of STEC O157 onto cattle hides in the lairage area accounted for a larger proportion of hide and carcass contamination than the initial level of STEC O157 found on hides when cattle left a feedlot. Hence, a further study of similar design, which includes the collection of hide samples from calves before and after transportation, would be required to determine if transportation or lairage have a greater impact on the hide contamination of calves at slaughter under New Zealand conditions, or whether hide contamination occurs mainly on farms.

Based on PFGE profiles of *E. coli* isolates, a cross-contamination of hides with *E. coli* from high- to low-risk calves was observed (more eminent for *E. coli* O26 than O157), in addition to a very high level of cross-contamination with *E. coli* isolates from different origins. Although no environmental samples were collected for the purpose of comparison, the cross-contamination of hides with genotypes different to those isolated from faecal samples of high-

and low-risk calves was most likely attributed to residual faecal contamination of vehicles and lairage yards, as study animals were not mixed with calves from other farms. Hide contaminations via the environment were described by Childs *et al.* [386] when STEC O157 isolates from transport trailer side walls and pen side rails on-plant matched genotypes of isolates found on hides of feedlot cattle at slaughter. Arthur *et al*. [283] reported that only 29% of STEC O157 isolates detected on hides of feedlot cattle at post-slaughter matched genotypes collected from hides before transportation. Combined with our observations, this indicates that a large proportion of hide cross-contamination in cattle (adult and calves) is due to residual faecal contamination of the environment.

In addition to transportation and lairage, there are also animal-related factors affecting the prevalence of hide contamination in cattle. For example, the presence of supershedders ($>10^4$ CFU/g faeces) in a pen can significantly increase the prevalence of hide contamination within a pen of feedlot cattle as has been described in a longitudinal study by Arthur *et al*. [404]. Similarly, Jacob *et al*. [405] found a significant correlation between hide prevalence within truckloads (a cohort of adult cattle transported and lairaged together) and the presence of high shedders ($>5 \times 10^4$ CFU/g faeces) in truckloads, indicating that the levels of faecal *E. coli* shed during transportation and lairage also have an impact on the prevalence of hide contamination among slaughter cattle.

4.5.3 Carcass contamination at pre- and post-intervention

An increased contamination of carcasses with *E. coli* from high- to low-risk calves and possibly from the environment and other animals was observed at the pre-intervention stage in this study. Slaughter practices have a large effect on the microbiological contamination of red meat carcass [303], with hides recognised as the main source of carcass contamination with *E. coli* (and other pathogens of public health concern) at beef processing [286, 288, 289, 406]. Apart from the animal component, there are several plant-specific factors, which also have an effect on the extent of carcass contamination, such as plant design, processing speed, degree of good handling practices, and skills of slaughtermen [407, 408]. Hence, the carcass contamination results presented here are specific for this particular slaughter plant and may not be representative for veal processing plants across New Zealand.

The increased prevalence associated with high- to low-risk group cross-contamination of hides was not evident on carcasses at the pre-boning stage, with only two out of six swab samples being positive for STEC O157 or STEC O26 at post-intervention. It is noteworthy that the overall prevalence of cross-contaminated hides and carcasses at pre- and post-intervention stages might have been higher as 15 out of 60 post-slaughter samples were not collected on the first run (carcass missed or carcass identification tag lost in hot water shower). Nevertheless, this study provided evidence that the intervention applied was effective to eliminate/reduce *E. coli* contamination of carcasses, although this study was not designed to evaluate the efficacy of the hot water wash used on the slaughter plant. Extensive research in pre- and post-slaughter intervention strategies has been conducted and is still ongoing to develop novel methods to reduce the prevalence/level of *E. coli* in/on live cattle and processed carcasses ([409, 410], cited in [411]). Steam vacuum, acidification, and hot water wash are intervention methods currently used on New Zealand beef/veal slaughter plants.

4.5.4 Counts and concentrations of *E. coli*

Similarly to the Prevalence study (Chapter 3), counts and concentrations of *E. coli* O157 and O26 were determined to provide the New Zealand red meat industry with quantitative data on the levels of *E. coli* entering the food chain via slaughtered calves, which will assist in quantitative risk assessments and developments of intervention strategies associated with food safety management. It is important to note that due to financial restrictions, direct culture and MPN analysis were not applied on samples from low-risk calves to assess counts and concentrations of *E. coli* O26, hence *E. coli* O26 data presented might be under- or overestimated. As only very limited literature was found on counts and concentrations of *E. coli* in calves, where possible, our results were compared with findings from previous studies on adult cattle, acknowledging the various different factors affecting the levels of *E. coli* in calves compared to adult slaughter cattle.

As already discussed in detail in Chapter 3, some care is needed when using real-time PCR and culture isolation methods, as other factors might influence the sensitivity and specificity, e.g. the presence and/or growth of background bacteria in enrichments, PCR detection of DNA from dead *E. coli*, and primer cross-reactions with other *Escherichia* species; culturability of *E. coli*, specificity of immunomagnetic beads, and growth of background

bacteria on culture isolation plates. In addition to direct culture, MPN analysis was used to detect *E. coli* present at low levels in post-slaughter samples. This enumeration method, however, also has some caveats as described by Brichta-Harhay *et al*. [412]. For example, the included enrichment step enhances the growth of the target pathogen but also the competing background flora present in the sample, which likely hinders the detection of the pathogen. Furthermore, the method is quite time consuming and could provide inconsistent results [288].

As anticipated, the highest aerobic counts of samples real-time PCR-positive for *E. coli* O157 and O26 (STEC and non-STEC) were observed in pre-slaughter faecal samples on-farm and on-plant, closely followed by post-slaughter hide samples. The lowest counts were found in pre- and post-intervention samples, showing that good hygienic dressing practices are essential to prevent hide to carcass contaminations.

Based on direct culture, the median concentrations of *E. coli* O157 and O26 (STEC and non-STEC) in faecal samples (on-farm and on-plant) were consistent with *E. coli* results in calves from the nationwide prevalence study (Chapter 3) and findings from a previous survey of North American dairy farms [187]. Bosilevac *et al*. [413] tested 1,995 hide samples from cattle in a survey of seven beef processing plants across the US and observed hide contaminations with STEC O157 ranging from 40 to 4,000 CFU/100 cm^2. These values are much lower compared to our findings in calves but could be explained by not having enumerated the concentrations specific for STEC O157 but of *E. coli* O157 as a serogroup. At pre-intervention, median concentrations of both *E. coli* O157 and O26 were 1.3 MPN/100 cm^2, similar to *E. coli* O157 MPN levels of post-evisceration and post-intervention samples (<1.5 MPN/100 cm^2) collected from beef in a study by Arthur *et al*. [406]. Although *E. coli* O26 concentrations or MPN results of post-intervention samples were not available, by comparing counts for *E. coli* O157 and O26 in hide and pre-intervention samples, it could be assumed results for *E. coli* O26 to be within the range of those observed for *E. coli* O157.

Both the level and prevalence of faecal shedding and hide contamination with *E. coli* can increase the prevalence of contaminated carcasses post-slaughter. Fegan *et al*. [414] suggested that the presence of a supershedder with an *E. coli* O157 count of 7.5×10^5 MPN/g faeces might have caused cross-contamination of three adjacent carcasses either through direct contact or by cross-contamination through equipment or personnel, as all carcasses

contained *E. coli* O157 isolates of indistinguishable PFGE profiles. Similarly, Fox *et al*. [415] identified a significant association between the presence of a high-shedder (>5 × 10^4 CFU/g faeces) in a truckload of cattle and the probability of isolating *E. coli* O157 from an individual carcass at pre-evisceration. In another study by Fegan *et al*. [388], one of 90 feedlot cattle shed *E. coli* O157 at a similarly high concentration ($\log_{10}$ 5.66 MPN/g faeces) after transportation to a slaughter plant ($\log_{10}$ 2.66 MPN/g before transportation) and *E. coli* O157 was isolated from this carcass at post-slaughter ($\log_{10}$ < –2.42 MPN/cm^2). These observations provide supporting evidence that microbial contamination of carcasses at dressing is linked with the level of *E. coli* shed by individual cattle.

4.5.5 Dynamic changes of *E. coli* prevalence and indications of different epidemiology between *E. coli* serotypes

Over the pre-testing and study period, an increase of prevalence of *E. coli* O157 on one low-risk farm within a period of three weeks was observed; from 10% (1/10) at first pre-testing to 63% (5/8) in the study week (second run), which was also a much higher prevalence compared to the other two low-risk farms on the first and third run (10% (1/10) and 27% (3/11), respectively). It is possible the rapid increase of prevalence was due to an acute infection and spread of *E. coli* O157 among calves on the farm, which would be supported by the comparatively large number of *E. coli* O157 isolated from on-farm and on-plant samples of calves from this low-risk farm (Figure 4.3). As indicated by the PFGE cluster numbers, it is possible that this was caused by a single clone of *E. coli* O157 but no PFGE data from pre-testing samples were available to support this. In addition, it is interesting this was observed for *E. coli* serogroup O157 only, even though *E. coli* O26 was also prevalent on the farm; and it also occurred only on this farm in the study. It can be hypothesised that *E. coli* O157 and O26 strains prevalent on this farm have a different epidemiology, as has been described for *E. coli* O26 and O103 serogroups [416]. O'Reilly *et al*. [416] quantified the transmission dynamics of *E. coli* O26 and O103 in the Scottish cattle population using modelling methods on available prevalence data. Their dynamic models revealed that these two serogroups have distinct transmission dynamics with different epidemiology and ecology.

A further indication of different epidemiology of *E. coli* O157 and O26 serogroups was observed on the third run of this study. Although real-time PCR prevalences of *E. coli* O157 and O26 in high-risk calves were high on-farm (O157: 81.8% (9/11), O26: 72.7% (8/11)) and

on-plant (O157: 81.8% (9/11), O26: 90.9% (10/11)), only *E. coli* O157 but no *E. coli* O26 were isolated. It is unlikely this was due to factors associated with culture isolation as *E. coli* O26 isolates were recovered from hide samples (but of different origins), but more plausibly an indication of differences in epidemiology of these two important *E. coli* serogroups.

4.6 Conclusions

Although the number of selected farms and calves was relatively small, this study provides evidence that transportation and lairage under New Zealand conditions is not associated with increased faecal shedding of *E. coli* O157 and O26 in calves at slaughter, but associated with increased cross-contamination of hides and carcasses with *E. coli* O26 from high- to low-risk calves and *E. coli* strains from undetermined sources. The study did not demonstrate an increased prevalence of contaminated carcasses through to the boning room, highlighting the need of good hygienic dressing practices and effective antimicrobial interventions during meat processing.

4.7 Acknowledgements

We thank all participating dairy farmers and livestock transport companies, as well as the slaughter plant manager and staff for their great collaboration and participation in this study; the Food Microbiology and Safety Team from AgResearch (Ruakura, Hamilton, New Zealand) for collection and processing of post-slaughter samples; John Mills (AgResearch, Ruakura) for his assistance with MPN calculations; Angie Reynolds (^{m}EpiLab, Hopkirk Research Institute, Massey University, Palmerston North, New Zealand) for her great assistance with processing pre-slaughter samples and coordination of the laboratory workload; and all other laboratory staff and students from ^{m}EpiLab for providing their help when needed.

As shown in the cohort study, carcasses can become faecally contaminated with STEC during meat processing and be a significant public health risk to consumers of red meat. The following chapter describes a prospective case-control study to gain epidemiological knowledge on the source and exposure pathways for sporadic STEC infections in New Zealand. The chapter has been published as a Research Article:

Jaros P, *et al.* (2013) A prospective case-control and molecular epidemiological study of human cases of Shiga toxin-producing *Escherichia coli* in New Zealand. *BMC Infectious Diseases* 13(1):450.

The full, published article is reproduced in Appendix 5.

Chapter 5

A prospective case-control and molecular epidemiological study of human cases of Shiga toxin-producing *Escherichia coli* in New Zealand

5.1 Abstract

Background

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 and related non-O157 STEC strains are enteric pathogens of public health concern worldwide, causing life-threatening diseases. Cattle are considered the principal hosts and have been shown to be a source of infection for both foodborne and environmental outbreaks in humans. The aims of this study were to evaluate possible risk factors for association with sporadic STEC infections in humans in New Zealand and to identify source and exposure pathways.

Methods

Any confirmed case of STEC infection notified to regional public health units from July 2011 to July 2012 and a random selection of controls from the New Zealand population were contacted by phone to participate in a national prospective case-control study. Data on risk factors for STEC infection were collected using a standardised questionnaire and evaluated by multivariate logistic regression analysis. Isolates from each case were subtyped using pulsed-field gel electrophoresis (PFGE) and Shiga toxin-encoding bacteriophage insertion (SBI) typing. Permutational multivariate analysis of variance was used to estimate the proportional contribution of exposure variables to the molecular variation of isolates.

Results

A total of 113 eligible cases and 506 controls were included in the study. Statistically significant animal and environmental risk factors for human STEC infections were identified, notably 'Cattle livestock present in meshblock' (the smallest geographical unit) (odds ratio (OR) 1.89, 95% CI 1.04–3.42), 'Contact with animal manure' (OR 2.09, 95% CI 1.12–3.90), and 'Contact with recreational waters' (OR 2.95, 95% CI 1.30–6.70). No food-associated risk

factors were identified as sources of STEC infection. *E. coli* O157:H7 caused 100/113 (88.5%) of clinical STEC infections in this study, and 97/100 isolates were available for molecular analysis. PFGE profiles of isolates revealed three distinctive clusters of genotypes, and these were strongly correlated with SBI type. The variable 'Island of residence' (North or South Island of New Zealand) was significantly associated with PFGE genotype ($p = 0.012$).

Conclusions

Our findings implicate environmental and animal contact, but not food, as significant exposure pathways for sporadic STEC infections in humans in New Zealand. Risk factors associated with beef and dairy cattle suggest that ruminants are the most important sources of STEC infection. Notably, outbreaks of STEC infections are rare in New Zealand and this further suggests that food is not a significant exposure pathway.

5.2 Introduction

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 and related non-O157 STEC strains are pathogens of public health concern worldwide. They can cause severe outbreaks of gastrointestinal illness with clinical symptoms ranging from diarrhoea and haemorrhagic colitis to the life-threatening haemolytic uraemic syndrome [4]. Ruminants, particularly cattle, are considered to be an important reservoir of STEC, shedding the pathogen via faeces [5-7], and are a primary source of foodborne and environmental outbreaks of STEC in humans [35, 36].

Food products of animal and plant origin have been confirmed as vehicles of disease transmission in case-control studies of STEC outbreaks and sporadic STEC infections; these included raw milk [8, 9, 38], unpasteurised cheese [10], undercooked hamburgers [24-26], sausages [22, 23], leafy lettuce [16] and unpasteurised apple cider [20]. Implicated food vehicles were most commonly contaminated directly or indirectly with ruminant faeces containing STEC before or after processing. Similarly, faecally contaminated recreational waters and water supplies have been identified as environmental sources of human STEC infections [102-104]. Exposures to farming environments have been reported as risk factors of sporadic STEC infections, particularly for young children [37, 38].

Improvements in surveillance of STEC infections, following their recognition as serious public health concerns, have resulted in an overall increasing trend of STEC notifications at the international level. Over the past decade, non-O157 STEC cases have been reported more frequently in the US and the EU [33, 69], a feature, which might be attributed to improved laboratory methods for isolation of non-O157 STEC serotypes and additional laboratory testing of specimens for non-O157 STECs.

Since 1993, when New Zealand's first case of STEC infection in humans was reported [314], the annual number of notified STEC cases has increased steadily. In 2012, 147 cases of STEC (3.3 cases per 100,000 population) were recorded in the national surveillance database (EpiSurv) used by regional public health units (PHU) to record epidemiological data from notified cases of communicable and other diseases [94]. All but five cases were confirmed by culture isolation and identified as STEC serotypes O157:H7 (83.8%) and non-O157 (16.2%). Although the majority of reported STEC cases in New Zealand are caused by serotype O157:H7, the percentage of non-O157 STEC cases has increased steadily over the past five years from 1.6% in 2008 to 16.2% in 2012.

While STEC infections in New Zealand appear as sporadic cases or small clusters, little is known about the relative importance of cattle as a reservoir, or the relative contribution of different exposure pathways to human cases of STEC.

The objectives of this study were to evaluate possible risk factors for association with sporadic STEC infections acquired in New Zealand and to identify source and exposure pathways for this disease.

5.3 Methods

5.3.1 Study design, definition of cases and controls

A national prospective case-control study was conducted in New Zealand from 18th July 2011 to 31st July 2012. A case was defined as a patient with (i) clinical symptoms of diarrhoea and/or haemolytic uraemic syndrome and/or thrombotic thrombocytopaenia purpura, (ii) an onset of clinical disease at a maximum of two weeks prior to being reported to a PHU, (iii) an infection most likely acquired in New Zealand, (iv) confirmed by isolation of STEC from a

clinical specimen, and (v) the primary STEC infection in a household. Study cases were interviewed by phone or in person by trained PHU staff using a standardised questionnaire on multiple risk factors potentially associated with STEC infections. STEC cases were excluded from the study if they did not fit the case definition described above, refused to participate in the study, or were unable to be interviewed (e.g. due to severity of illness).

Study controls, intended to be representative of the national demography, were selected from the New Zealand population using random landline dialling from the New Zealand phone directory. Monthly quotas of controls were recruited by a professional survey provider (UMR Research, Wellington, New Zealand) contacting households in the third week of every month. In each household, the individual with the last birthday was chosen as the study participant. If the person was not available, a time was arranged to call back for the interview. An eligible control had to be free of symptoms of diarrhoea or any other gastrointestinal disease at the time of interview, and in the two weeks prior to the interview. A computer-assisted telephone interview was conducted by a trained team of assigned interviewers using the same questionnaire as that used for cases.

Informed consent was obtained from all study participants before being interviewed. For study cases and controls aged <18 years, a parent or adult caregiver served as the interview respondent after their consent was acquired.

5.3.2 Questionnaire

A standardised questionnaire (Appendix 6) was used to collect data from study cases and controls concerning potential risk factors for infection in the two weeks before onset of disease (cases) and the telephone interview (controls). The questionnaire covered demographic characteristics and exposure categories such as food consumed (treated/raw milk and products thereof, various raw/pink meats, fish, raw fruit and vegetables, and purchased fruit juices), dining locations, supply of drinking water (town supply, private bore, roof run-off, creek, tanker truck), contact with recreational waters, hunting activities, contacts with animals and humans, recent travels, and medications taken (antibiotic and antacid). To investigate the spatial distribution of study participants, while protecting privacy, cases and controls were asked to name the nearest school to their home to assign their geographical

locality. The month and year of interview was recorded to investigate the seasonality of disease. The questionnaire was cognitive and pilot tested.

5.3.3 Sample sizes of cases and controls

Based on a predicted sample size of 150–170 cases (expected number of cases based on STEC cases notified nationally in two preceding years) and an attempted case to control ratio of 1:3, it was proposed to recruit a total of 450–510 controls. Epi Info™ software [390] was used to calculate the sample size for cases and to perform power calculations for three different expected frequencies of exposure among controls. There was sufficient statistical power (at least 80%) at a confidence level of 95% to detect an odds ratio of 3.0, using 5%, 20% and 80% as the expected frequencies of exposure among controls. Hence, the sample size of controls was set at 506 and included oversampling of children 0–4 years of age (n = 200) to provide a similar predicted ratio of cases and controls (1:3) as this age group showed the highest number of reported STEC cases in the past. A monthly quota of 42 controls was interviewed by the survey provider.

5.3.4 STEC isolates of study cases

Available STEC isolates of confirmed clinical cases, including data on serotype, presence of virulence genes (*ehx*A, *eae*, *stx*1, *stx*2) and pulsed-field gel electrophoresis (PFGE, restriction enzyme *Xba*I) profiles, were obtained from the Enteric Reference Laboratory (ERL, Institute of Environmental Science & Research Ltd, Upper Hutt, New Zealand). Each isolate was further screened for the presence of virulence gene subtype *stx*2c at the ^{m}EpiLab as described in Chapter 4 under *Laboratory methods*, and submitted to Washington State University, Pullman, USA, for Shiga toxin (Stx)-encoding bacteriophage insertion (SBI) typing [267, 269].

5.3.5 Ethical approval

This study was approved by the Multi-region Ethics Committee, Wellington, New Zealand, on 17 June 2011, reference number MEC/11/04/043.

5.3.6 Data management and statistical analysis

R software (version 2.15.2) [345] was used for all statistical analysis, with significance set at $p < 0.05$. Datasets of cases and controls were screened for completeness prior to analysis. Descriptive statistics were calculated for each study group. To account for potential confounding from imperfect frequency matching on age, the variable 'Age' was categorised by grouping 'pre-school children' (0–4 years), 'children/students' (5–19 years), and 'adults' (>19 years).

To illustrate the spatial distribution of study participants, New Zealand Transverse Mercator coordinates (NZTM2000) of named schools were plotted, using R packages 'maptools' [349] and 'spatstat' [350]. Based on the spatial distribution of cases and controls, a relative risk surface of STEC cases for New Zealand was produced, using R package 'sparr' [351]. To account for spatial heterogeneity, an adaptive estimate was utilised for case and control densities with an average smoothing bandwidth of 50 km. Areas with values >0.0 indicate increased relative risks of STEC infection. For comparison, cattle densities were mapped by regions of New Zealand; using the sum of beef and dairy cattle numbers from 2011 [338] divided by the area (km^2) of each region.

In addition to the data generated by the case-control questionnaires, information on ruminant livestock numbers from a national livestock database [417] was used in two separate analyses. Firstly, additional variables were generated, representing whether particular species of livestock (dairy cattle, beef cattle, sheep, and deer) were farmed in meshblocks (the smallest geographic unit of statistical data collected for Statistics New Zealand) in which the cases and controls resided. These additional variables (presence/absence, numbers and density of each species) were used in the logistic regression analysis of the case-control dataset.

Secondly, in order to extend the analysis of the relationship between ruminant livestock (dairy cattle, beef cattle, sheep, and deer) and the risk of STEC, a separate logistic regression analysis was conducted at the meshblock level. The relationship between ruminant livestock (presence/absence, numbers and density of each species) and the risk of STEC notification in all meshblocks of New Zealand was assessed. In essence, this analysis used the cases from the case-control study, but extended the control set to consider the entire population of New Zealand.

For both logistic regression analyses, ruminant livestock data from 2009 were used as they represented the most reliable recent data that could be linked to geographical boundaries (meshblocks) and the most recent human census data. The last population census (2006) estimated a national human population of 4,027,527.

Multivariate logistic regression model building

Questionnaire answers of "unknown" or "not sure" were treated as missing values of the exposure variables. Exposure variables were analysed using univariate and multivariate logistic regression to identify risk factors associated with sporadic STEC cases. Exposure variables with Wald test or Likelihood ratio tests p-values <0.20 in univariate analysis were tested for correlation, and included in an initial multivariate model if their correlation values were < |0.30|.

To generate a preliminary multivariate model, stepwise backward- and forward-elimination of least significant variables and those with correlation values of ≥ |0.30|, respectively, was used, while eliminated variables were assessed for confounding. The confounding effect was determined by a change of >30% in a variable coefficient in the model after another variable was dropped from or added to the model. Variables with confounding effect were retained in the model even if they were non-significant. Biologically plausible interactions between variables were assessed to generate the final multivariate model.

To adjust for a proportion of missing values in relevant variables such as 'Contact with animal manure' (cases: n = 13, controls: n = 7), 'Contact with children wearing nappies' (cases: n = 6, controls: n = 4), and 'Contact with person vomiting/ having gastrointestinal disease' (cases: n = 10, controls: n = 16), multiple imputations by chained equations [418] were applied on the final multivariate model using R package 'mice' [419]. Likelihood ratio tests and the le Cessie-van Houwelingen normal test statistics [352] were applied to evaluate the model's significance and goodness-of-fit, respectively, using R package 'rms'[353]. Models were compared using the Akaike information criterion (AIC), a measure of the relative goodness of fit. Odds ratios of model variables including 95% confidence intervals were calculated in Microsoft Excel 2010.

For the extended analysis of the relationship between ruminant livestock and the risk of STEC at the meshblock level, a second multivariate logistic regression model was built. In this analysis the number of STEC cases out of the population in each meshblock was the outcome variable (a two-column vector of the number of cases out of the population in each meshblock) and variables representing each ruminant species per meshblock were considered as exposure variables (presence/absence, numbers and density).

Population attributable fractions (PAF)

To assess the proportion of sporadic STEC disease in the study population attributable to a specific exposure, the variable's population attributable fraction (PAF_i) was computed. PAF of variables associated with increased risk of STEC infection were estimated using the following formula [420, 421]:

$$PAF_i = \frac{p_i(aOR_i - 1)}{aOR_i} \times 100\%$$

where p_i is the proportion of all study cases within a categorical variable and a reference category denoted by $i = 1$, and aOR_i is the adjusted variable-specific odds ratio derived from the final multivariate model. Medians and 95% credible intervals were computed from 1,000 simulations as described in Stafford *et al.* [420].

Molecular analysis of *E. coli* O157:H7 isolates

PFGE profiles of the clinical *E. coli* O157:H7 isolates were analysed and compared using BioNumerics software (version 6.6) [394] to create a dendrogram applying UPGMA (unweighted pair group method with arithmetic mean) cluster analysis using the Dice similarity coefficient, with a band matching tolerance of 1%.

Fisher's exact test was used to evaluate the association between SBI genotypes and exposure variables considered in the multivariate logistic regression analysis. A distance matrix of isolates' PFGE profiles was generated in BioNumerics and each isolate was linked with exposure variables, to analyse the molecular relatedness of isolates and estimate the proportional contribution of these variables to the molecular variation. Multi-dimensional

scaling plots and permutational multivariate analysis of variance (PERMANOVA+, version 1.0.4) were used for this analysis using Primer 6 (version 6.1.14) [422].

5.4 Results

5.4.1 Study population, spatial and temporal epidemiology

A total of 123 STEC cases meeting the case definition were notified to PHUs during the study period. Eight cases refused to participate in the study, thus resulting in a 93.5% response rate. Two potential cases were excluded, one due to a high probability of having acquired the infection overseas and the other due to severe illness. Therefore, 113 STEC cases were included in this study, of which 75 (66.4%) were interviewed by phone and 35 (31.0%) were visited by PHU staff; the interviewing methods of three cases were unknown. The majority of STEC cases (83.2%, 94/113) were interviewed within 0–5 days of notification (12 cases within 6–9 days, four cases within 10–12 days, and three cases within 21 days). Of these 113 STEC cases, 100 (88.5%) were *E. coli* O157:H7 and 13 (11.5%) were non-O157 STEC.

To recruit 506 controls, including 200 0–4 year-old children, a total of 7,864 phone calls were made. Contact was established for 66.8% (5,254/7,864) of phone calls and of those contacted, 62.2% (3,266/5,254) were interested in participating in the study (response rate). From 3,266 interested respondents, 84.5% (2,760/3,266) were not eligible mainly because of not fitting the required monthly quota of 0–4 year-olds (93.6% (2,583/2,760)), or not meeting the selection criteria.

Males comprised 52.2% (59/113) of cases and 42.9% (217/506) of controls. The median age of cases and controls were 7.0 years (interquartile range (IQR), 2.0–29.0) and 11.5 years (IQR 3.0–58.0), respectively. The age and spatial distribution of cases and controls are shown in Figure 5.1.

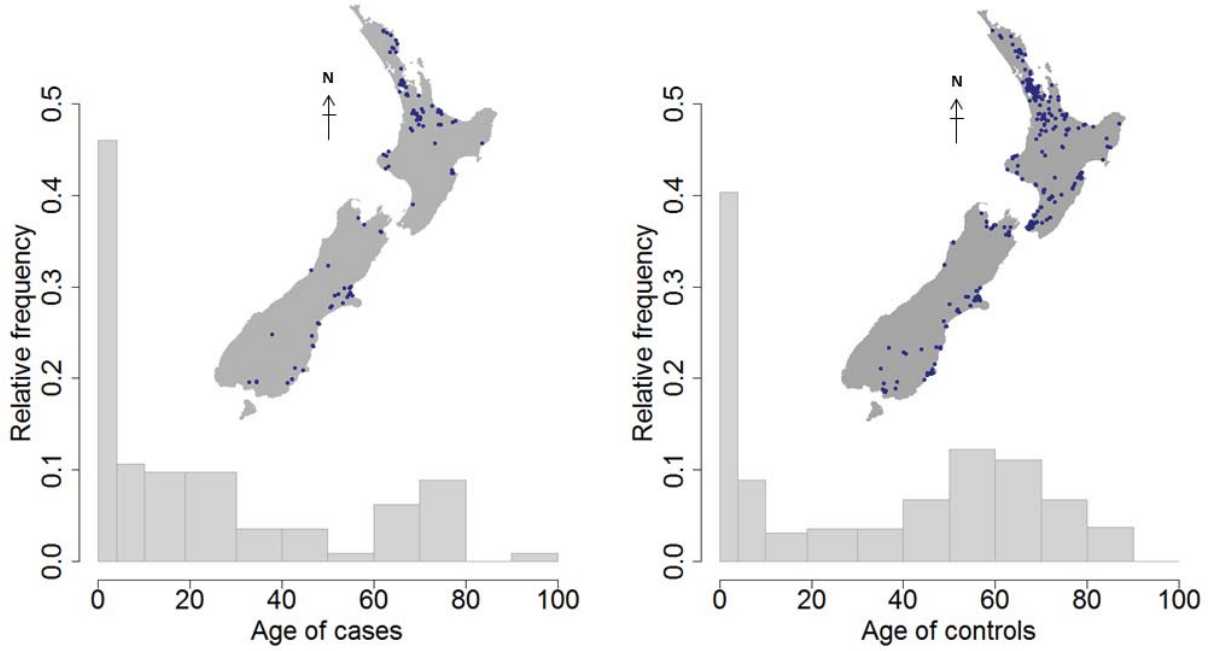

Figure 5.1: Age and spatial distribution of STEC cases (n = 113) and controls (n = 506) across New Zealand.

The proportional distributions of participants stratified by age categories were: 46.0% cases (52/113) and 40.3% controls (204/506) for 0–4 year-old pre-school children; 20.4% cases (23/113) and 12.1% controls (61/506) for 5–19 year-old children/students; and 33.6% cases (38/113) and 47.6% controls (241/506) for >19 year-old adults.

The temporal distribution of cases during the study period showed a peak in summer/autumn (January until April), with no cases reported in July 2011 (Figure 5.2).

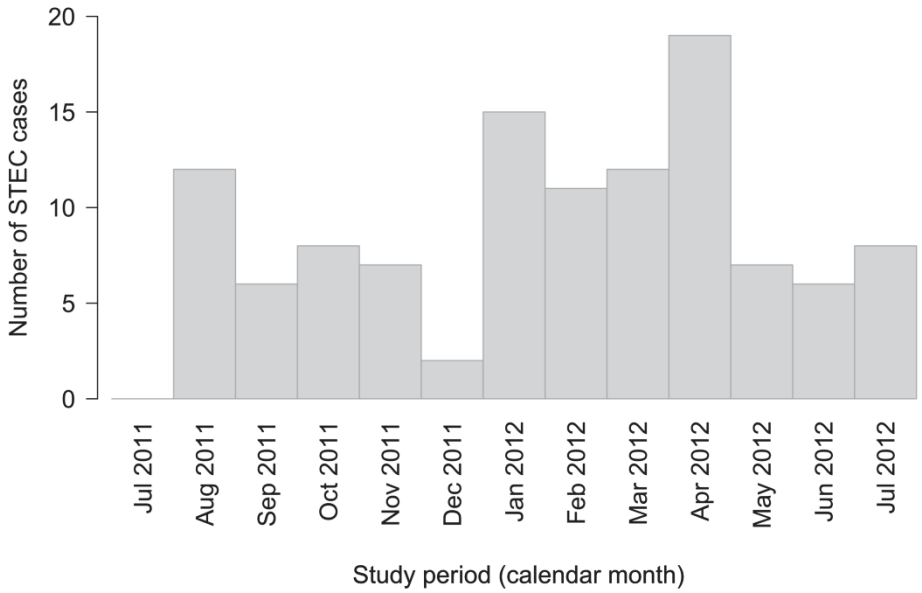

Figure 5.2: Temporal distribution of sporadic STEC cases (n = 113) from July 2011 to July 2012.

Based on the spatial distribution of cases and controls across New Zealand (Figure 5.1), increased relative risk estimates of STEC infections were observed in regions such as Northland, Waikato, Bay of Plenty, Taranaki, Canterbury, and Southland, while reduced risks were found in high density urban areas in the Auckland and Wellington regions (Figure 5.3, A). For comparison, areas with high ruminant livestock densities are shown in Figure 5.3, B and Appendix 7.

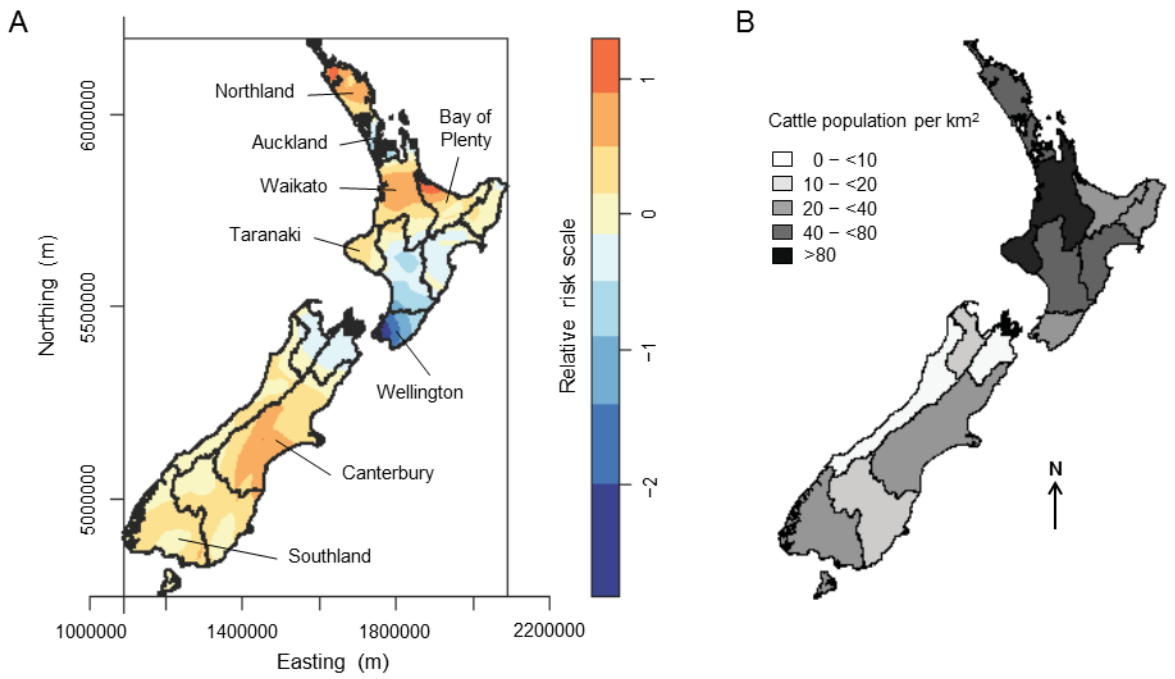

Figure 5.3: (A) Relative risk estimates of sporadic STEC infection across New Zealand and (B) cattle density from 2011. (A) The bivariate kernel density plot shows estimates of relative risks of STEC infection across regions in New Zealand. Values >0 on the relative risk scale indicate increased risk of infection. (B) Total cattle density (dairy and beef animals/km^2) from 2011 is shown to indicate areas with high cattle densities. Similar plots of the densities of dairy, beef, sheep, and deer are provided in the supplementary material (Appendix 7).

5.4.2 Risk factors

Univariate and bivariate logistic regression results (adjusted for age categories) are provided in the supplementary material (Appendix 8 and Appendix 9, respectively). Statistically significant risk factors of the final multivariate logistic regression model with imputations, including confounding variables ('Eating seafood', 'Dining outside home', 'Water supply to home from private bore/ spring/ creek/ or stream', 'Contact with children wearing nappies', and 'Taking antacids') are presented in Table 5.1. The equivalent final model without imputation is provided in the supplementary material (Appendix 10).

Table 5.1: Multivariate logistic regression model showing risk factors for sporadic cases of STEC infections in New Zealand in a prospective case-control study (113 study cases and 506 controls).

Variable	Coefficient (SE)[a]	Adjusted odds ratio (95% CI)[b]	p-value[c]
Other household member having contact with animals other than household pets[d]*			
for 0–4 year-old	1.39 (0.42)	4.03 (1.78–9.13)	0.001
for 5–19 year-old	–0.77 (0.67)	0.47 (0.13–1.72)	0.251
for >19 year-old	0.30 (0.49)	1.35 (0.51–3.56)	0.541
Cattle livestock present in meshblock	0.64 (0.30)	1.89 (1.04–3.42)	0.037
Contact with animal manure	0.74 (0.32)	2.09 (1.12–3.90)	0.021
Contact with recreational waters	1.08 (0.42)	2.95 (1.30–6.70)	0.010
Travelled to areas in New Zealand with interrupted or no main water supply	0.89 (0.43)	2.43 (1.04–5.65)	0.040
Handling raw offal	–0.94 (0.35)	0.39 (0.20–0.78)	0.008
Drinking refrigerated fruit juice from supermarket	–1.37 (0.32)	0.25 (0.14–0.47)	<0.001
Visiting childcare/kindergarten/ or school	–0.92 (0.31)	0.40 (0.22–0.73)	0.003
Eating raw vegetables	–0.65 (0.33)	0.52 (0.27–0.99)	0.046
Eating seafood	–0.52 (0.27)	0.59 (0.35–1.02)	0.057
Dining outside home	–0.44 (0.29)	0.65 (0.37–1.14)	0.133
Water supply to home from private bore/spring/ creek/ or stream	0.62 (0.39)	1.85 (0.86–4.00)	0.117
Contact with children wearing nappies	–0.33 (0.31)	0.72 (0.39–1.33)	0.298
Taking antacids	–0.84 (0.58)	0.43 (0.14–1.34)	0.147

Likelihood ratio test = 153.70 (df=18, p <0.001)

[a] Standard error.

[b] 95% confidence interval.

[c] p-values were computed based on 50 imputations.

[d] This variable was modelled using a multiplicative interaction term comprising the variables 'Other household member having contact with animals other than household pets' and 'Age'.
It can be interpreted as follows: a child 0–4 years of age is at significantly higher risk of being an STEC case, if another household member had contact with animals other than household pets, compared to a child of the same age without this risk factor.

* p-value = 0.064 for the variable 'Other household member having contact with animals other than household pets' without the interaction term.

Animal and environmental exposures were identified as risk factors for sporadic STEC infections including; 'Other household member having contact with animals other than household pets' for pre-school children aged 0–4, 'Cattle livestock present in meshblock', 'Contact with animal manure', 'Contact with recreational waters', and 'Travelled to areas in New Zealand with interrupted or no main water supply'. Food items such as 'Drinking refrigerated fruit juice from supermarket' and 'Eating raw vegetables' were identified as

having a protective effect rather than being risk factors for STEC infections. When the final multivariate logistic regression model was applied to *E. coli* O157:H7 cases only, the strength of associations and significance of variables remained relatively unchanged (data not shown), except for the variable 'Contact with recreational waters', which became non-significant (adjusted odds ratio 2.13, 95% CI 0.84–5.42, p = 0.112). This could be explained by a higher percentage of non-O157 cases (30.8%, 4/13) being exposed to this risk factor compared to O157 cases (11.0%, 11/100).

The final multivariate logistic regression model was also applied to cases only contacted by phone as 35/113 cases (31.0%) had home visits for interviews, which was considered as possible source of bias. The changes of strength of associations and significance of variables are presented in Table 5.2.

Table 5.2: Multivariate logistic regression model showing risk factors for sporadic cases of STEC infections in New Zealand based on phone interviews of 75 study cases and 506 controls.

Variable	Coefficient (SE)[a]	Adjusted odds ratio (95% CI)[b]	p-value[c]
Other household member having contact with animals other than household pets[d]*			
for 0–4 year-old	1.66 (0.47)	5.24 (2.09–13.16)	<0.001
for 5–19 year-old	–1.52 (0.94)	0.22 (0.03–0.36)	0.104
for >19 year-old	0.51 (0.61)	1.66 (0.50–5.51)	0.410
Cattle livestock present in meshblock	0.98 (0.36)	2.66 (1.32–5.39)	0.007
Contact with animal manure	0.52 (0.39)	1.68 (0.78–3.62)	0.186
Contact with recreational waters	1.15 (0.50)	3.16 (1.19–8.36)	0.021
Travelled to areas in New Zealand with interrupted or no main water supply	0.48 (0.59)	1.61 (0.51–5.09)	0.420
Handling raw offal	–0.90 (0.45)	0.40 (0.17–0.99)	0.047
Drinking refrigerated fruit juice from supermarket	–1.35 (0.39)	0.26 (0.12–0.55)	0.001
Visiting childcare/kindergarten/ or school	–0.76 (0.36)	0.47 (0.23–0.95)	0.036
Eating raw vegetables	–0.61 (0.39)	0.54 (0.25–1.16)	0.116
Eating seafood	–0.77 (0.34)	0.46 (0.24–0.91)	0.025
Dining outside home	–0.69 (0.34)	0.50 (0.26–0.97)	0.042
Water supply to home from private bore/spring/ creek/ or stream	0.89 (0.44)	2.42 (1.03–5.71)	0.043
Contact with children wearing nappies	–0.38 (0.39)	0.68 (0.32–1.46)	0.324
Taking antacids	–1.05 (0.79)	0.35 (0.08–1.64)	0.184

Likelihood ratio test = 139.61 (df=18, p <0.001)

[a] Standard error.

[b] 95% confidence interval.

[c] p-values were computed based on 50 imputations.

[d] This variable was modelled using a multiplicative interaction term comprising the variables 'Other household member having contact with animals other than household pets' and 'Age'.
It can be interpreted as follows: a child 0–4 years of age is at significantly higher risk of being an STEC case, if another household member had contact with animals other than household pets, compared to a child of the same age without this risk factor.

* p-value = 0.041 for the variable 'Other household member having contact with animals other than household pets' without the interaction term.

Variables such as 'Contact with animal manure', 'Travelled to areas in New Zealand with interrupted or no main water supply' and 'Eating raw vegetables' became non-significant, which could be explained by a higher percentage of home-visited cases being exposed to these risk factors compared to cases contacted by phone (contact with animal manure: 37.9% (11/29; 6 unknown) vs. 25.0% (17/68; 7 unknown); travelled to areas in New Zealand with interrupted or no main water supply: 17.6% (6/34; 1 unknown) vs. 9.5% (7/74; 1 unknown)).

In contrast, the confounding variables ‘Dining outside home’ and ‘Water supply to home from private bore/spring/ creek/ or stream’ became significant; a lower percentage of home-visited cases (14.3%, 5/35) compared to cases contacted by phone (23.3%, 17/73; 2 unknown) would explain the change.

For the multivariate analysis considering cases and the whole population at the meshblock level, the univariate logistic model identified significant associations between STEC and dairy cattle, beef cattle, and sheep, where variables with different functional forms were considered including presence/absence, numbers of animals and densities per km^2. The best fitting variables, in terms of AIC, were presence/absence of cattle and sheep. These included presence of beef cattle (odds ratio (OR) 2.45, 95% CI 1.65–3.59, Wald test p-value <0.001, AIC = 1556.9), presence of dairy cattle (OR 2.14, 95% CI 1.27–3.42, p = 0.003, AIC = 1567.5), presence of all cattle (OR 2.40, 95% CI 1.62–3.52, p <0.001, AIC = 1557.6), and presence of sheep (OR 1.98, 95% CI 1.29–2.97, p = 0.001, AIC = 1565.7). When considered in multivariate models, there was strong confounding and collinearity between these variables but only species combinations of beef cattle with dairy cattle (Likelihood ratio test p-value <0.001, AIC = 1558.9), and dairy cattle with sheep (p = 0.005, AIC = 1566.6) provided biologically meaningful results. According to lowest AIC, the variable presence of beef cattle fitted the best.

5.4.3 Population attributable fractions (PAF)

PAF of exposure variables associated with increased risk for sporadic STEC infections (Table 5.1) are summarised in Table 5.3.

Table 5.3: Population attributable fractions (PAF) of identified risk factors. PAF (in %) with 95% credible intervals (CrI in %) are shown for exposures associated with increased risks for sporadic STEC infections in humans in New Zealand.

Variable	Cases (n)	Proportion of cases (p)	Adjusted odds ratio[a]	PAF (95% CrI)[b]
For children 0-4 years old: Other household member having contact with animals other than household pets				
No	28	0.549	Ref	-
Yes	23	0.451	4.03	16.82 (9.0–23.7)
Cattle livestock present in meshblock				
No	74	0.655	Ref	-
Yes	39	0.345	1.89	18.20 (0.6–29.4)
Contact with animal manure				
No	66	0.660	Ref	-
Yes	34	0.340	2.09	17.47 (4.4–27.7)
Contact with recreational waters				
No	97	0.866	Ref	-
Yes	15	0.134	2.95	9.41 (2.7–16.5)
Travelled to areas in New Zealand with interrupted or no main water supply				
No	96	0.865	Ref	-
Yes	15	0.135	2.43	8.17 (0.7–15.7)
Water supply to home from private bore/spring/creek/ or stream				
No	89	0.802	Ref	-
Yes	22	0.198	1.85	9.46 (–2.5–18.8)

[a] Adjusted odds ratios were derived from multivariate logistic regression analysis model using multiple imputations by chained equations (Table 5.1).

[b] PAF and 95% CrI were computed based on 1,000 simulations.

Ref = reference level for comparison.

The interaction term 'Other household member having contact with animals other than household pets' for 0–4 year-old children, 'Cattle livestock present in meshblock' and 'Contact with animal manure' showed the highest estimated proportions that could be attributed to STEC infections in the study population.

5.4.4 Molecular analysis of *E. coli* O157:H7 isolates

E. coli O157:H7 and non-O157 STECs, as confirmed by isolation, caused 100/113 (88.5%) and 13/113 (11.5%) of the STEC infections, respectively. The non-O157 STECs were of serogroups O26, O84, O103, O123, O176, O180, and ONT (O serogroup not typable). Only 97/100 O157:H7 isolates and their PFGE profiles were available for molecular analysis; PFGE profiles of non-O157 STEC isolates were not available. The most frequent SBI types of *E. coli* O157:H7 isolates were 1 (55/97, 56.7%), 3 (17/97, 17.5%), and 5 (20/97, 20.6%); equivalent to SBI genotypes AY2, WY12, and ASY2c/ASWY2c/SY2c, respectively, according to the recently proposed coding system by Shringi *et al.* [269]. All isolates of SBI type 1 (AY2) carried the *stx*2a gene, while all SBI type 3 (WY12) had both the *stx*2a and *stx*1 genes; all SBI type 5 (ASY2c/SY2c) contained only the *stx*2c gene.

PFGE profiles of the 97 human *E. coli* O157:H7 isolates were compared (Figure 5.4).

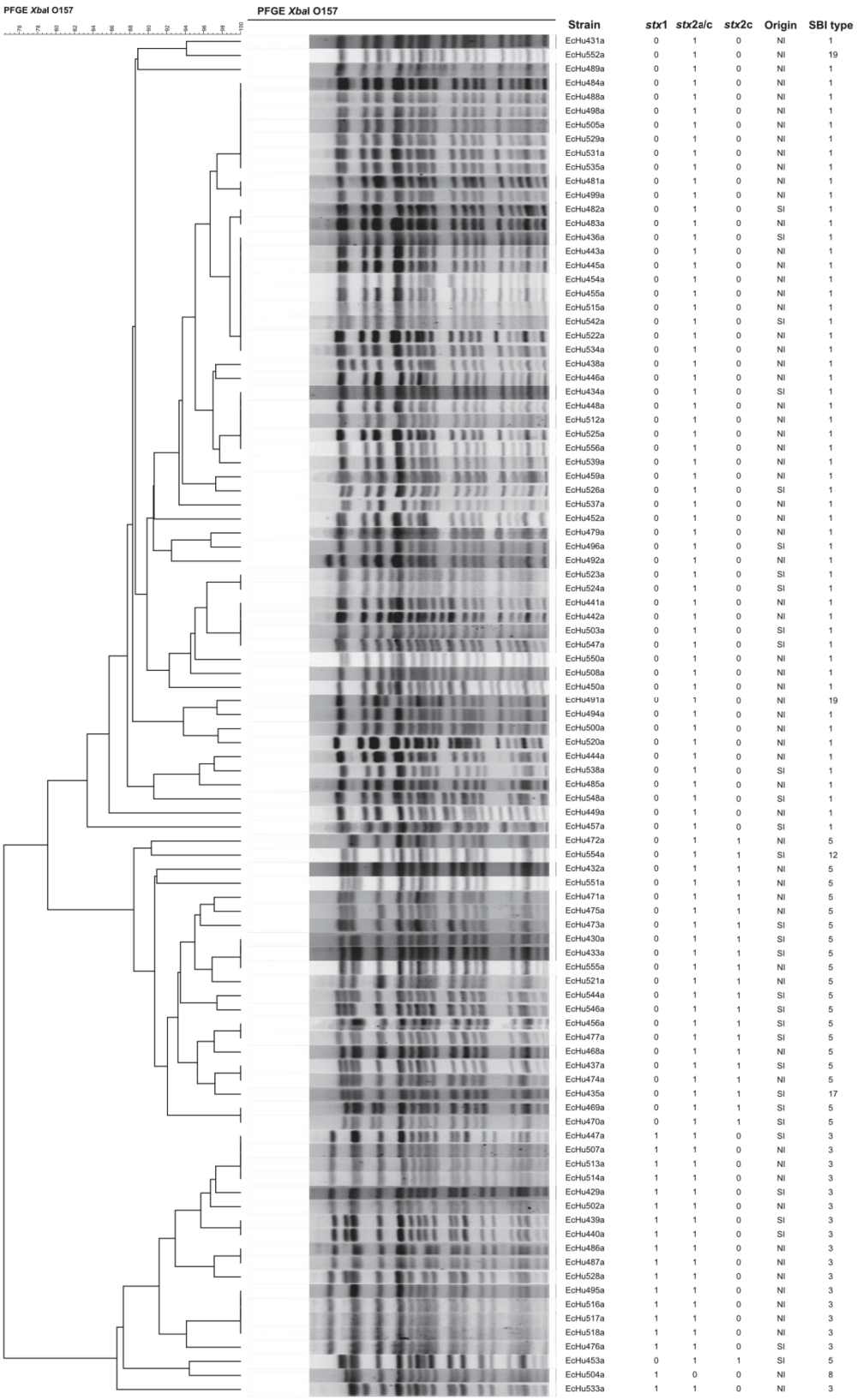

Figure 5.4: Comparison of PFGE profiles from 97 human *E. coli* O157:H7 isolates. PFGE profile comparison performed using UPGMA cluster analysis, Dice similarity coefficient, and 1% band matching tolerance. *stx*1, *stx*2a/c, *stx*2c virulence genes encoding for Shiga toxins present (1) or absent (0). Island of residence (Origin) presented as North Island (NI) or South

Island (SI) of New Zealand, and genotypes of isolates as Shiga toxin (Stx)-encoding bacteriophage insertion (SBI) types.

The two small clusters of indistinguishable PFGE profiles (two clusters of seven and eight isolates) were not concurrent in space and time and therefore do not present clusters of infections or small outbreaks.

Four statistically significant relationships were observed between SBI types and exposure variables considered in the multivariate logistic regression analysis of the case-control study and are presented in Table 5.4.

Table 5.4: Number of SBI types in *E. coli* O157:H7 isolates shown for statistically significant exposure variables considered in the multivariate logistic regression analysis of the case-control study.

	SBI type							
Variable	1	3	5	8	12	17	19	p-value
Age category								0.009
0–4 year-old	24	4	13	1	1	1	0	
5–19 year-old	10	5	6	0	0	0	2	
>19 year-old	21	8	1	0	0	0	0	
Island of residence								0.017
North Island	41	12	8	1	0	0	2	
South Island	14	5	12	0	1	1	0	
Season								0.034
Spring	14	1	2	0	0	0	0	
Summer	13	3	7	0	0	0	1	
Autumn	20	10	3	1	0	0	0	
Winter	8	3	8	0	1	1	1	
Contact with animal manure								0.047
No	29	14	8	0	1	0	2	
Yes	19	2	9	0	0	1	0	

SBI type 5 isolates were overrepresented in 0–4 year-old children, associated with the South Island and direct exposure to animal manure, while SBI type 3 isolates were overrepresented in autumn.

The molecular relatedness between PFGE profiles of *E. coli* O157:H7 isolates considering SBI types, age of cases and island of residence is shown in Figure 5.5.

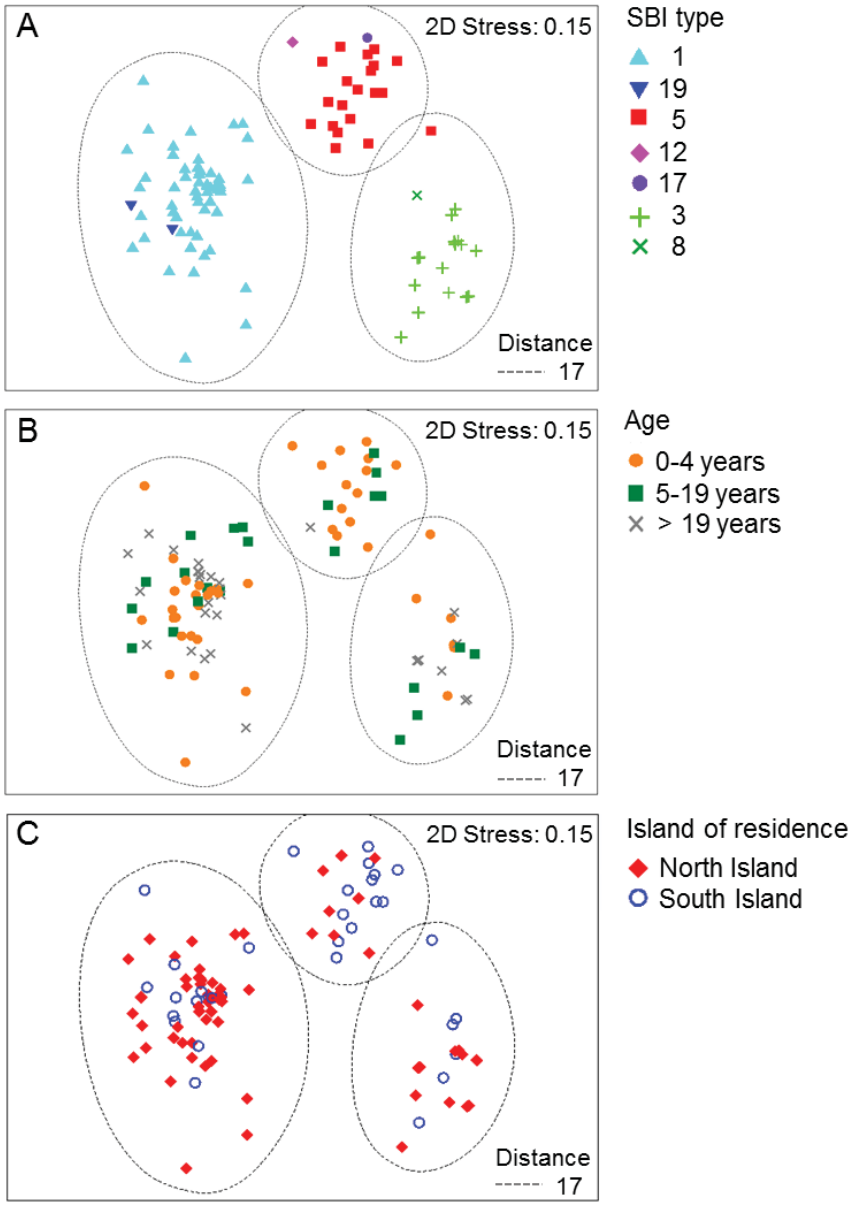

Figure 5.5: Multi-dimensional scaling plots (MDS). MDS showing the genotypic clustering of *E. coli* O157:H7 human isolates and (A) Shiga toxin-encoding bacteriophage insertion (SBI) types; (B) age categories; and (C) island of residence. The three clusters of isolates share a genetic difference of 17% (Distance = 17) based on the isolates' PFGE profiles.

Three very distinct clusters were observed, which were in strong association with SBI types 1, 3, and 5 (Figure 5.5, A). Although less apparent, the cluster containing SBI type 5 was more prevalent in pre-school children (0–4 years) compared to the other two age categories (Figure

5.5, B). The cluster containing SBI type 5 was also more prevalent in the South Island, while SBI types 1 and 3 were found more frequently in the North Island (Figure 5.5, C). Other exposure variables (e.g. 'Season' or 'Contact with animal manure') were used in MDS plots but no obvious patterns could be observed.

PERMANOVA analysis results in Table 5.5 show the proportional contribution of variables to the molecular variation of PFGE profiles of the *E. coli* O157:H7 isolates.

Table 5.5: PERMANOVA analysis of *E. coli* O157:H7 isolates. Results showing the proportional contribution of variables in the molecular variation of PFGE profiles from human *E. coli* O157:H7.

Variable	Df[a]	Mean square	p-value	Perms[b]	Estimated component of variation (%)
Island of residence	1	859.0	0.012	999	22.3
Residuals	95	187.5			77.7

[a] Degrees of freedom.
[b] Number of permutations.

Other than SBI type, only island of residence explained a significant amount of the variation in PFGE profiles in multivariate models.

5.5 Discussion

This study was designed to identify risk factors associated with domestically-acquired sporadic STEC infections in humans in New Zealand. The results strongly suggest that direct exposure to animal and/or environmental sources of infection, most likely originating from dairy and beef livestock, is the most important contributor to the burden of sporadic STEC cases observed in New Zealand. No food items were identified as risk factors for sporadic STEC cases in this study.

5.5.1 New Zealand – an agricultural country

To interpret our findings in context, it is essential to recognise that agriculture is New Zealand's largest primary industry sector contributing to approximately 48% of New Zealand's export earnings in 2009 [423]. In 2011, 6.1 million dairy cattle, 3.8 million beef

cattle, 31.1 million sheep, and 1.0 million deer were recorded in New Zealand. By contrast the estimated human population was approximately 4.4 million with 14% living in rural areas and only about 1.2% working in the agricultural industry [338]. Pastoral agriculture is the predominant land use in New Zealand with dairy cattle farming in the flatter and/or wetter areas in Northland, Waikato, Taranaki, and Manawatu in the North Island; and Canterbury, West Coast, Otago, and Southland in the South Island; while sheep and beef cattle farming are practiced in hill and high country areas across both islands.

5.5.2 Spatial and temporal epidemiology

The highest number of reported STEC infections in this study was in the youngest age category (children aged 0–4 years), which is consistent with New Zealand's health surveillance reports [424-426], and the number of cases peaked in summer/autumn (January until April) [427].

The seasonality of cases is likely to be associated with environmental exposure during the warmer season, such as increased outdoor activities in recreational waters potentially contaminated with STEC from ruminant livestock, but could also be related to the seasonal variation in the prevalence of faecal shedding of STEC in cattle. These phenomena were observed in The Netherlands [377], Great Britain [177], and also in New Zealand during a recent two-year cross-sectional study conducted at four slaughter plants across the country [428] (Chapter 3). Similarly, a recent multinational systematic review of seasonality in human zoonotic enteric diseases [93] confirmed a strong summer peak for STEC incidence.

The spatial distribution of sporadic STEC cases across New Zealand has suggested that infections might be associated with farming [317, 425, 426]. We observed increased relative risks of STEC infections in dairy farming regions (Northland, Waikato, Taranaki, Canterbury, and Southland), however, it was not possible to consider dairy and beef farming separately in the analysis of the case-control data due to the strong collinearity between these two variables. As all dairy farms in the meshblocks occupied by cases and controls also had beef cattle, hence the two variables were combined into a single variable comprising all cattle. The best fitting variable in the second analysis, which considered the entire population at the meshblock level, was the presence of beef cattle in the meshblock. Associations between STEC infections and areas with higher densities of cattle have been observed in previous

studies conducted in The Netherlands [377], Finland [429], Scotland [430], Sweden [431], and Canada [432, 433], providing evidence of direct or indirect contact with cattle as a likely source of infection.

5.5.3 Risk factors

Since reporting of STEC commenced in New Zealand in 1997, cases have occurred sporadically or as small clusters throughout the country, suggesting highly dispersed animal and/or environmental exposures rather than STEC-contaminated food as likely sources of infection. In this study, animal and environmental contacts were identified as significant risk factors for sporadic STEC infections. A child 0–4 years of age was at significantly higher risk, if another household member had contact with animals other than household pets, compared to a child of the same age without this risk factor. This finding is biologically plausible and is more likely to occur in rural than urban settings; for example, when household members working on a farm could be a source of infection. Pre-school children might be exposed to contaminated work clothing and footwear harbouring pathogenic organisms. For example, *Campylobacter* was recovered from loose debris shaken off protective overalls worn on broiler farms [434]. Household occupation contact with farm animals (sheep or lambs) was also the major risk factor for *Salmonella* Brandenburg infection in a previous New Zealand case-control study where the infection also particularly affected rural children [435].

In addition, infants and pre-school children exhibit high frequencies of hand-to-mouth and object-to-mouth behaviour indoors and outdoors and this will inevitably increase the risk of ingesting pathogens from clothing, surfaces, objects, hands, and soil [436, 437]. This is particularly apparent when children are raised in a farming environment, where they are more likely to be exposed to zoonotic and soil-associated pathogens than in urban areas. Therefore, practicing good hand-hygiene and supervising activities of infants and toddlers could help to reduce the risk of ingesting pathogens.

Although ‘Living in a rural area’ was not statistically significant in our model, there was sufficient evidence that risk factors related to agricultural or rural characteristics were associated with sporadic STEC infection, and these would have confounded the strong univariate relationship with rurality. Similar associations between rurality and notification

rates of both STEC and cryptosporidiosis in New Zealand have been observed by Thorburn [438], reporting higher rates in rural areas.

Exposures to farming environments were reported as risk factors for sporadic STEC infections in case-control studies conducted in England [334], North America [24], Germany [38], and Argentina [37]. A prospective case-control study by Locking *et al.* [68] identified contact, or likely contact, with animal manure as a strong risk factor for sporadic STEC O157 infection in Scotland, while a retrospective case-control study by Voetsch *et al.* [439] observed direct or indirect contact with cattle manure as a leading source of sporadic STEC O157 infections in North America. We also identified contact with animal manure as a significant risk factor in addition to cattle being present in the meshblock in which the case resided. Both variables were associated with the largest population attributable fractions and, when combined with the spatial analysis and the strong correlation between the presence of cattle, and particularly beef cattle and STEC cases at the meshblock level, it indicates that contact with cattle faeces is the major exposure pathway for infection in New Zealand.

'Travelling to areas of New Zealand with interrupted or no main water supply' and 'Contact with recreational waters' emerged as significant environmental risk factors for sporadic STEC infections; activities, which increasingly occur in the summer period. Previous studies conducted in Finland [336] and North America [104, 440] reported an association between gastrointestinal illnesses, including STEC, after exposure to recreational waters during summer. Such an association could also explain some of the observed seasonality of STEC cases as discussed above. In addition, an estimated 14% of New Zealand's population is not served by community drinking-water supplies [441] but retrieves drinking water from private springs and bores, streams and creeks, or roof runoffs. This risk applies particularly to residents of rural areas. Considering the large ruminant livestock population in New Zealand, ground water and particularly surface water in rural areas are potentially contaminated with ruminant faeces containing STEC.

We found no evidence to suggest that sporadic STEC cases in New Zealand were associated with exposure to STEC-contaminated food products, while 'Drinking refrigerated fruit juice from supermarket', 'Eating raw vegetables' and 'Eating seafood' were negatively correlated with disease. An inverse effect of fruit and vegetables has also been reported in previous case-control studies conducted in Australia [442] and Scotland [68] and merits further

investigation. The association seems biologically plausible compared to other food products, as they are associated with health benefits such as antimicrobial properties against human pathogens in berries [443] and sweet potato leaves [444]. An alternative explanation might be the association between fruit and vegetable consumption and the participants' choice of healthy eating. This apparent protective effect might also be caused by recall bias, as discussed under *Sources of bias*.

5.5.4 Molecular epidemiology of *E. coli* O157:H7

The molecular analysis of PFGE profiles from human *E. coli* O157:H7 isolates revealed three distinctive clusters of genotypes, each represented by a specific SBI type. SBI types are defined based on the insertion site of the Stx-associated bacteriophage and the presence or absence of *stx* genes in the bacterial genome, which encode for the Shiga toxin proteins. *stx*2c is a subtype of the *stx*2 gene and characteristic for isolates of SBI type 5. The observed clustering was significantly associated with 'Island of residence', indicating that SBI type 5 was more prevalent in the South Island, whereas SBI types 1 and 3 were more common in the North Island. This distinct geographical difference in genotype distribution was also observed in a recent molecular study including 28 bovine and 209 human *E. coli* O157:H7 isolates originating from both islands of New Zealand [445]. The distinct between-island distributions of genotypes found among bovine and human isolates indicate localised transmission between cattle and humans. SBI type 5 accounted for 20.6% of human isolates in that study, which is much greater than its frequency in other international studies [269, 446]. This is consistent with a limited historical introduction of this strain into New Zealand and subsequent evolution.

A significant relationship between SBI types and age categories of cases was observed, in particular between SBI type 5 and 0–4 year-old children. It can be hypothesised, if this genotype possesses host-adapted characteristics to affect specifically the immature gastrointestinal tract of children, or whether the observed association is due to SBI type 5 being a more persistent environmental contaminant, to which very young children are more likely to be exposed than adults. Recent studies investigating differential virulence of STEC O157 strains have suggested that STEC O157 strains carrying *stx*2c alone are likely to be less pathogenic compared to strains carrying combinations of *stx*2c and *stx*1 as shown in a piglet model [205], or less potent on human kidney cell lines and in mouse models [447].

The molecular variation of PFGE profiles of the isolates was explained by only one explanatory variable: 'Island of residence', which was consistent with the observed clustering of isolates. Together with significant associations observed between SBI types and both 'Season' and 'Contact with animal manure', these findings provide further evidence of an animal/environmental-associated pathway of sporadic STEC infection in New Zealand.

5.5.5 Sources of bias

The two week window of exposure might have resulted in some recall bias due to difficulty remembering previous exposures. However, this time period was chosen to cover the likely incubation period for STEC (3–12 days) while lists of possible answers facilitated recall of consumed food items, contact with animal species and environmental exposures. Observational studies of this type can also introduce recall bias due to the cases being more likely to recall events than the non-affected controls [448]. This effect could explain the apparent protective association seen for consumption of a range of foods, where recall was less complete amongst controls, as found in a previous New Zealand case-control study of similar populations [435]. In addition, 31% of cases were interviewed differently to the other cases and controls, which might have introduced some systematic differences between them, though such a bias is unlikely to have had an important effect on the findings.

There was evidence of some selection bias in the control population as a result of using random landline dialling for recruitment. Based on the national census data, the older age group of controls was overrepresented compared to the younger age groups. This might be because younger age groups favouring mobile technology over landlines, or their tendency to reside in relatively fewer households with a larger number of individuals. Nonetheless, little bias was observed in the distribution of ethnicities, gender, and rural/urban living among controls, compared to national census data.

The exclusion of potentially eligible cases had a negligible effect on the findings. Only one case probably acquired their infection overseas so was excluded. There were no apparent outbreaks or clusters of concurrent cases observed during the study period, indicating that secondary infections occur only relatively infrequently in New Zealand suggesting that this study was effectively one of sporadic cases.

The number of confirmed cases reported through the disease surveillance system is likely to be an underestimation of the true incidence of human STEC infections in New Zealand. Scallan *et al.* [449] and Tam *et al.* [450], using different approaches, have estimated under-ascertainment fractions of STEC cases in the US and the UK. Asymptomatic or mild cases are unlikely to present to medical practitioners and not all stool samples received at diagnostic laboratories are routinely tested for *E. coli* O157:H7 and non-O157 STECs in New Zealand. In addition, the majority of diagnostic laboratories test stool samples of STEC cases for *E. coli* O157:H7 only, which could explain the current predominance of STEC O157. Therefore risks presented could be underestimated, or different measures of association could apply compared to findings in this study.

5.6 Conclusions

Our findings strongly indicate that environmental and animal contact, but not food, are important exposure pathways for sporadic cases of human STEC infection in New Zealand. There are strong indications that dairy cattle and beef cattle are the most important sources of STEC and contact with manure from these animals represents an important exposure pathway. Notably, outbreaks of STEC infections are rare in New Zealand and this further suggests that food is not a significant exposure pathway.

5.7 Acknowledgements

We thank the Public Health Units across New Zealand for their collaboration and participation in this study; the Health Intelligence team at ESR (Ali Borman, Ruth Pirie, Kerry Sexton) with study preparations and technical support; UMR for recruiting and interviewing study controls; ERL for providing STEC isolates and PFGE profiles (Brent Gilpin, ESR, Christchurch, New Zealand); Charlotte Bolwell (Hopkirk Research Institute, Massey University, Palmerston North, New Zealand) for providing the meshblock data and livestock numbers; Dr John Holmes (Ministry of Health, Wellington, New Zealand) for advice in study design; Martyn Kirk (National Centre for Epidemiology and Population Health, Australian National University, Canberra, Australia) for advice in questionnaire and study design; and the Ministry for Primary Industries (Wellington, New Zealand) for funding this study.

Findings from the prospective case-control study strongly indicated that cattle are a source of STEC infection in humans in New Zealand. To support these observations and assess evidence for localised transmission of STEC from cattle to humans, the following chapter describes the comparison of *E. coli* O157:H7 (STEC O157) isolates from bovine and human sources in New Zealand using molecular typing methods. The chapter has been published as a Research Article:

Jaros P, *et al.* (2014) Geographic divergence of bovine and human Shiga toxin-producing *Escherichia coli* O157:H7 genotypes, New Zealand. *Emerging Infectious Diseases*, doi: 10.3201/eid2012.140281 (e-published ahead of print).

The full, e-published article is reproduced in Appendix 11.

Chapter 6

Geographic divergence of bovine and human Shiga toxin-producing *Escherichia coli* O157:H7 genotypes in New Zealand

6.1 Abstract

Shiga toxin-producing *Escherichia* coli (STEC) O157:H7 is a zoonotic pathogen of public health concern worldwide. This study compared the local and large-scale geographical distributions of STEC O157:H7 genotypes from bovine and human sources, as determined by pulsed-field gel electrophoresis and Shiga toxin-encoding bacteriophage insertion (SBI) typing. Isolates from the North and South Islands of New Zealand were compared with isolates from Australia and the United States using multivariate methods. STEC O157:H7 population structure differed significantly between North and South Island of New Zealand and showed evidence of finer-scale spatial structuring consistent with highly localised transmission, rather than disseminated foodborne outbreaks. There were marked differences in the SBI type distributions in New Zealand, Australia and the United States. Furthermore, our findings provide evidence for the historical introduction of a subset of globally-circulating STEC O157:H7 strains into New Zealand, with ongoing evolution and localised transmission between cattle and humans.

6.2 Introduction

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 and related non-O157 STEC strains are important zoonotic pathogens, which can cause severe gastrointestinal illness in humans with clinical symptoms ranging from diarrhoea and haemorrhagic colitis to the life-threatening haemolytic uraemic syndrome [26, 59]. Ruminants are asymptomatic carriers of STEC, shedding the pathogen via faeces, and are considered a primary source of foodborne and environmental outbreaks of STEC infection in humans [451].

Annual incidence rates for STEC infections in New Zealand have been among the highest in the world. In 2013, 207 clinical STEC cases (4.6 cases per 100,000 population) were reported [31]. Consistent with observations in previous years, O157:H7 was the predominant serotype among confirmed STEC cases (89.4%; 194/217 [316]). Since reporting commenced in 1997, the annual number of STEC notifications has increased steadily [31]. Although the spatial distribution of STEC cases in New Zealand suggests an association with farming and other "rural" activities, limited epidemiological data are available on the particular transmission pathways of STEC from cattle populations to humans.

The objectives of this research were (i) to compare the population structure and geographical distribution of STEC O157:H7 genotypes isolated from bovine and human sources in New Zealand, (ii) to assess evidence for localised transmission of STEC from cattle to humans in New Zealand, and (iii) to compare the genotype distribution of New Zealand isolates with those isolated in Australia (historically, the predominant source of imported New Zealand cattle [452]) and the United States.

To investigate the molecular divergence of isolates, two molecular typing methods were used in this study: Shiga toxin (Stx)-encoding bacteriophage insertion (SBI) typing and pulsed-field gel electrophoresis (PFGE) profiling. Although PFGE can provide an indication of genomic similarities, it is not able to provide a reliable measure of genetic relatedness of isolates, and the visual assessment of bands on an agarose gel to create PFGE profiles can result in misclassification bias [266]. By using two methods, and examining the concordance between them, the combined genotyping datasets could be used to assess structuring and patterns of diversity among STEC O157:H7 isolates of bovine and human origin in New Zealand.

6.3 Materials and methods

6.3.1 Human isolates and data

A total of 363 STEC O157:H7 isolates from clinical STEC cases occurring between 2008 and 2011 in New Zealand were obtained from the national Enteric Reference Laboratory (ERL, Institute of Environmental Science & Research Ltd, Upper Hutt, New Zealand), along with PFGE profiles (restriction enzyme XbaI) and geographic data associated with the clinical

cases (North or South Island of New Zealand, and region within island); 76.6% (278/363) of the isolates originated from the North Island. Isolates included in this study represented 71.3% (363/509) of notified and confirmed STEC O157 cases between 2008 and 2011 [453], which were reported as sporadic cases or household clusters of two STEC infections and not associated with confirmed foodborne outbreaks.

6.3.2 Bovine faecal isolates and data

Forty bovine faecal STEC O157:H7 isolates were used from two preceding studies conducted at beef slaughter plants in New Zealand in 2008 [324], and 2009 to 2011 [428] (Chapter 3). Animal data on origin (North or South Island, region, farm location) and molecular data on the presence of virulence genes (*ehx*A, *eae*, *stx*1, *stx*2, and subtype *stx*2c) were available. The isolates were collected from 26 calves and 14 adult cattle, originating from 35 different farms, mostly from the North Island (80.0%; 32/40).

6.3.3 Bovine meat isolates and data

Bovine meat isolates (n = 235) included those collected from routine mandatory testing of samples at beef processing plants from across New Zealand from 2008 to 2011. Only PFGE profiles (*Xba*I) of STEC O157:H7 isolates were available for this study, which were obtained from ERL. Geographic data associated with meat sample location (regions in North and South Islands) were obtained from the Ministry for Primary Industries. The majority of isolates (85.5%; 201/235) originated from beef slaughter plants in the North Island. Virulence profiles of the isolates were not available.

6.3.4 PCRs for detection of virulence genes

All human isolates were re-grown on Columbia horse blood agar (Fort Richard Laboratories, Auckland, New Zealand). Bacterial DNA was extracted from five colonies using 2% Chelex beads solution (Chelex® 100 Resin, Bio-Rad) and analysed in two PCR assays using an automated real-time thermocycler (Rotor Gene 6200HRM, Corbett Research).

Firstly, a multiplex PCR assay was performed using previously published primer sequences to detect the presence of virulence genes encoding for enterohaemolysin (*ehx*A) [245], intimin

(*eae*) [245], and Shiga toxins (*stx*1 and *stx*2) [342]. Primers for detection of genes *stx*1 and *stx*2 did not differentiate between subtypes of toxins. The final 25 µl PCR reaction volume contained 2x PCR buffer (Express qPCR SuperMix, Invitrogen), 2 µmol/L of each primer, 2.0 µl DNA, and 2.5 µl sterile water. The amplification programme included an initial enzyme-activation step of 5 min at 94°C, which was followed by 40 cycles of, 20 s at 94°C, 20 s at 64°C and 20 s at 72°C, followed in turn by a final extension of 5 min at 72°C. The PCR products were detected by electrophoresis using a 2% (wt/vol) agarose gel (Agarose low EEO, AppliChem, Germany), stained with ethidium bromide and visualised under ultraviolet illumination.

Secondly, *stx*2-positive isolates were further tested to determine if the *stx*2 gene present was the genetic subtype *stx*2c. The presence of the *stx*2c gene was detected using previously published primer sequences [267, 269]. The final 20 µl PCR reaction volume contained 2x PCR buffer (Express qPCR SuperMix, Invitrogen), 2 µmol/L of each primer, 2.0 µl DNA, and 6.0 µl sterile water. The PCR included an initial enzyme-activation step of 5 min at 94°C, followed by 35 cycles of, 20 s at 94°C, 20 s at 55°C; no extensions were used. The amplified PCR product was detected as described above.

6.3.5 Molecular typing methods

All human and bovine faecal isolates were genotyped using SBI typing [267, 269]; SBI typing is a multiplex PCR method screening specific *stx*-associated bacteriophage insertion sites and *stx* genes (*stx*1, and genetic subtypes *stx*2a and *stx*2c of *stx*2). The characters A, W, Y, S and 1, 2a, 2c represent bacteriophage insertion sites *argW*, *wrbA*, *yehV*, *sbcB*, and Shiga toxin genes *stx*1, *stx*2a, *stx*2c, respectively [269, 454]. All bovine faecal isolates were subtyped using PFGE (*Xba*I) following the standardised laboratory protocol published by PulseNet International [393].

6.3.6 Ethical approval

The use of isolates from notified STEC cases in New Zealand was approved by the Multi-region Ethics Committee, Wellington, New Zealand, on 19 March 2012; reference number MEC/11/04/043.

6.3.7 Data management and statistical analysis

For initial analysis, SBI types were grouped into four categories of three predominant SBI types AY2a, WY12a, ASY2c/SY2c, and other SBI types (which included the less common types AS12c, AS2c, ASWY2c, ASY12c, ASY2a2c, AWY12a, AWY2a, SWY2c, and Y2c). SBI types SY2c and ASY2c were grouped together because both were relatively common and shared the same virulence gene profile.

Although bovine meat isolates were not SBI-typed, a close correlation between PFGE profile and SBI type was observed for the human and bovine faecal samples. Based on the PFGE/SBI clusters, the most likely SBI type was inferred from the PFGE profiles for the meat isolates by taking the following approach. Firstly, BioNumerics software [394] was used to compare PFGE profiles of human and bovine faecal isolates by carrying out a UPGMA (unweighted pair group method with arithmetic mean) cluster analysis using the Dice similarity coefficient, with a band matching tolerance of 1%. Secondly, the UPGMA cluster analysis was applied on PFGE profiles of bovine meat isolates. The dominant SBI types in human and bovine faecal isolates were used to assign 'SBI-like' types (AY2a, WY12a, and ASY2c/SY2c) to clusters with similar PFGE band patterns in bovine meat isolates.

Chi-square and Fisher's exact test for count data were used to evaluate associations between SBI type (AY2a, WY12a, ASY2c/SY2c, and other SBI types) and island for bovine faecal, bovine meat, and human isolates, using R software [345]. P-values for region and year and SBI types in human and bovine meat isolates were computed by simulating 10^8 tables from the null hypothesis (independence) and comparing with the test statistic from the observed data.

Population differentiation among human and bovine meat isolates was assessed using analysis of molecular variance (AMOVA) applied to haplotypes of isolates' PFGE profiles (generated in BioNumerics) using Arlequin software (version 3.5.1.2) [455]. A multilevel hierarchy was used for the AMOVA model; assessing population differentiation between island, between regions within island, and within regions. Regions with <5 isolates were excluded from the analysis. A matrix of pairwise F_{ST} values was computed by comparing the PFGE haplotype frequency distributions for each pair of regions (using Arlequin (version 3.5.1.2)). F_{ST} is an index of population differentiation, measuring the variance between subpopulations relative to

the total variance, and ranges from 0 (no divergence) to 1 (complete divergence). The computed pairwise F_{ST} matrix, representing genetic distances between the regional populations of STEC O157:H7, was illustrated graphically as a NeighborNet tree in SplitsTree software (version 4.12.6) [456].

To illustrate the molecular relatedness and genotypic clustering of isolates, distance matrices of PFGE profiles of human and bovine meat isolates (generated in BioNumerics) were linked with explanatory variables (SBI type and region) to create multi-dimensional scaling (MDS) plots using Primer 6 software [422]. Regions with <5 isolates were excluded from the analysis.

Published frequency distributions of SBI types in 205 and 143 cattle, and 79 and 179 human STEC O157:H7 sourced from Australia and the United States [270], respectively, were compared to frequency distributions of SBI types among bovine and human STEC O157:H7 isolates from New Zealand to assess the population structure of New Zealand isolates.

To evaluate genetic similarities of human and bovine faecal isolates, proportional similarity indices (PSI) were computed based on the frequency distributions of SBI types in humans and cattle from all three countries. PSI is a similarity measure, estimating the area of congruence between two frequency distributions [457], and ranges from 0 (distributions with no common SBI types) to 1 (highest possible similarity between distributions). Bootstrapped 95% confidence intervals for PSI values were calculated according to the percentile method described by Efron and Tibshirani [458], using 2,000 iterations. No grouping of SBI types was applied for PSI calculations. To illustrate the international geographic divergence of isolates, differences in PSI values (1 – PSI) were used to construct a NeighborNet tree in SplitsTree software.

6.4 Results

6.4.1 Genotype diversity

All 403 human and bovine faecal isolates were positive for *ehx*A, *eae*, and *stx*2 (except one *ehx*A-negative human isolate); of these, 61 (15.1%) were also positive for *stx*1. The different

virulence profiles of isolates, each represented by a dominant SBI type, are shown in Table 6.1.

Table 6.1: Virulence profiles and SBI types of Shiga toxin-producing *Escherichia coli* O157:H7 isolates collected from human cases and faecal samples of slaughter cattle in New Zealand between 2008 and 2011*.

				Virulence genes					SBI type	
Species	No. of isolates	NI	SI	*ehx*A	*eae*	*stx*2	*stx*2c	*stx*1	Dominant (no., %)	Other (no., %)
Bovine	6	6	0	+	+	+	–	+	WY12a (6, 100.0)	–
	10	2	8	+	+	+	+	–	ASY2c (7, 70.0), SY2c (2, 20.0)	AS2c (1, 10.0)
	24	24	0	+	+	+	–	–	AY2a (22, 91.7)	AWY2a (2, 8.3)
Human	51	43	8	+	+	+	–	+	WY12a (49, 96.1)	AWY12a (2, 3.9)
	1	0	1	–	+	+	–	+	WY12a (1, 100.0)	
	94	54	40	+	+	+	+	–	ASY2c (69, 73.4)	SWY2c (3, 3.2)
									SY2c (15, 16.0)	ASWY2c (2, 2.1)
										AS2c (2, 2.1)
										Y2c (2, 2.1)
										ASY2a2c (1, 1.1)
	214	179	35	+	+	+	–	–	AY2a (210, 98.1)	AWY2a (4, 1.9)
	3	2	1	+	+	+	+	+	ASY12c (2, 66.7)	AS12c (1, 33.3)

*NI, North Island of New Zealand; SI, South Island of New Zealand; +, gene present; – , gene absent; *ehx*A gene encodes for enterohaemolysin; *eae* gene encodes for intimin; *stx*2, primers for detection of this gene did not differentiate between subtypes of Shiga toxin type 2; *stx*2c gene encodes for Shiga toxin subtype 2c; *stx*1, primers for detection of this gene did not differentiate between subtypes of Shiga toxin type 1; SBI, Shiga toxin-encoding bacteriophage insertion.

The predominant SBI types AY2a, WY12a, and ASY2c/SY2c accounted for 55.0% (22/40), 15.0% (6/40), and 22.5% (9/40) of the studied bovine faecal isolates, respectively. Similarly, in human isolates, SBI types AY2a, WY12a, and ASY2c/SY2c, were detected in 57.9% (210/363), 13.8% (50/363), and 23.1% (84/363) of the isolates, respectively. The distributions of AY2a, WY12a, ASY2c/SY2c and other SBI types varied by year ($p = 0.037$) (Figure 6.1).

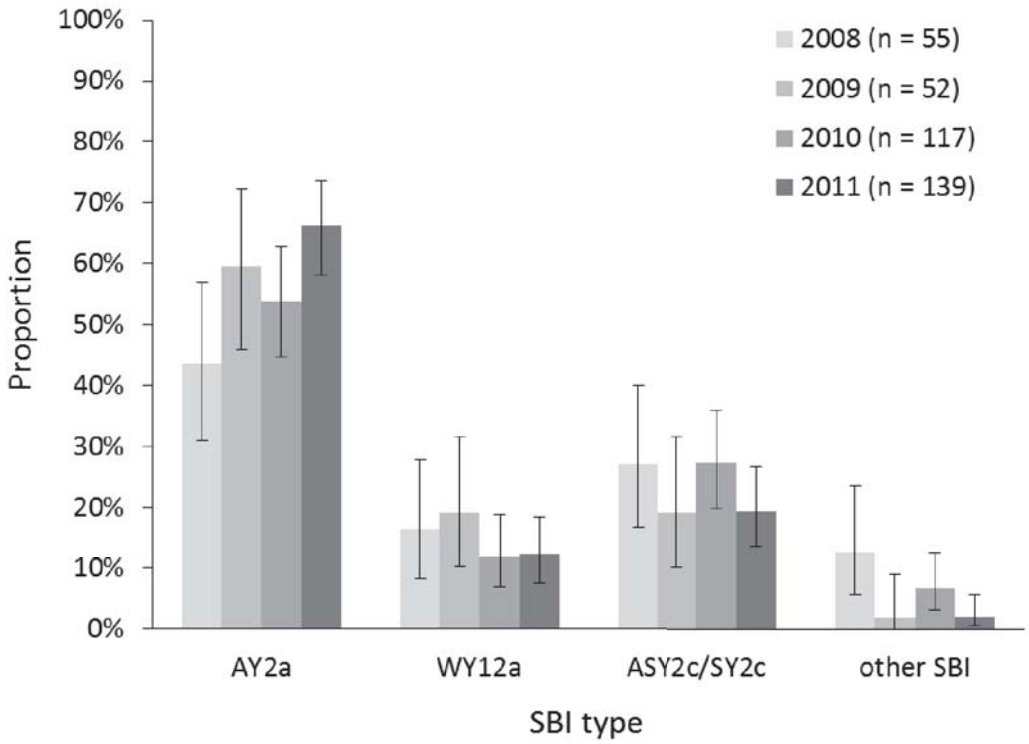

Figure 6.1: Proportional distributions of Shiga toxin-encoding bacteriophage insertion (SBI) types AY2a, WY12a, ASY2c/SY2c of 363 human Shiga toxin-producing *Escherichia coli* O157:H7 isolates from clinical cases in New Zealand occurring between 2008 and 2011, stratified by year. Error bars indicate 95% confidence intervals.

Based on the genotype calibration of PFGE profiles of bovine meat isolates, SBI-like types AY2a, WY12a, and ASY2c/SY2c were prevalent in 64.7% (152/235), 23.4% (55/235), and 11.9% (28/235) of the isolates, respectively. There was a marginally non-significant association between SBI-like type and year ($p = 0.052$).

6.4.2 Between-island comparisons

The distribution of SBI types observed differed between North and South Islands in bovine faecal and human isolates, with SBI types AY2a and WY12a more prevalent in the North Island, and ASY2c/SY2c more common in the South Island (Table 6.2). Similarly, a significant difference in the prevalence distribution of SBI-like types between islands was observed in bovine meat isolates (Table 6.2).

Table 6.2: Frequency distribution of predominant SBI genotypes (AY2a, WY12a, and ASY2c/SY2c) of Shiga toxin-producing *Escherichia coli* O157:H7 isolates collected from human cases, bovine faeces and bovine meat samples, stratified by island of New Zealand. P-values refer to differences between frequency distributions of SBI types and islands of New Zealand (Chi-square and Fisher's exact test)*.

		Prevalence (%)		
Isolate type	SBI type	North Island (no./total)	South Island (no./total)	P-value
Human	AY2a	62.9 (175/278)	41.2 (35/85)	<0.001
	WY12a	14.7 (41/278)	10.6 (9/85)	
	ASY2c/SY2c	17.6 (49/278)	41.2 (35/85)	
	other	4.7 (13/278)	7.1 (6/85)	
Bovine faecal	AY2a	68.8 (22/32)	0.0 (0/8)	<0.001
	WY12a	18.8 (6/32)	0.0 (0/8)	
	ASY2c/SY2c	3.1 (1/32)	100.0 (8/8)	
Bovine meat	AY2a-like	68.2 (137/201)	44.1 (15/34)	<0.001
	WY12a-like	24.4 (49/201)	17.6 (6/34)	
	ASY2c/SY2c-like	7.5 (15/201)	38.2 (13/34)	

*SBI, Shiga toxin-encoding bacteriophage insertion.

6.4.3 Within-island comparisons

Using a three-level hierarchy of island, region within island and within region, the AMOVA results revealed that most of the molecular variation (>98%) resided between isolates within regions (based on PFGE haplotypes). However, for the human isolates, a small but highly significant proportion of the molecular variation was estimated to be between regions within islands (1.03% variation, $p < 0.001$), providing evidence for highly localised geographical structuring. Having allowed for between region variation, island was no longer a significant source of variation for the human isolates ($p = 0.212$). In contrast, a very small but significant amount of molecular variation was apparent between islands among the bovine meat isolates (0.38% variation, $p = 0.017$), while the proportion of variation between regions within islands was non-significant (0.34% variation, $p = 0.121$). The population differentiation and geographic clustering of STEC O157:H7 genotypes of human cases and bovine meat samples from regions of both islands of New Zealand are illustrated in Figure 6.2.

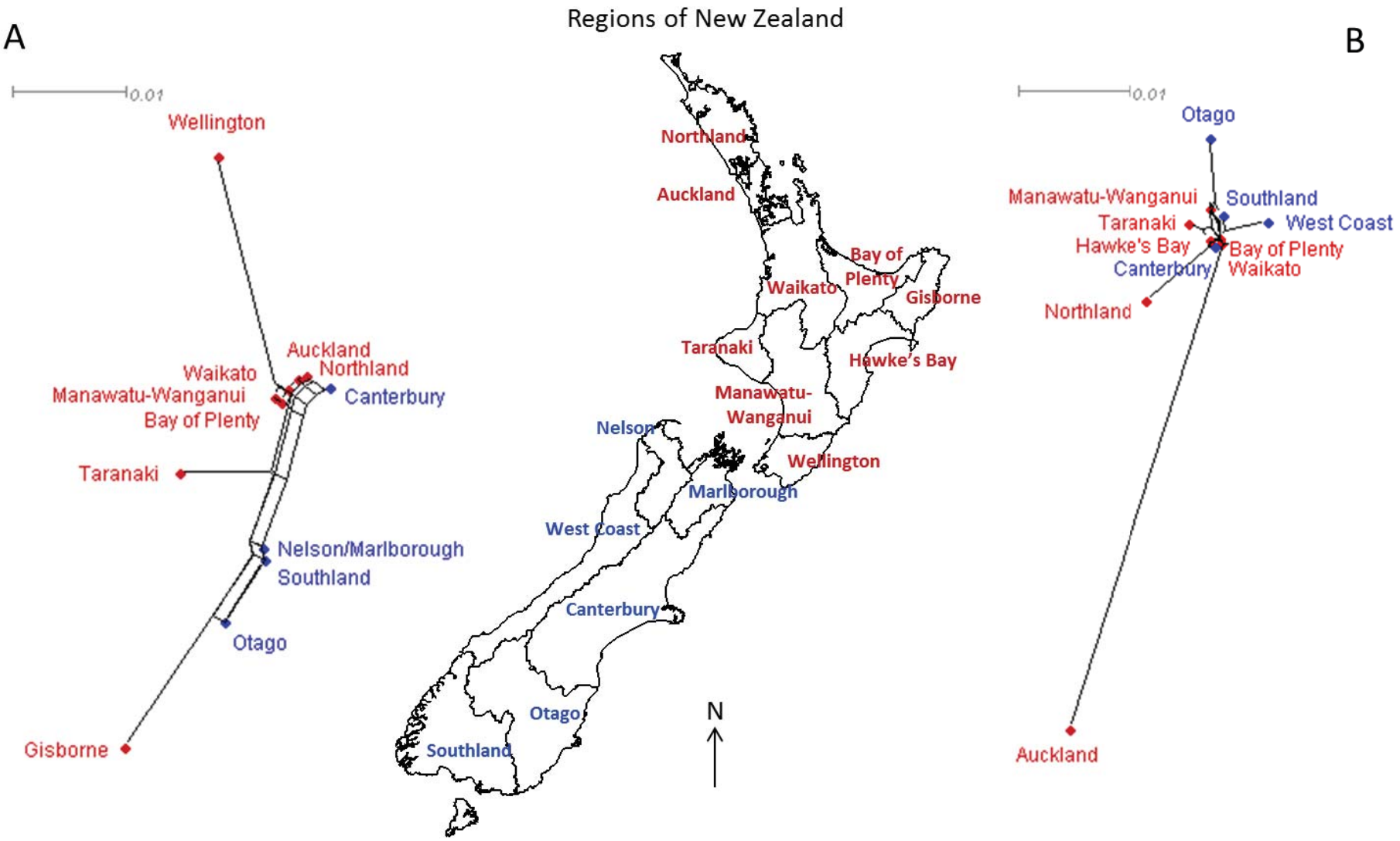

Figure 6.2: NeighborNet trees showing population differentiation of Shiga toxin-producing *Escherichia coli* O157:H7 isolates from (A) human cases (n = 355, 8 isolates excluded) and (B) bovine meat samples (n = 233, 2 isolates excluded) from different regions in the North Island (red) and the South Island (blue) of New Zealand, as depicted on the map of New Zealand. The distances indicate population differentiation measured as pairwise F_{ST} values.

Consistent with the AMOVA results, there was evidence of within island clustering of human isolates. Two main clusters were observed representing North and South Island regions, with the exception of Canterbury, which clustered with North Island regions, and Wellington, Taranaki, and Gisborne being North Island outliers. Among human cases, the highest population differentiation of STEC O157:H7 genotypes was observed between the regions of Wellington (n = 15) and Gisborne in the North Island (pairwise F_{ST} value of 0.071), followed by Wellington and Otago (F_{ST} = 0.060); the isolates from Gisborne (n = 12) included two household clusters of two human cases each. For bovine meat isolates, no obvious structuring was apparent, however Auckland region (n = 5) appeared as a strong North Island outlier. Consequently, the most distinct difference in genotypes was observed between the regions of Auckland and Otago (F_{ST} = 0.060), closely followed by Auckland and Northland (F_{ST} = 0.057).

The molecular relatedness between PFGE profiles of human isolates, considering SBI type and region of origin as explanatory variables, is shown in Figure 6.3.

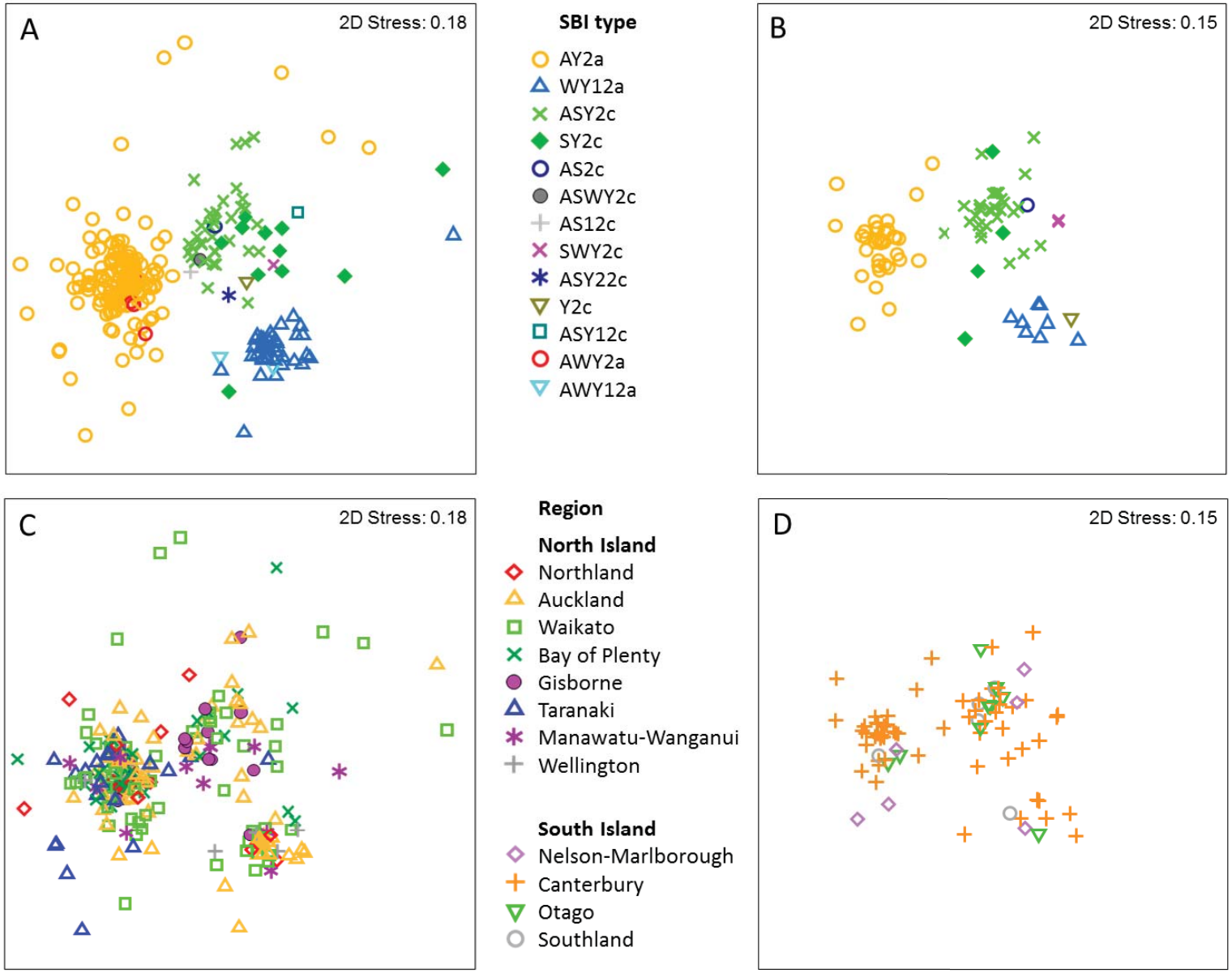

Figure 6.3: Multi-dimensional scaling plots (MDS) showing the genotypic clustering of human Shiga toxin-producing *Escherichia coli* O157:H7 isolates originating from the North Island (n = 274, 4 isolates excluded) and the South Island (n = 81, 4 isolates excluded) of New Zealand, based on the isolates' pulsed-field gel electrophoresis profiles. Clusters associated with Shiga toxin-encoding bacteriophage insertion (SBI) types and regions are presented for isolates from the North Island (A and C) and the South Island (B and D), respectively.

PFGE profiles showed genotypic clustering, which was strongly associated with SBI types AY2a, WY12a, ASY2c/SY2c, even after stratifying by island of origin (Figure 6.3, A and B). Clusters containing SBI type AY2a and ASY2c/SY2c were the predominant genotypes in the Taranaki and Gisborne regions in the North Island, respectively (Figure 6.3, C); the association between SBI type and region of origin was statistically significant ($p < 0.001$). A similar genotypic clustering of regions was observed in bovine meat isolates from the North and South Islands (Appendix 12).

6.4.4 International comparison

Within each country, similar frequency patterns of SBI types were observed in cattle and human cases, however, there were distinct differences in the population structure of SBI types between countries (Figure 6.4).

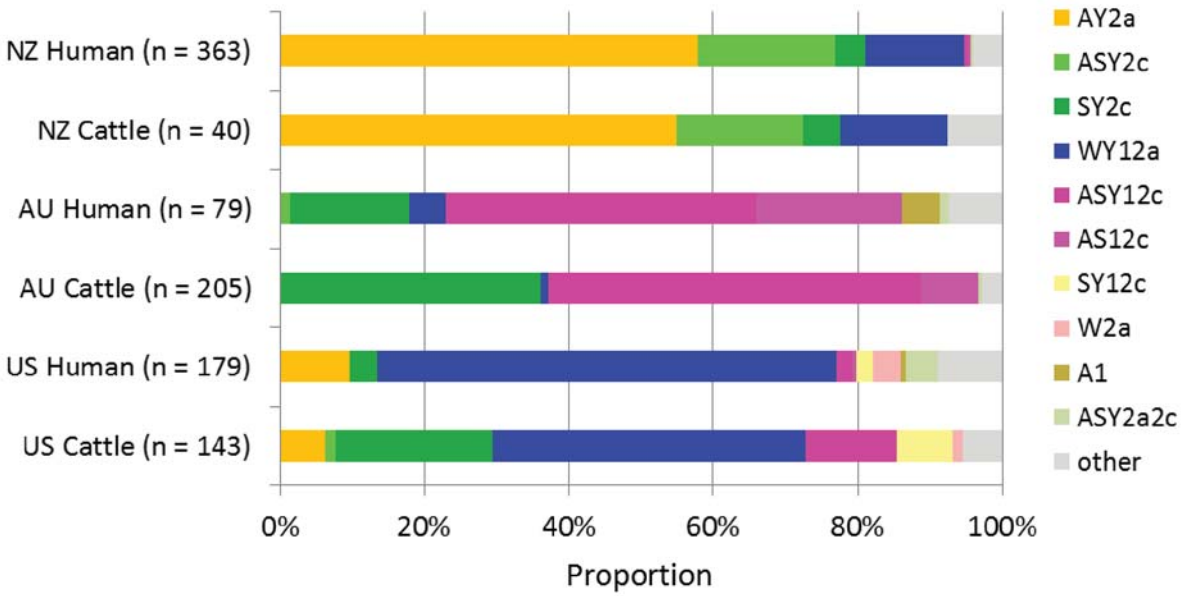

Figure 6.4: Proportional distributions of Shiga toxin-encoding bacteriophage insertion types of Shiga toxin-producing *Escherichia coli* O157:H7 isolates sourced from cattle and human in New Zealand (NZ), Australia (AU), and the United States (US).

New Zealand bovine and human genotypes shared the highest similarity (PSI value of 0.92, 95% CI 0.74–0.93) followed by Australia (PSI 0.69, 95% CI 0.57–0.79) and the United States (PSI 0.61, 95% CI 0.51–0.69) (Appendix 13). The observed differences in proportional similarities of SBI types between cattle and human of all three countries are illustrated in Figure 6.5.

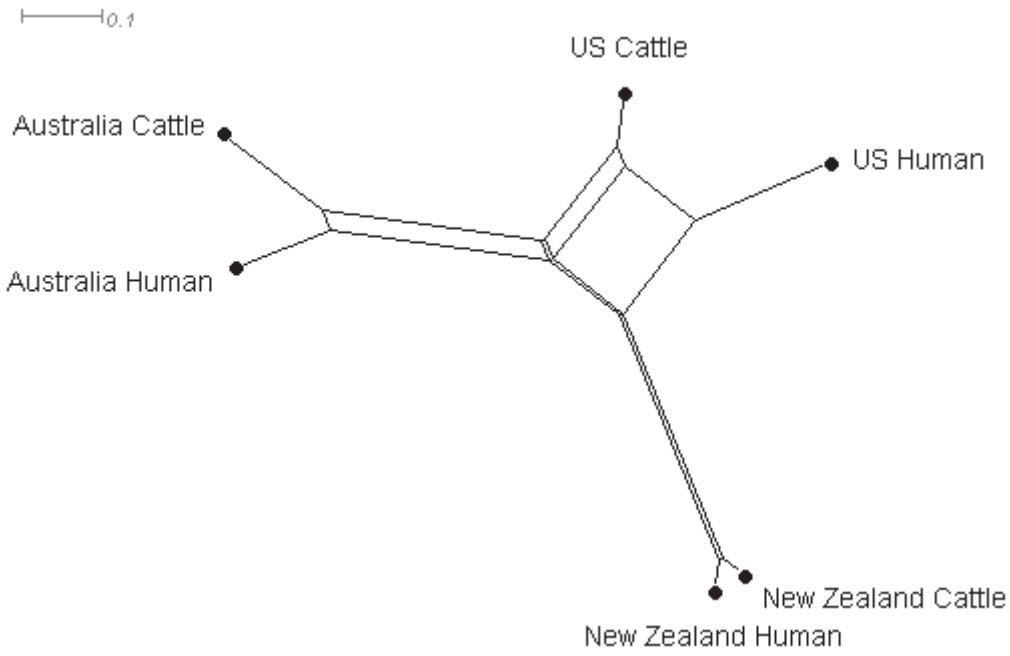

Figure 6.5: NeighborNet tree showing geographic divergence of bovine and human Shiga toxin-producing *Escherichia coli* O157:H7 isolates sourced from New Zealand (40 cattle, 363 human), Australia (205 cattle, 79 human), and the United States (US) (143 cattle, 179 human). The distance indicates the difference in proportional similarity of Shiga toxin-encoding bacteriophage insertion (SBI) types among the isolates.

6.5 Discussion

This study assessed the molecular epidemiological evidence for transmission of STEC from cattle to humans in New Zealand and the relationship between population structure and geography, at multiple spatial scales. The molecular analysis of bovine and human STEC O157:H7 isolates revealed a concordant geographical variation of genotypes (SBI types) in both populations. In addition, there were marked differences between the North and South Islands of New Zealand, consistent with localised transmission of STEC between cattle and humans.

The evidence of localised transmission of STEC between cattle and humans has advanced our understanding of the epidemiology of sporadic STEC infections in New Zealand, which are consistent with environmental/animal-associated sources of infection rather than more disseminated foodborne outbreaks [459] (Chapter 5). Measures to prevent direct contact with

animal faecal material in the environment include the wearing of protective clothing, increased hand washing; and targeted education of the population at risk on possible sources of STEC infection.

6.5.1 Between-island divergence

The North and South Islands of New Zealand are separated by the Cook Strait, a geographic barrier of >20 km, which might contribute to the island-associated differences in distribution of genotypes observed in this study, by restricting the movement of carrier animals between islands. Although there are large cattle populations on each island (about 6.6 million in the North Island and 3.5 million in the South Island [1]), cattle livestock movements between the islands from farm-to-farm or farm-to-slaughter (>42,400 cattle from North Island to South Island; and >64,600 cattle from South Island to North Island per year [460]) are relatively low, and likely to have a limited influence on the distinct distribution of genotypes across both islands.

Although none of the bovine meat isolates were SBI typed, the PFGE data showed a strong island-associated distribution of bovine STEC O157:H7 genotypes, equivalent to the patterns observed in faecal isolates from cattle and humans. As bovine meat isolates were recovered from carcass swabs and bulk meat samples collected at beef processing plants, it could be hypothesised that fresh beef meat might be an important exposure pathway for humans. However, although various food sources (including beef) were considered as potential risk factors during a nationwide prospective case-control study on sporadic STEC infections in humans, food was not identified as a significant exposure pathway of infections in New Zealand [459] (Chapter 5).

6.5.2 Regional divergence

Significant genetic variation was observed among human isolates at the regional level, indicating a more localised spatial clustering of STEC O157:H7 genotypes. Strong regional variation in the prevalence of zoonotic diseases has been observed previously in New Zealand. For example, there is marked regional variation in the distribution of serotypes in human cases of salmonellosis, with *Salmonella enterica* ser. Brandenburg associated with sheep and human cases in the southern regions of the South Island [461], whereas the wild-

bird associated *S. enterica* ser. Typhimurium DT 160 is distributed more evenly across the whole country [462]. *S. enterica* ser. Brandenburg has not been found to be endemic in any other regions in New Zealand, and it is likely the spatial pattern of disease is influenced by environmental factors, such as the presence and density of local maintenance hosts or the local climate.

Although cattle are considered the most likely maintenance host of STEC O157:H7, and the association between human cases and cattle density suggests spill-over from cattle to humans as the most important pathway [459] (Chapter 5), the pathogen has frequently been isolated from sheep [176, 463, 464] and deer [193, 209] overseas. Cookson *et al*. [322, 323] identified STEC serotypes of public health concern in sheep from the lower North Island of New Zealand but did not isolate STEC O157:H7. No nationwide studies of sheep or deer have been undertaken in New Zealand, hence sheep cannot be ruled out as potential maintenance hosts for region-specific populations of STEC O157:H7.

The regional clustering of STEC O157:H7 genotypes observed in human isolates leads to another hypothesis that other (yet unidentified) hosts could be reservoir/maintenance hosts in the epidemiology of STEC, with the possibility that cattle are only serving as 'bridging hosts' at the human-animal interface [465], causing STEC infections in humans. For example, starlings have been implicated as biological vectors in the dissemination of STEC among dairy farms in Ohio (USA) [466, 467], indicating that wildlife might play an important role in the epidemiology and ecology of STEC.

6.5.3 International divergence

A relatively high prevalence of SBI types AY2a and ASY2c was observed in human and bovine faecal isolates from New Zealand. These findings are in strong contrast to the Australian study by Mellor *et al.* [270], where SBI type AY2a was not identified (0/284, $p < 0.001$) and accounted for only 8.1% (26/322) of the isolates from the United States (human and cattle combined); SBI type ASY2c was prevalent in less than 1.0% of combined isolates in both countries. These differences in frequency distributions of SBI types indicate marked differences in the population structure of SBI types between countries. Australia and New Zealand are neighbouring countries but separated by the Tasman Sea; a distance of about 1,250 km. Based on historical data, Australia has been the predominant source of imported

New Zealand cattle, mainly in the 19th century [452]. Hence, the distinct geographical divergence of STEC O157:H7 genotypes between both countries is somewhat puzzling and would suggest a limited historical introduction of STEC O157:H7 from Australia or elsewhere into New Zealand and a subsequent evolution in the New Zealand host population. Alternatively, the observed divergence of genotypes between Australia and New Zealand could be the result of genetic drift and/or selection driven by different environmental factors, such as climate, types of feed, husbandry systems, or animal genetics.

In this study, the highest PSI was observed between cattle and human isolates from New Zealand, followed by Australia, and the United States. These findings provide evidence for a close association between cattle and human populations of isolates, which is consistent with the transmission of STEC from cattle to humans. This is in agreement with the national case-control study on clinical STEC cases in New Zealand, which identified variables related to beef and dairy cattle as significant risk factors [459] (Chapter 5).

6.6 Conclusions

The molecular analysis of STEC O157:H7 isolates from cattle and clinical cases revealed distinct geographical prevalence distributions of both bovine and human isolates between the North and South Islands of New Zealand, suggesting localised transmission of STEC between cattle and humans. Furthermore, a different distribution of STEC O157:H7 genotypes compared to that observed overseas suggests a historical introduction of a subset of the globally-circulating STEC O157:H7 strains into New Zealand.

6.7 Acknowledgments

We thank Muriel Dufour, Brent Gilpin, Kari Gobius, and Glen Mellor for their contributions; and Charlotte Bolwell for very helpful comments on this manuscript.

This work was supported by the Meat Industry Association of New Zealand (Inc), Wellington; and the Ministry for Primary Industries, Wellington, New Zealand.

Chapter 7

General discussion

This thesis has encompassed two main research areas; to advance our knowledge on the epidemiology of Shiga toxin-producing *Escherichia coli* (STEC) serogroups O157 and O26 in New Zealand slaughter cattle, and to investigate the source attribution of human STEC infections in New Zealand. For the first time, valuable data have been generated on the nationwide prevalence of STEC O157 and O26 faecal carriage in slaughter cattle (very young (bobby) calves and adult cattle) and the levels of *E. coli* and STEC entering the food chain via slaughtered animals (Chapter 3). The identification of calves as more prevalent carriers of STEC compared to adult cattle led to the subsequent investigation of the impact of transportation and lairage on the faecal shedding and carcass contamination with *E. coli* O157 and O26 (STEC and non-STEC) in calves by conducting a cohort study (Chapter 4). It was the first of its kind for this age group of slaughter animals and findings highlighted critical control points in the contamination of veal carcasses with *E. coli* O157 and O26. Rather than food, environmental and animal contact were identified as significant exposure pathways for sporadic STEC infections in humans in a nationwide prospective case-control study (Chapter 5), suggesting ruminants as the most important sources of STEC infection in New Zealand. Further evidence for the localised transmission of STEC between cattle and human in New Zealand was provided in a molecular epidemiological study (Chapter 6), comparing the geographical distribution of genotypes of bovine and human STEC O157 isolates.

As many aspects of the studies were deliberated extensively in the discussions of each study chapter, including methodologies and limitations, the following sections discuss some of the main findings of each study and how the study outcomes are interrelated.

7.1 Prevalence of STEC in New Zealand cattle (Chapter 3)

To understand the epidemiology of STEC O157 and O26 and their importance to public health in New Zealand, the prevalence study was conducted to assess the significance of cattle

as a reservoir of these pathogens across New Zealand and generate basic but essential epidemiological data for the design of further epidemiological studies. Compared to previous surveys in slaughter cattle conducted in the North Island of New Zealand [321, 324], the study provided evidence of STEC O157 and STEC O26 prevalence on both islands of New Zealand, hence cattle present a possible source of STEC infection in humans through animal contact, contaminated food, or environmental contamination. Although STEC O26 was not isolated from adult cattle in the South Island, *E. coli* O26 real-time PCR-positive faecal samples were identified, hence it can be assumed that STEC O26 is present in adult cattle in the South Island. It is possible that some STEC O26 isolates were missed due to their sensitivity to supplements of cefixime and potassium tellurite in CT-RMAC plates [235] or that some cells were viable but non-culturable [237].

7.1.1 Calves – a major source of STEC

Calves were identified as more prevalent carriers of STEC than adult cattle at slaughter and should therefore be considered as a significant source of STEC entering the food chain. Furthermore, calves shed STEC O26 at a higher prevalence compared to STEC O157. It is possible that the gastrointestinal environment of calves is more suitable for colonisation of STEC O26 with *eae* subtype β compared to STEC O157 (*eae* subtype γ), as various subtypes of *eae* may determine host specificity and tissue tropism of different STEC [468-470]; a hypothesis, which could be explored in further studies. In addition to a naïve gut and a less developed gastrointestinal tract of calves, other external factors such as husbandry systems and animal management could account for a higher susceptibility for STEC colonisation and thereby higher prevalence of STEC in calves, as has been reported in studies from overseas [166, 263, 378-380]. It is common practice on New Zealand dairy farms to keep calves of similar age in groups in barns/sheds and send bobby calves for slaughter within the first seven days *post partum*. As animal management and husbandry systems vary between dairy farms in New Zealand, the farm prevalence of STEC in calves could also differ significantly. Further information on animal management practices of calves on dairy farms are required to identify risk factors that contribute to higher prevalence of STEC among calves, particularly bobby calves, which enter the food chain.

7.1.2 Farm level prevalence

A higher STEC prevalence was observed for dairy farms compared to beef farms. However, as only a very small number of animals were tested per farm at slaughter (mostly one or two), the farm-level prevalence values are grossly underestimated and represent estimates of the minimum farm-level prevalence. It is likely that the large proportion of calf samples representing dairy farms (75.0%) accounted for the higher farm-level prevalence observed for dairy farms (6.0%), although the difference in STEC prevalence between the two farming types could also be influenced by animal management, husbandry, and environmental factors. A farm survey of dairy and beef farms in New Zealand would help identifying potential farm-related risk factors that might contribute to higher STEC prevalence estimates at the farm level.

7.1.3 Seasonality

The monthly collection of faecal samples over a two-year study period enabled the observation of temporal trends of STEC O157 and O26 in adult cattle. Consistent with findings in previous studies from overseas [176, 177, 377], the STEC O157 prevalence peaked in summer/autumn. However, we also observed a second peak in spring and it can be speculated whether this increase in faecal shedding of STEC O157 is associated with the seasonal calving on dairy farms across the country. The phenomenon of seasonal shedding is still not understood and different factors related with animals and/or the environment have been proposed to explain it. These include the increased presence of young stock (high shedders) over summer periods [165, 183, 184]; physiological changes within the host animal due to extended day-light [471]; and the pathogen's increased replication in the environment (e.g. feed or sediments in water troughs) exposing cattle more frequently to levels of infectious doses of STEC O157 [184]. Compared to the observed bimodal shedding pattern of STEC O157, STEC O26 peaked only in summer. A national survey of 338 cattle farms in Scotland has also observed seasonal patterns for *E. coli* serogroups O26, O103, and O145, with highest percentage of positive farms prevalent in the summer period [319]. Hence, these observations raise the question of whether there are differences in the ecology of STEC O157 and O26, if STEC O26 have a different host-pathogen interaction compared to STEC O157, or if this seasonality is affected by specific genotypes. Whole genome sequencing or other molecular genotyping methods equivalent to SBI would provide further molecular data (e.g.

identification of some markers) to better understand the seasonality of STEC O157 and STEC O26 in adult cattle.

7.2 Carcass contaminations with *E. coli* at calf processing (Chapter 4)

Several outbreaks of STEC infection in humans overseas have been associated with the consumption of undercooked and cross-contaminated meat and meat products, most likely faecally contaminated at slaughter [22, 23, 25, 26]. Having identified calves compared to adult cattle as more likely shedders of STEC at slaughter (Chapter 3), the transport and lairage study was conducted to evaluate the impact of transportation and lairage on the rectal carriage of *E. coli* O157 and O26 in calves at slaughter and the levels of carcass contamination through meat processing; it was the first study of its kind conducted in slaughter animals of such young age. Data collected from the prevalence study (Chapter 3) and this study will be used to inform decision making in the meat industry to minimise the public health risks associated with the consumption of potentially STEC-contaminated red meat produced in New Zealand.

7.2.1 Contamination of hides and carcasses at pre-intervention

Despite the relatively small number of selected farms and calves used in the study, the results indicated that transportation and lairage do not increase the faecal shedding of *E. coli* O157 and O26 in calves at slaughter, but result in increased cross-contamination of hides and carcasses post-slaughter with *E. coli* O26 from high- to low-risk animals and *E. coli* (O157 and O26) from unknown sources. Hides have been identified as the major source of adult cattle carcass contamination with STEC O157 and other pathogens of public health concern [283-289]. Although transportation and lairage have been associated with increased hide contamination at slaughter [300-302], this could not be determined in the study as comparable hide samples were not collected from calves pre- and post-transportation given the resources available. Similarly, sources of unknown *E. coli* O157 and O26 strains detected on hides and carcasses at pre- and post-intervention could not be identified, as environmental samples were not collected from either transport trucks or the lairage area. Therefore, further studies are required to determine if hide contamination occurs predominantly on farm, or during transportation and lairage in order for the design of effective strategies to control/reduce the microbial load on hides and the subsequent potential contamination of veal.

In addition to cross-contamination of carcasses at pre-intervention (after dehiding) with *E. coli* from undetermined sources, an increased cross-contamination from high- to low-risk calves was observed for *E. coli* O26, but could not be confirmed for *E. coli* O157. It is likely this was associated with the higher faecal prevalence of *E. coli* O26 in calves, similar to observations in the prevalence study (Chapter 3) where the culture prevalence of *E. coli* O26 at slaughter was significantly higher compared to *E. coli* O157.

7.2.2 Contamination at post-intervention

Despite the increased cross-contamination of hides with *E. coli* O26 from high- to low-risk calves, this increased prevalence was not evident on carcasses at post-intervention, highlighting the need of effective interventions during meat processing to reduce *E. coli* contamination of carcasses and the potential risks of foodborne STEC infection in humans, as complete prevention of carcass contamination at post-slaughter is difficult to achieve. However, commonly used interventions such as steam, hot water, acetic acid or lactic acid treatment only generate bacterial reductions of less than two orders of magnitude on carcasses [304], emphasising that the residual contamination of carcasses is dependent on the bacterial load at pre-intervention and associated with the level of hide contamination. Hence, control strategies should also focus on the reduction of hide contamination of animals at pre-slaughter to enhance the microbiological safety of meat produced.

7.2.3 *E. coli* genotypes

Although STEC are the main concern for foodborne disease in humans, all types of *E. coli* O157 and O26 isolates were collected in the study to investigate the population dynamics of these two serogroups. Similar to isolates obtained from calves in the prevalence study (Chapter 3), most of *E. coli* O157 isolates carried STEC-associated virulence factors (*stx*1 and/or *stx*2, *eae*, and *ehx*A) and a large variety of genotypes was observed among *E. coli* O26 isolates. However in this study, only a few isolates were STEC O26 (*stx*1, *eae*, *ehx*A) and aEPEC O26 (*eae*, *ehx*A), while a large proportion of isolates were rhamnose-fermenting *E. coli* O26 and harboured *eae* only or none of the virulence factors tested (*stx*, *eae*, *ehx*A). It is noteworthy that rhamnose-fermenting *E. coli* O26 isolates lacking *stx*, *eae*, and *ehx*A were more prevalent in this study compared to the prevalence study (Chapter 3). As this study was conducted in the Waikato region, a large dairy farming area in the central part of the North

Island of New Zealand, it is possible that this rhamnose-fermenting *E. coli* O26 genotype is specific for the region or the participating farms. Although this clonal group does not possess common STEC-associated virulence factors, it should not be underestimated as bacteria can acquire virulence factors via horizontal gene transfer [84] and may evolve to more virulent strains of public health importance.

7.3 Source and exposure pathways of STEC infections in humans (Chapter 5)

With no large outbreaks of STEC being reported in New Zealand, the spatial distribution of sporadic STEC cases or small clusters of STEC infections has suggested that infections might be associated with farming [94, 317]. The national prospective case-control study was conducted to assess the relative importance of cattle as a source of STEC infection and gain epidemiological knowledge on the source and exposure pathways for domestically-acquired sporadic STEC infections.

7.3.1 Risk factors

The public health significance of STEC in New Zealand is high. Although the number of annual STEC notifications is comparatively low (217 confirmed STEC cases in 2013 [316]), it has increased steadily over the past two decades. Based on previous case investigations, contact with pets, farm animals or animal manure, and consumption of contaminated food were reported as most likely sources of infection, however, only some of these putative sources could be confirmed as potential risk factors for infection in our study. Food products of animal origin have been confirmed as vehicles of disease transmission in case-control studies of sporadic STEC infections overseas [9, 10, 24, 38], but no food product (e.g. dairy products, beef or beef products, raw fruit or vegetables) could be identified as a significant risk factor for disease in this study.

'Contact with animal manure', 'Living in a meshblock with cattle being present', 'Contact with recreational waters', or 'Travelling to areas with interrupted or no main water supply', for example, were identified as risk factors for STEC disease, providing strong evidence that sporadic STEC infections in New Zealand are associated with exposure to animal and/or environmental sources of infection, most likely originating from dairy and beef livestock.

Other researchers have also identified exposures to farming environments as risk factors for sporadic STEC infections in case-control studies conducted in Argentina [37], North America [24], England [334], and Germany [38], while contact with animal/cattle manure have been identified as risk factors for sporadic STEC O157 infections in Scotland [68], and North America [439].

The risk factor analysis was conducted on STEC cases including all STEC serogroups. As the majority (88.5%) of cases were caused by STEC O157, strength of associations and significance of variables remained relatively unchanged, when the final multivariate logistic regression model was applied to STEC O157 cases only (except that variable 'Contact with recreational waters' became non-significant). Due to a very low number of non-O157 STEC cases (n = 13), a separate risk factor analysis was not conducted in this study but could reveal further important information whether risk factors for disease for STEC O157 and non-O157 STEC differ. There is only one case-control study that has reported risk factors for sporadic STEC infection, in which the majority (85%) of cases were infected with strains of non-O157 STEC serogroups [38], describing age-specific risk factors. 'Having touched ruminant' and 'Consuming raw milk' were identified as risk factors in children <3 years of age, while only food products (i.e., mutton, and raw spreadable sausages) were associated with disease in cases aged ≥10 years. To identify season-specific risk factors and potentially elucidate the seasonal occurrence of sporadic STEC infections, a risk factor analysis stratified by season could be conducted but was not considered in this study due to the small number of cases per season.

7.3.2 Seasonality

Similar to previous surveillance reports, the incidence of sporadic STEC cases followed a seasonal pattern and peaked in summer/autumn [427]. It is likely the seasonality of STEC disease is related to the seasonal variation in the prevalence of faecal shedding of STEC in cattle, as it was observed in the prevalence study (Chapter 3) and has been reported in other studies overseas [177, 377]. In addition, some of the seasonality of STEC cases could be explained by the increased outdoor activities in recreational waters (recreational waters was a risk factor; e.g. creeks, streams, and rivers) during the warmer summer months, which are potentially contaminated with ruminant faecal material containing STEC from pasture run off.

7.3.3 Molecular findings

Molecular typing methods such as PFGE and SBI were applied to STEC O157 isolates from cases to support findings from the risk factor analysis. Significant associations were observed between SBI types and both 'Season' and 'Contact with animal manure', providing further evidence of an animal/environmental-associated pathway of sporadic STEC infection in New Zealand. Furthermore, three dominant SBI genotypes were observed, including 1 (AY2a), 3 (WY12a), and 5 (ASY2c/ASWY2c/SY2c), of which SBI type 5 was overrepresented in 0–4 year-old children. As SBI type 5 harbours only *stx*2c, it can be hypothesised that this genotype may possess host-adapted characteristics to affect specifically the immature gastrointestinal tract of children, compared to other genotypes. Although differential virulence of STEC O157 strains have been studied in a piglet model [205] suggesting that STEC O157 strains possessing *stx*2c alone are likely to be less pathogenic compared to strains harbouring *stx*2c and *stx*1, investigating a larger number of SBI-typed STEC O157 isolates from <5 year-old children would certainly provide more information as to whether SBI type 5 is a predominant genotype affecting young children.

In addition, SBI type 5 was associated with the South Island of New Zealand, while SBI types 1 and 3 were more prevalent in the North Island. A subset of bovine STEC O157 isolates (collected in the prevalence study (Chapter 3) and used in the molecular epidemiological study (Chapter 6)) was SBI typed in advance and therefore available for a molecular comparison with SBI types from humans in this study. The preliminary results of bovine SBI types also showed a predominant prevalence of SBI type 5 in the South Island, and SBI types 1 and 3 in the North Island, indicating localised transmission of STEC O157 between cattle and humans.

7.4 Transmission of STEC O157 between cattle and humans (Chapter 6)

Findings from the prospective case-control study (Chapter 5) strongly indicated that cattle are a source of STEC infection in humans in New Zealand. To support these observations and assess evidence for localised transmission of STEC from cattle to humans, bovine and human STEC O157 isolates were compared in a molecular epidemiological study, investigating the population structure and geographical distribution of genotypes from bovine and human

sources in New Zealand using SBI and PFGE typing methods. Furthermore, New Zealand genotypes were compared with those from overseas to illustrate the international divergence of STEC O157 genotypes circulating in bovine and human populations in different parts of the world.

7.4.1 Selection of isolates

STEC isolates of serogroup O157 were chosen for the study as this serogroup causes the majority of clinical STEC infections in New Zealand; only a few STEC O26 cases have been reported in the past. To increase the number of bovine faecal STEC O157 isolates (collected in the prevalence study (Chapter 3)), additional faecal isolates were used from a preceding survey of calves at two New Zealand slaughter plants [324]. Human isolates were selected to correspond with the sampling period from which bovine faecal isolates were obtained to match the likely exposure period of humans to STEC from cattle.

The molecular analysis of bovine STEC O157 isolates prevalent in New Zealand was extended in this study by including bovine meat isolates retrieved from carcass swabs and bulk meat samples at slaughter plants (post-intervention) during routine mandatory testing (National Microbiological Database (NMD) microbiological monitoring programme). The bovine meat isolates were also selected based on the sampling period of cattle at slaughter. Interestingly, the majority of these meat isolates originated from slaughter plants in the North Island and could be associated with the higher faecal prevalence of STEC O157 observed in calves and adult cattle in the North Island compared to the South Island (Chapter 3). It can be argued whether these meat isolates were a subset of isolates, which were able to survive intervention methods applied on at the slaughter plants, contributing to a lower prevalence distribution of strains found in meat samples. Current intervention methods used at New Zealand beef slaughter plants are steam vacuum, acidification, and hot water wash. Lee *et al.* [472] have studied resistance to acid, freeze-thaw, heat, osmotic, oxidative, and starvation stresses in STEC O157 isolates from clinical cases in Japan and concluded that strains possessing Shiga toxin-encoding genes *stx*1 and *stx*2, or *stx*2 and *stx*2c were more likely to be stress resistant than other genotypes. This could, to some extent, explain the higher prevalence of WY12-like SBI type (carrying *stx*1 and *stx*2) observed among bovine meat isolates (23.4%) compared to faecal isolates from cattle (15.0%) and humans (13.8%).

7.4.2 Between-island divergence

Molecular findings of the human case-control study indicated (Chapter 5) a distinct between-island distribution of SBI genotypes among bovine faecal, bovine meat, and human isolates in this study; AY2a and WY12a genotypes were more prevalent in the North Island and ASY2c/SY2c more prevalent in the South Island, indicating a localised transmission of STEC from cattle to humans. Although the bovine meat isolates were not SBI typed, the assigning of SBI-like genotypes based on PFGE/SBI clusters of bovine faecal and human isolates was shown to be valid, as a similar between-island distribution of SBI genotypes was observed. The island-associated differences in distribution of SBI genotypes could be attributable to the Cook Strait, a geographic barrier separating the North and South Islands of New Zealand, restricting the movements of carrier animals between the islands but could also be the result of genetic drift and/or selection driven by different environmental factors, such as climate and/or husbandry systems, or potentially the presence of an intermediate host(s).

7.4.3 Regional divergence

Analysis of molecular variance (AMOVA) was used on PFGE profiles of bovine meat and human isolates to assess the population differentiation between islands, between regions within island, and within regions, but was not applied on bovine faecal isolates as the number of isolates was considered too small to provide meaningful results. The strong regional variation observed among human isolates indicated a more localised spatial clustering of genotypes, suggesting the presence of a localised maintenance host(s). The application of AMOVA on a larger number of bovine faecal isolates from different regions across New Zealand would possibly have provided more information to confirm cattle as the most likely maintenance host of STEC O157. Another possibility is the presence of other potential (yet unidentified) maintenance hosts. STEC O157 has frequently been isolated from sheep [176, 463, 464], deer [193, 209], and other wildlife overseas [200, 202, 203, 466, 467]. Cernicchiaro *et al.* [213] described in their study that European starlings (*Sturnus vulgaris*) contributed to the transmission of STEC O157 between dairy farms in northern Ohio (US), identifying starlings as potential vectors for STEC O157 transmission between farms. These reports highlight the need of nationwide studies on the epidemiology of STEC O157 in sheep and farmed deer, and other potential maintenance hosts, such as starlings, wild rabbits, feral goats and pigs, to better understand the epidemiology of STEC infections in New Zealand.

7.4.4 International divergence

The international comparison of SBI types of bovine faecal and human isolates from New Zealand, Australia, and the US showed a marked difference in the population structure of SBI types between the countries, with fewer SBI types associated with New Zealand isolates compared to a larger frequency distribution of SBI types among US isolates. The proportional similarity index (PSI) illustrated the close association between cattle and human STEC O157 populations and was very high for New Zealand isolates, providing further evidence of transmission of STEC from cattle to humans. The lower frequency distribution of SBI genotypes among New Zealand isolates could be the result of a limited introduction of clones of STEC O157 into New Zealand with imported cattle from Australia in the 19th century [452], or the evolution of selected genotypes driven by different environmental factors, such as climate, types of feed, husbandry systems, or animal genetics. Further studies would be needed to investigate the origins and evolution of the currently circulating genotypes in New Zealand.

7.5 Implications of research

Findings from this PhD research have advanced our knowledge on the epidemiology of STEC O157 and O26 in New Zealand slaughter cattle. The studies have provided important data but also highlighted areas of high importance for further research. In consequence, the Ministry for Primary Industries (MPI) and the Meat Industry Association (MIA) have funded a further PhD project in collaboration with ^{m}EpiLab, Massey University, to conduct further observational studies on dairy farms in New Zealand with extended focus on the currently most important STEC serogroups O157, O26, O45, O103, O111, O121, and O145.

Furthermore, outcomes of the case-control study (Chapter 5) have been used by the organisation 'Rural Women New Zealand' to produce a newsletter and inform the community on the risks associated with STEC infections in New Zealand.

7.6 Areas for further research

Studies presented in this thesis have provided basic but important epidemiological data and should therefore be regarded as foundation studies. There are several areas that warrant

further research to advance our understanding of the epidemiology of STEC under New Zealand conditions, including the following:

- Case-control study of New Zealand dairy farms to identify risk factors associated with higher prevalence of STEC among calves, particularly bobby calves, which enter the food chain;

- A repeated cross-sectional study of dairy and beef farms across New Zealand investigating within-farm and between-farm prevalence of STEC, to establish the true prevalence of STEC and to identify farm-related risk factors that contribute to higher STEC prevalence estimates at the farm level;

- A longitudinal study on selected dairy and beef farms across New Zealand to describe the dynamics of STEC infections in different age groups of animals on farms, including between-animal transmission of STEC infections;

- Investigating the role of 'supershedders' on dairy and beef farms in a longitudinal study;

- Nationwide cross-sectional studies in sheep and deer to assess their importance as ruminant reservoirs of STEC and potential sources of STEC infections in humans;

- Investigating the presence of STEC in other potential intermediate hosts, e.g. starlings, and the identification of other reservoir/maintenance or spill-over hosts for STEC to better understand the epidemiology of STEC;

- Investigating the origins of current STEC lineages circulating in New Zealand using whole genome sequencing and phylodynamic and phylogeographic techniques.

7.7 Concluding statement

Findings presented in this thesis have already advanced our understanding of the epidemiology of STEC O157 and O26 in New Zealand and will have major implications for both the public health and the red meat industry. Development of interventions to reduce or

minimise the exposures to STEC from animal and environmental sources will help to lower the occurrence of STEC infections in New Zealand, while improvements in the controls of STEC in food processing will enhance New Zealand's reputation overseas and help minimising risks associated with the export of red meat and red meat products. Despite this, further epidemiological investigations are needed, especially on cattle farms, to give further insight into the complex ecology of STEC in animals and the environment, as many aspects of this zoonotic pathogen are still unknown.

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Appendices

Appendix 1: Univariate logistic regression analysis of risk factors for calves being tested real-time PCR-positive (rt PCR pos) for *E. coli* O157 or O26 (STEC and non-STEC).

	O157					O26				
Variable	rt PCR pos	rt PCR neg	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value	rt PCR pos	rt PCR neg	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Sex										
Male	128	384	Ref	-	-	167	345	Ref	-	-
Female	35	148	–0.34 (0.21)	0.71 (0.47–1.08)	0.109	65	118	0.13 (0.18)	1.14 (0.80–1.62)	0.475
Breed					0.819*					0.135*
Friesian	108	361	Ref	-	-	168	301	Ref	-	-
Jersey	53	167	0.06 (0.19)	1.06 (0.73–1.55)	0.759	62	158	–0.35 (0.18)	0.70 (0.50–1.00)	0.048
Other breeds	2	4	0.51 (0.87)	1.67 (0.30–9.25)	0.556	2	4	–0.11 (0.87)	0.90 (0.16–4.94)	0.900
Farm type										
Dairy	163	526	Ref	-	-	228	461	Ref	-	-
Beef	0	6	–14.39 (594.16)	0 (0–2.77)	0.981	4	2	1.4 (0.87)	4.04 (0.74–22.24)	0.108
Island										
North	120	375	Ref	-	-	167	328	Ref	-	-
South	43	157	–0.16 (0.20)	0.86 (0.58–1.27)	0.440	65	135	–0.06 (0.18)	0.95 (0.67–1.34)	0.754
Plant					<0.001*					<0.001*
A	78	207	Ref	-	-	126	159	Ref	-	-
B	42	168	–0.41 (0.218)	0.66 (0.43–1.02)	0.060	41	169	–1.18 (0.211)	0.31 (0.20–0.46)	<0.001
C	33	67	0.27 (0.251)	1.31 (0.80–2.14)	0.285	31	69	–0.57 (0.247)	0.57 (0.35–0.92)	0.022
D	10	90	–1.22 (0.359)	0.29 (0.15–0.60)	0.001	34	66	–0.43 (0.243)	0.65 (0.40–1.05)	0.076
Month					0.865*					0.006*
July	33	99	Ref	-	-	57	75	Ref	-	-
August	97	318	–0.09 (0.23)	0.92 (0.58–1.44)	0.702	138	277	–0.42 (0.20)	0.66 (0.44–0.98)	0.039
September	33	115	–0.15 (0.28)	0.86 (0.50–1.50)	0.595	37	111	–0.82 (0.26)	0.44 (0.26–0.73)	0.001
Presence of the other serotype										
No	75	388	Ref	-	-	144	388	Ref	-	-
Yes	88	144	1.15 (0.19)	3.16 (2.20–4.54)	<0.001	88	75	1.15 (0.18)	3.16 (2.20–4.54)	<0.001

[a] SE = Standard error. [b] CI = 95% confidence interval. * p-value of variable as whole using Likelihood ratio test. Ref = reference level for comparison.

Appendix 2: Univariate logistic regression analysis of risk factors for adult cattle being tested real-time PCR-positive (rt PCR pos) for *E. coli* O157 or O26 (STEC and non-STEC).

	O157					O26				
Variable	rt PCR pos	rt PCR neg	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value	rt PCR pos	rt PCR neg	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Sex					0.016*					0.852*
Male	5	168	Ref	-	-	12	161	Ref	-	-
Male castrated	33	322	1.24 (0.49)	3.44 (1.32–8.98)	0.012	26	329	0.06 (0.36)	1.06 (0.52–2.16)	0.872
Female	25	342	0.90 (0.50)	2.46 (0.92–6.53)	0.072	30	337	0.18 (0.35)	1.19 (0.60–2.39)	0.617
Breed					0.095*					0.896*
Friesian	21	255	Ref	-	-	20	256	Ref	-	-
Jersey	2	73	–1.10 (0.75)	0.33 (0.08–1.45)	0.143	5	70	–0.09 (0.52)	0.91 (0.33–2.52)	0.863
Angus	27	287	0.13 (0.30)	1.14 (0.63–2.07)	0.661	22	292	–0.04 (0.32)	0.96 (0.51–1.81)	0.910
Hereford	6	153	–0.74 (0.47)	0.48 (0.19–1.21)	0.118	14	145	0.21 (0.36)	1.24 (0.61–2.52)	0.560
Other breeds	7	64	0.28 (0.46)	1.33 (0.54–3.26)	0.536	7	64	0.34 (0.46)	1.40 (0.57–3.45)	0.465
Age (in years)					0.542*					0.147*
≤2.25	33	405	Ref	-	-	26	412	Ref	-	-
2.5–3.0	8	154	–0.45 (0.41)	0.64 (0.29–1.41)	0.267	15	147	0.48 (0.34)	1.62 (0.83–3.14)	0.155
3.25–3.75	4	71	–0.37 (0.54)	0.69 (0.24–2.01)	0.498	4	71	–0.11 (0.55)	0.89 (0.30–2.63)	0.837
≥4.0	18	202	0.09 (0.31)	1.09 (0.60–1.99)	0.770	23	197	0.62 (0.30)	1.85 (1.03–3.32)	0.040
Farm type										
Dairy	17	213	Ref	-	-	17	213	Ref	-	-
Beef	46	619	–0.07 (0.29)	0.93 (0.52–1.66)	0.809	51	614	0.04 (0.29)	1.04 (0.59–1.84)	0.891
Island										
North	29	417	Ref	-	-	38	408	Ref	-	-
South	34	415	0.16 (0.26)	1.18 (0.70–1.97)	0.532	30	419	–0.26 (0.25)	0.77 (0.47–1.26)	0.300

Appendix 2 continued

	O157					O26				
Variable	rt PCR pos	rt PCR neg	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value	rt PCR pos	rt PCR neg	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Plant					0.259*					0.335*
A	13	199	Ref	-	-	14	198	Ref	-	-
B	16	218	0.12 (0.39)	1.12 (0.53–2.39)	0.763	24	210	0.48 (0.35)	1.62 (0.81–3.21)	0.171
C	24	225	0.49 (0.36)	1.63 (0.81–3.29)	0.171	15	234	–0.10 (0.38)	0.91 (0.43–1.92)	0.798
D	10	190	–0.22 (0.43)	0.81 (0.35–1.88)	0.617	15	185	0.14 (0.39)	1.15 (0.54–2.44)	0.722
Season					<0.001*					0.513*
Spring	17	138	Ref	-	-	14	141	Ref	-	-
Summer	11	261	–1.07 (0.40)	0.34 (0.16–0.75)	0.007	24	248	–0.03 (0.35)	0.97 (0.49–1.94)	0.942
Autumn	31	242	0.04 (0.32)	1.04 (0.56–1.95)	0.903	16	257	–0.47 (0.38)	0.63 (0.30–1.32)	0.220
Winter	4	191	–1.77 (0.57)	0.17 (0.06–0.52)	0.002	14	181	–0.25 (0.39)	0.78 (0.36–1.69)	0.526
Presence of the other *E. coli* serotype										
No	48	779	Ref	-	-	53	779	Ref	-	-
Yes	15	53	1.52 (0.33)	4.59 (2.41–8.74)	<0.001	15	48	1.52 (0.33)	4.59 (2.41–8.74)	<0.001

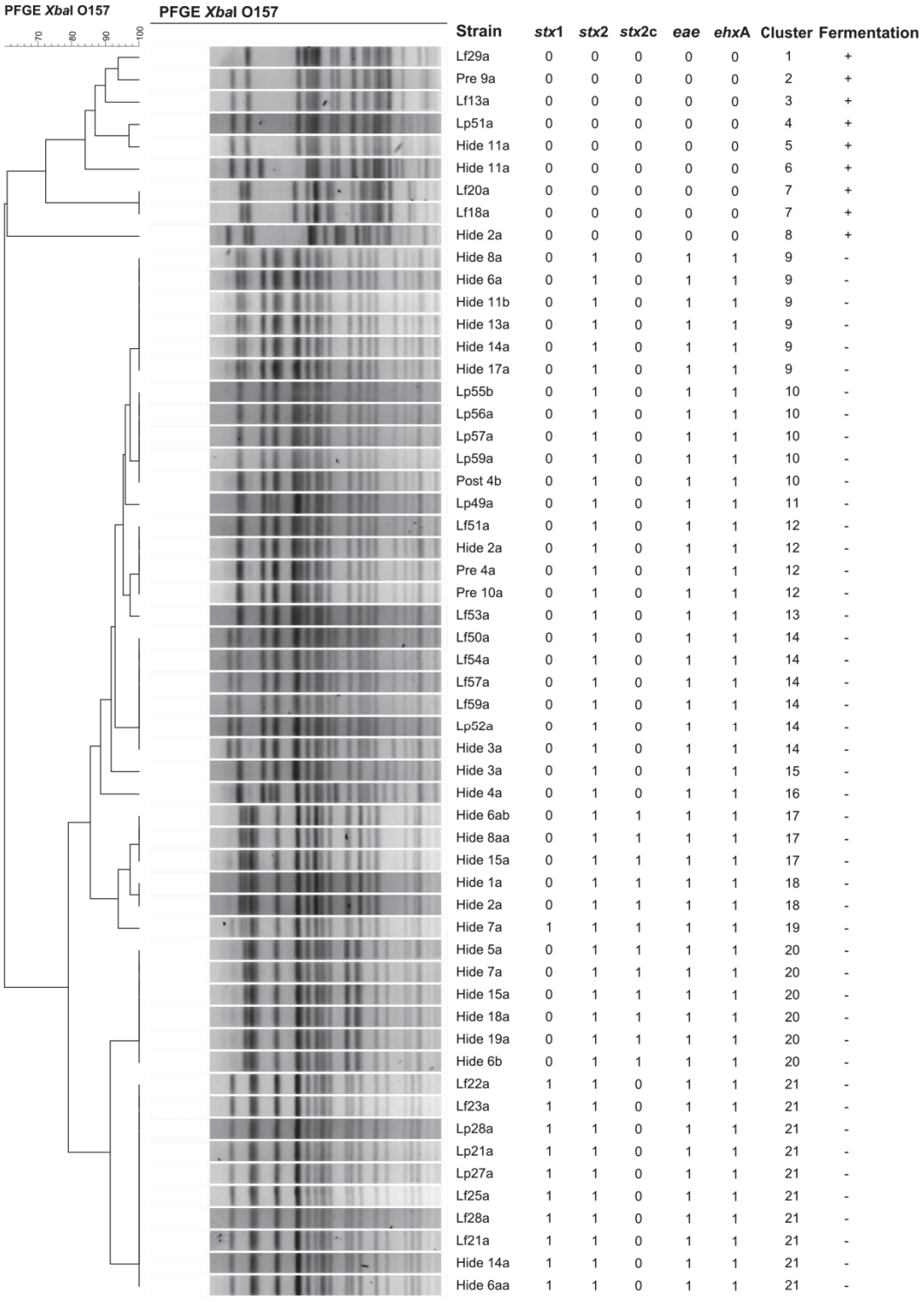
[a] SE = Standard error.

[b] CI = 95% confidence interval.

* p-value of variable as whole using Likelihood ratio test.

Ref = reference level for comparison.

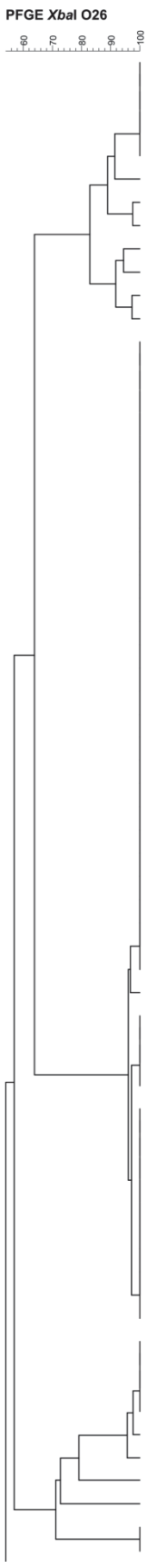
Appendix 3: PFGE profile comparison of *E. coli* O157 isolates.



Strain	*stx*1	*stx*2	*stx*2c	*eae*	*ehx*A	Cluster	Fermentation
Lf29a	0	0	0	0	0	1	+
Pre 9a	0	0	0	0	0	2	+
Lf13a	0	0	0	0	0	3	+
Lp51a	0	0	0	0	0	4	+
Hide 11a	0	0	0	0	0	5	+
Hide 11a	0	0	0	0	0	6	+
Lf20a	0	0	0	0	0	7	+
Lf18a	0	0	0	0	0	7	+
Hide 2a	0	0	0	0	0	8	+
Hide 8a	0	1	0	1	1	9	-
Hide 6a	0	1	0	1	1	9	-
Hide 11b	0	1	0	1	1	9	-
Hide 13a	0	1	0	1	1	9	-
Hide 14a	0	1	0	1	1	9	-
Hide 17a	0	1	0	1	1	9	-
Lp55b	0	1	0	1	1	10	-
Lp56a	0	1	0	1	1	10	-
Lp57a	0	1	0	1	1	10	-
Lp59a	0	1	0	1	1	10	-
Post 4b	0	1	0	1	1	10	-
Lp49a	0	1	0	1	1	11	-
Lf51a	0	1	0	1	1	12	-
Hide 2a	0	1	0	1	1	12	-
Pre 4a	0	1	0	1	1	12	-
Pre 10a	0	1	0	1	1	12	-
Lf53a	0	1	0	1	1	13	-
Lf50a	0	1	0	1	1	14	-
Lf54a	0	1	0	1	1	14	-
Lf57a	0	1	0	1	1	14	-
Lf59a	0	1	0	1	1	14	-
Lp52a	0	1	0	1	1	14	-
Hide 3a	0	1	0	1	1	14	-
Hide 3a	0	1	0	1	1	15	-
Hide 4a	0	1	0	1	1	16	-
Hide 6ab	0	1	1	1	1	17	-
Hide 8aa	0	1	1	1	1	17	-
Hide 15a	0	1	1	1	1	17	-
Hide 1a	0	1	1	1	1	18	-
Hide 2a	0	1	1	1	1	18	-
Hide 7a	1	1	1	1	1	19	-
Hide 5a	0	1	1	1	1	20	-
Hide 7a	0	1	1	1	1	20	-
Hide 15a	0	1	1	1	1	20	-
Hide 18a	0	1	1	1	1	20	-
Hide 19a	0	1	1	1	1	20	-
Hide 6b	0	1	1	1	1	20	-
Lf22a	1	1	0	1	1	21	-
Lf23a	1	1	0	1	1	21	-
Lp28a	1	1	0	1	1	21	-
Lp21a	1	1	0	1	1	21	-
Lp27a	1	1	0	1	1	21	-
Lf25a	1	1	0	1	1	21	-
Lf28a	1	1	0	1	1	21	-
Lf21a	1	1	0	1	1	21	-
Hide 14a	1	1	0	1	1	21	-
Hide 6aa	1	1	0	1	1	21	-

PFGE profile comparison of *E. coli* O157 isolates (n = 56) performed using UPGMA cluster analysis, Dice similarity coefficient, and 1% band matching tolerance. *stx*1, *stx*2, *stx*2c (encoding for Shiga toxins), *eae* (encoding for intimin), and *ehx*A (encoding for enterohaemolysin) virulence genes present (1) or absent (0). PFGE cluster numbers ranging from 1 to 21. Fermentation of sorbitol present (+) or absent (–). Lf, Lp, Hide, Pre, and Post represent samples taken on-farm, on-plant, from hide, at pre-intervention, and post-intervention, respectively.

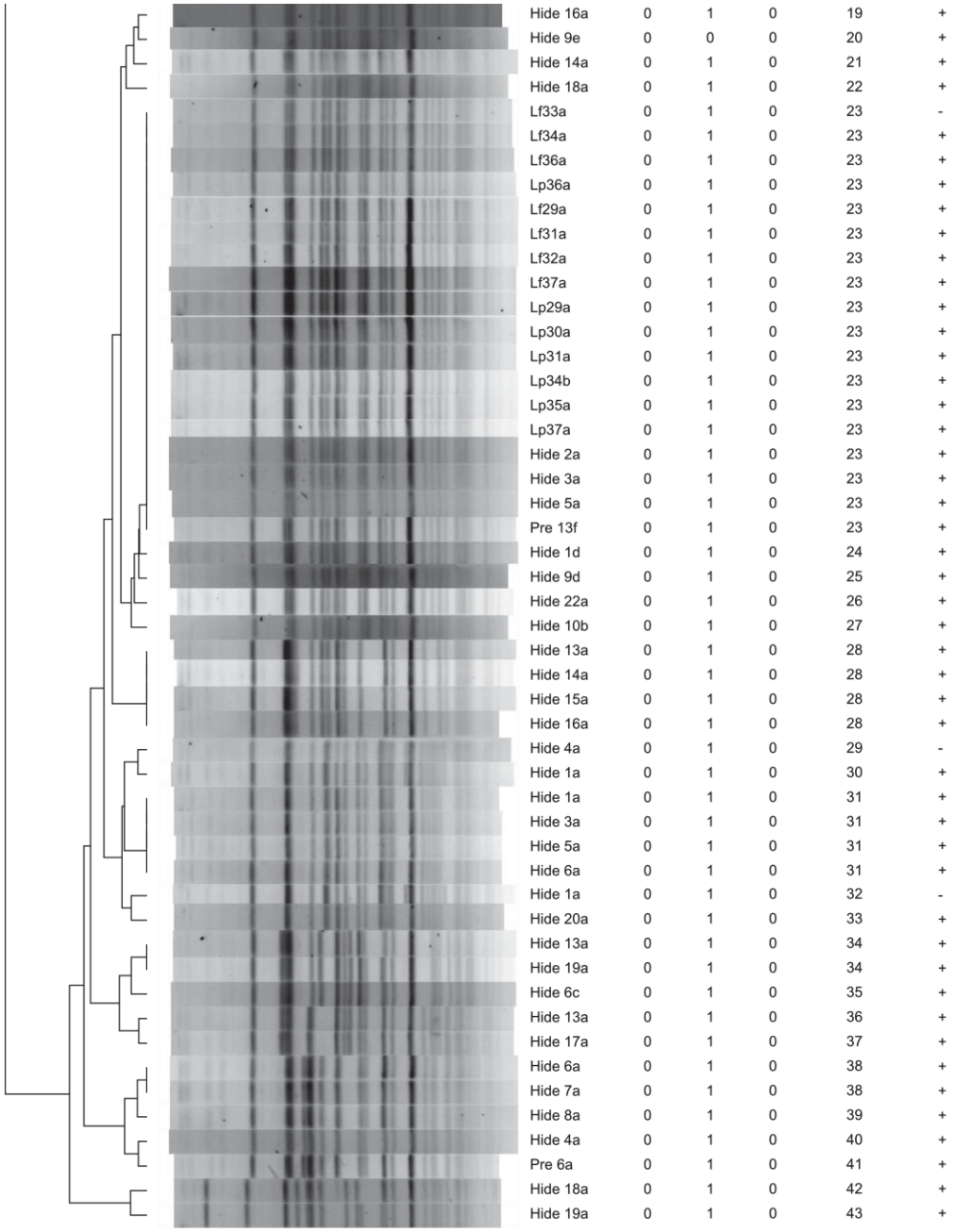
Appendix 4: PFGE profile comparison of *E. coli* O26 isolates.



PFGE *Xba*I O26

Strain	*stx*1	*eae*	*ehx*A	Cluster	Fermentation
Hide 11a	0	1	1	1	-
Hide 15a	0	1	1	1	-
Hide 1a	0	1	1	1	-
Hide 8e	0	1	1	1	-
Pre 13a	0	1	1	1	-
Lf22a	0	1	1	2	-
Lf30a	0	1	0	3	-
Hide 5d	0	1	1	4	-
Hide 9a	0	1	1	5	-
Hide 12a	0	1	1	6	-
Hide 10a	0	1	1	7	-
Hide 10a	0	1	1	8	-
Lf11a	0	0	0	9	+
Lf12a	0	0	0	9	+
Lf15a	0	0	0	9	+
Lf16a	0	0	0	9	+
Lf17a	0	0	0	9	+
Lf18a	0	0	0	9	+
Lf19a	0	0	0	9	+
Lf20a	0	0	0	9	+
Lp11a	0	0	0	9	+
Lp12a	0	0	0	9	-
Lp13a	0	0	0	9	-
Lp16a	0	0	0	9	+
Lp17a	0	0	0	9	+
Lp18a	0	0	0	9	+
Hide 9a	0	0	0	9	+
Hide 10b	0	0	0	9	+
Pre 10a	0	0	0	9	+
Post 7a	0	0	0	9	+
Hide 2a	0	0	0	9	+
Hide 3a	0	0	0	9	+
Hide 5h	0	0	0	9	+
Hide 7a	0	0	0	9	+
Hide 18a	0	0	0	9	+
Pre 3a	0	0	0	9	+
Pre 11a	0	0	0	9	+
Pre 15a	0	0	0	9	+
Hide 4a	0	0	0	9	+
Post 3a	0	0	0	9	+
Lf13a	0	0	0	10	+
Hide 12a	0	0	0	11	+
Hide 16a	0	0	0	11	+
Pre 17a	0	0	0	11	+
Post 12a	0	0	0	11	+
Lf14a	0	0	0	12	+
Lp14a	0	0	0	12	+
Lp15a	0	0	0	12	+
Lp19a	0	0	0	12	+
Lp2a	0	0	0	12	+
Hide 6b	0	0	0	12	+
Hide 13c	0	0	0	12	+
Hide 8a	0	0	0	12	+
Pre 9a	0	0	0	12	+
Post 19a	0	0	0	12	+
Hide 14a	1	1	1	13	-
Hide 17a	1	1	1	13	-
Pre 14a	1	1	1	13	-
Post 8a	1	1	1	13	-
Pre 15a	1	1	1	14	-
Hide 5a	1	1	1	15	-
Hide 12b	1	1	1	16	-
Hide 9a	1	1	1	17	-
Hide 5e	1	1	1	18	-
Pre 4a	1	1	1	18	-

Appendix 4 continued



PFGE profile comparison of *E. coli* O26 isolates (n = 115) performed using UPGMA cluster analysis, Dice similarity coefficient, and 1% band matching tolerance. *stx*1 (encoding for Shiga toxin), *eae* (encoding for intimin), and *ehx*A (encoding for enterohaemolysin) virulence genes present (1) or absent (0). PFGE cluster numbers ranging from 1 to 43. Fermentation of rhamnose present (+) or absent (–). Lf, Lp, Hide, Pre, and Post represent samples taken on-farm, on-plant, from hide, at pre-intervention, and post-intervention, respectively.

Appendix 5: Published article.

Jaros et al. BMC Infectious Diseases 2013, 13:450
http://www.biomedcentral.com/1471-2334/13/450

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

A prospective case–control and molecular epidemiological study of human cases of Shiga toxin-producing *Escherichia coli* in New Zealand

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Abstract

Background: Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 and related non-O157 STEC strains are enteric pathogens of public health concern worldwide, causing life-threatening diseases. Cattle are considered the principal hosts and have been shown to be a source of infection for both foodborne and environmental outbreaks in humans. The aims of this study were to investigate risk factors associated with sporadic STEC infections in humans in New Zealand and to provide epidemiological information about the source and exposure pathways.

Methods: During a national prospective case–control study from July 2011 to July 2012, any confirmed case of STEC infection notified to regional public health units, together with a random selection of controls intended to be representative of the national demography, were interviewed for risk factor evaluation. Isolates from each case were genotyped using pulsed-field gel electrophoresis (PFGE) and Shiga toxin-encoding bacteriophage insertion (SBI) typing.

Results: Questionnaire data from 113 eligible cases and 506 controls were analysed using multivariate logistic regression. Statistically significant animal and environmental risk factors for human STEC infections were identified, notably 'Cattle livestock present in meshblock' (the smallest geographical unit) (odds ratio 1.89, 95% CI 1.04–3.42), 'Contact with animal manure' (OR 2.09, 95% CI 1.12–3.90), and 'Contact with recreational waters' (OR 2.95, 95% CI 1.30–6.70). No food-associated risk factors were identified as sources of STEC infection. *E. coli* O157:H7 caused 100/113 (88.5%) of clinical STEC infections in this study, and 97/100 isolates were available for molecular analysis. PFGE profiles of isolates revealed three distinctive clusters of genotypes, and these were strongly correlated with SBI type. The variable 'Island of residence' (North or South Island of New Zealand) was significantly associated with PFGE genotype ($p = 0.012$).

Conclusions: Our findings implicate environmental and animal contact, but not food, as significant exposure pathways for sporadic STEC infections in humans in New Zealand. Risk factors associated with beef and dairy cattle suggest that ruminants are the most important sources of STEC infection. Notably, outbreaks of STEC infections are rare in New Zealand and this further suggests that food is not a significant exposure pathway.

Keywords: Prospective case–control study, Sporadic STEC infections, New Zealand, Risk factors, Source attribution, Cattle, Molecular epidemiology, Pathways of infection, Population attributable fractions

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Background

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 and related non-O157 STEC strains are pathogens of public health concern worldwide. They can cause severe outbreaks of gastrointestinal illness with clinical symptoms ranging from diarrhoea and haemorrhagic colitis to the life-threatening haemolytic uraemic syndrome [1]. Ruminants, particularly cattle, are considered to be an important reservoir of STEC, shedding the pathogen via faeces [2-4], and are a primary source of foodborne and environmental outbreaks of STEC in humans [5,6].

Food products of animal and plant origin have been confirmed as vehicles of disease transmission in case–control studies of STEC outbreaks and sporadic STEC infections; these included raw milk [7-9], unpasteurised cheese [10], undercooked hamburgers [11-13], sausages [14,15], leafy lettuce [16] and unpasteurised apple cider [17]. Implicated food vehicles were most commonly contaminated directly or indirectly with ruminant faeces containing STEC before or after processing. Similarly, faecally contaminated recreational waters and water supplies have been identified as environmental sources of human STEC infections [18-20]. Exposures to farming environments have been reported as risk factors of sporadic STEC infections, particularly for young children [8,21].

Improvements in surveillance of STEC infections, following their recognition as serious public health concerns, have resulted in an overall increasing trend of STEC notifications at the international level. Over the past decade, non-O157 STEC cases have been reported more frequently in the USA and the EU [22,23], a feature that might be attributed to improved laboratory methods for isolation of non-O157 STEC serotypes and additional laboratory testing of specimens for non-O157 STECs.

Since 1993, when New Zealand's first case of STEC infection in humans was reported [24], the annual number of notified STEC cases has increased steadily. In 2012, 147 cases of STEC (3.3 cases per 100,000 population) were recorded in the national surveillance database (EpiSurv) used by regional public health units (PHU) to record epidemiological data from notified cases of communicable and other diseases [25]. All but five cases were confirmed by culture isolation and identified as STEC serotypes O157:H7 (83.8%) and non-O157 (16.2%). Although the majority of reported STEC cases in New Zealand are caused by serotype O157:H7, the percentage of non-O157 STEC cases has increased steadily over the past five years from 1.6% in 2008 to 16.2% in 2012.

While STEC infections in New Zealand appear as sporadic cases or small clusters, little is known about the relative importance of cattle as a reservoir, or the relative contribution of different exposure pathways to human cases of STEC.

The primary objective of this study was the identification of risk factors associated with sporadic STEC infections acquired in New Zealand so as to gain epidemiological knowledge on the source and exposure pathways for this disease. A second objective was to conduct a molecular epidemiological investigation of STEC isolates from clinical cases.

Methods

Study design, definition of cases and controls

A national prospective case–control study was conducted in New Zealand from 18th July 2011 to 31st July 2012. A case was defined as a patient with (i) clinical symptoms of diarrhoea and/or haemolytic uraemic syndrome and/or thrombotic thrombocytopaenia purpura, (ii) an onset of clinical disease at a maximum of two weeks prior to being reported to a PHU, (iii) an infection most likely acquired in New Zealand, (iv) confirmed by isolation of STEC from a clinical specimen, and (v) the primary STEC infection in a household. Study cases were interviewed by phone or in person by trained PHU staff using a questionnaire on multiple risk factors potentially associated with STEC infections.

Study controls, intended to be representative of the national demography, were selected randomly from the New Zealand population. An eligible control had to be free of symptoms of diarrhoea or any other gastrointestinal disease at the time of interview, and in the two weeks prior to the interview. Monthly quotas of controls were recruited by a professional survey provider (UMR Research, Wellington, New Zealand) using random landline dialling from the New Zealand phone directory. Controls were contacted in the third week of every month. In each household, the individual with the last birthday was chosen as the study participant. A computer-assisted telephone interview was conducted by a trained team of assigned interviewers using the same questionnaire as that used for cases.

Informed consent was obtained from all study participants before being interviewed. For study cases and controls aged <18 years, a parent or adult caregiver served as the interview respondent after their consent was acquired.

Questionnaire

A standardised questionnaire (Additional file 1) was used to collect data from study cases and controls concerning potential risk factors for infection in the two weeks before onset of disease (cases) and the telephone interview (controls). The questionnaire covered demographic characteristics and exposure categories such as food consumed (treated/raw milk and products thereof, various raw/pink meats, fish, raw fruit and vegetables, and purchased fruit juices), dining locations, supply of drinking water (town supply, private bore, roof run-off, creek, tanker truck),

contact with recreational waters, hunting activities, contacts with animals and humans, recent travels, and medications taken (antibiotic and antacid). To investigate the spatial distribution of study participants, while protecting privacy, cases and controls were asked to name the nearest school to their home to assign their geographical locality. The month and year of interview was recorded to investigate the seasonality of disease. The questionnaire was cognitive and pilot tested.

Sample sizes of cases and controls

Epi Info™ software [26] was used to calculate the sample size for cases and to perform power calculations for three different expected frequencies of exposure among controls. Based on a predicted sample size of 150–170 cases (expected number of cases based on STEC cases notified nationally in two preceding years) and an attempted case to control ratio of 1:3, there was sufficient statistical power (at least 80%) at a confidence level of 95% to detect an odds ratio of 3.0, using 5%, 20% and 80% as the expected frequencies of exposure among controls. Hence, the sample size of controls was set at 506 and included oversampling of children 0–4 years of age ($n = 200$) to provide a similar predicted ratio of cases and controls (1:3) as this age group showed the highest number of reported STEC cases in the past. A monthly quota of 42 controls was interviewed by the survey provider.

STEC isolates of study cases

Clinical cases were confirmed by culture isolation of STEC from clinical specimens submitted to medical laboratories or the Enteric Reference Laboratory (ERL, Institute of Environmental Science & Research Ltd, Upper Hutt, New Zealand). STEC isolates were submitted to ERL for serotyping, testing for the presence of virulence genes (*ehx*A, *eae*, *stx*1, *stx*2), and genotyping using pulsed-field gel electrophoresis (PFGE, restriction enzyme *Xba*I). Isolates were sent to the Molecular Epidemiology and Public Health Laboratory (™EpiLab, Hopkirk Research Institute, Massey University, Palmerston North, New Zealand) for screening for the presence of virulence gene subtype *stx*2c. In addition, *E. coli* O157:H7 isolates were genotyped using Shiga toxin (Stx)-encoding bacteriophage insertion (SBI) typing (Prof. Thomas E. Besser and colleagues at Washington State University, Pullman, USA) [27,28].

Ethical approval

This study was approved by the Multi-region Ethics Committee, Wellington, New Zealand, on 17 June 2011; reference number MEC/11/04/043.

Data management and statistical analysis

R software (version 2.15.2) [29] was used for all statistical analysis, with significance set at $p < 0.05$.

Datasets of cases and controls were screened for completeness prior to analysis. Descriptive statistics were calculated for each study group. To account for potential confounding from imperfect frequency matching on age, the variable 'Age' was categorised by grouping 'pre-school children' (0–4 years), 'children/students' (5–19 years), and 'adults' (>19 years).

To illustrate the spatial distribution of study participants, New Zealand Transverse Mercator coordinates (NZTM2000) of named schools were plotted, using R packages 'maptools' [30] and 'spatstat' [31]. Based on the spatial distribution of cases and controls, a relative risk surface of STEC cases for New Zealand was produced, using R package 'sparr' [32]. To account for spatial heterogeneity, an adaptive estimate was utilised for case and control densities with an average smoothing bandwidth of 50 km. Areas with values >0.0 indicate increased relative risks of STEC infection. For comparison, cattle densities were mapped by regions of New Zealand; using the sum of beef and dairy cattle numbers from 2011 [33] divided by the area (km^2) of each region.

In addition to the data generated by the case–control questionnaires, information on ruminant livestock numbers from a national livestock database [34] was used in two separate analyses. Firstly, additional variables were generated, representing whether particular species of livestock (dairy cattle, beef cattle, sheep, and deer) were farmed in meshblocks (the smallest geographic unit of statistical data collected for Statistics New Zealand) in which the cases and controls resided. These additional variables (presence/absence, numbers and density of each species) were used in the logistic regression analysis of the case–control dataset.

Secondly, in order to extend the analysis of the relationship between ruminant livestock (dairy cattle, beef cattle, sheep, and deer) and the risk of STEC, a separate logistic regression analysis was conducted at the meshblock level. The relationship between ruminant livestock (presence/absence, numbers and density of each species) and the risk of STEC notification in all meshblocks of New Zealand was assessed. In essence, this analysis used the cases from the case–control study, but extended the control set to consider the entire population of New Zealand.

For both logistic regression analyses, ruminant livestock data from 2009 were used as they represented the most reliable recent data that could be linked to geographical boundaries (meshblocks) and the most recent human census data. The last population census (2006) estimated a national human population of 4,027,527.

Multivariate logistic regression model building

Questionnaire answers of "unknown" or "not sure" were treated as missing values of the exposure variables. Exposure variables were analysed using univariate and

multivariate logistic regression to identify risk factors associated with sporadic STEC cases. Exposure variables with Wald test or Likelihood ratio tests p-values <0.20 in univariate analysis were tested for correlation, and included in an initial multivariate model if their correlation values were < ±0.30.

To generate a preliminary multivariate model, stepwise backward- and forward-elimination of least significant variables and those with correlation values of ≥ ±0.30, respectively, was used, while eliminated variables were assessed for confounding. The confounding effect was determined by a change of >30% in a variable coefficient in the model after another variable was dropped from or added to the model. Variables which demonstrated confounding were retained in the model even if they were non-significant. Biologically plausible interactions between variables were assessed to generate the final multivariate model.

To adjust for a proportion of missing values in relevant variables such as 'Contact with animal manure' (cases: $n = 13$, controls: $n = 7$), 'Contact with children wearing nappies' (cases: $n = 6$, controls: $n = 4$), and 'Contact with person vomiting/having gastrointestinal disease' (cases: $n = 10$, controls: $n = 16$), multiple imputations by chained equations [35] were applied on the final multivariate model using R package 'mice' [36]. Likelihood ratio tests and the le Cessie-van Houwelingen normal test statistics [37] were applied to evaluate the model's significance and goodness-of-fit, respectively, using R package 'rms' [38]. Models were compared using the Akaike information criterion (AIC), a measure of the relative goodness of fit.

For the extended analysis of the relationship between ruminant livestock and the risk of STEC at the meshblock level, a second multivariate logistic regression model was built. In this analysis the number of STEC cases out of the population in each meshblock was the outcome variable (a two-column vector of the number of cases out of the population in each meshblock) and variables representing each ruminant species per meshblock were considered as exposure variables (presence/absence, numbers and density).

Population attributable fractions (PAF)

To assess the proportion of sporadic STEC disease in the study population attributable to a specific exposure, the variable's population attributable fraction (PAF_i) was computed. PAF of variables associated with increased risk of STEC infection were estimated using the following formula [39,40]:

$$PAF_i = \frac{p_i(aOR_i - 1)}{aOR_i} \times 100\%,$$

where p_i is the proportion of all study cases within a categorical variable and a reference category denoted by $i = 1$, and aOR_i is the adjusted variable-specific odds ratio derived from the final multivariate model. Medians and 95% credible intervals were computed from 1,000 simulations as described in Stafford *et al.* [39].

Molecular analysis of *E. coli* O157:H7 isolates

PFGE profiles of the clinical *E. coli* O157:H7 isolates were analysed and compared using BioNumerics software (version 6.6) [41] to create a dendrogram applying UPGMA (unweighted pair group method with arithmetic mean) cluster analysis using the Dice similarity coefficient, with a band matching tolerance of 1%.

Fisher's exact test was used to evaluate the association between SBI genotypes and exposure variables considered in the multivariate logistic regression analysis. A distance matrix of isolates' PFGE profiles was generated in BioNumerics and linked with exposure variables, to analyse the molecular relatedness of isolates and estimate the proportional contribution of these variables to the molecular variation. Multidimensional scaling plots and permutational multivariate analysis of variance (PERMANOVA+, version 1.0.4) were used for this analysis using Primer 6 (version 6.1.14) [42].

Results

Study population, spatial and temporal epidemiology

A total of 123 STEC cases meeting the case definition were notified to PHUs during the study period. Eight cases refused to participate in the study, thus resulting in a 93.5% response rate. Two potential cases were excluded, one due to a high probability of having acquired the infection overseas and the other due to severe illness. Therefore, 113 STEC cases were included in this study, of which 75 (66.4%) were interviewed by phone and 35 (31.0%) were visited by PHU staff; the interviewing methods of three cases were unknown. The majority of STEC cases (83.2%, 94/113) were interviewed within 0–5 days of notification (12 cases within 6–9 days, four cases within 10–12 days, and three cases within 21 days). Of these 113 STEC cases, 100 (88.5%) were *E. coli* O157:H7 and 13 (11.5%) were non-O157 STEC.

To recruit 506 controls, including 200 0–4 year-old children, a total of 7864 phone calls were made. Contact was established for 66.8% (5254/7864) of phone calls and of those contacted, 62.2% (3266/5254) were interested in participating in the study (response rate). From 3266 interested respondents, 84.5% (2760/3266) were not eligible mainly because of not fitting the required monthly quota of 0–4 year-olds (93.6% (2583/2760)), or not meeting the selection criteria.

Males comprised 52.2% (59/113) of cases and 42.9% (217/506) of controls. The median age of cases and controls were 7.0 years (interquartile range 2.0–29.0) and 11.5 years (IQR 3.0–58.0), respectively. The age and

spatial distribution of cases and controls are shown in Figure 1.

The proportional distributions of participants stratified by age categories were: 46.0% cases (52/113) and 40.3% controls (204/506) for 0–4 year-old pre-school children; 20.4% cases (23/113) and 12.1% controls (61/506) for 5–19 year-old children/students; and 33.6% cases (38/113) and 47.6% controls (241/506) for >19 year-old adults.

The temporal distribution of cases during the study period showed a peak in summer/autumn (January until April), with no cases reported in July 2011 (Figure 2).

Based on the spatial distribution of cases and controls across New Zealand (Figure 1), increased relative risk estimates of STEC infections were observed in regions such as Northland, Waikato, Taranaki, Canterbury, and Southland, while reduced risks were found in high density urban areas in the Auckland and Wellington regions (Figure 3A). For comparison, areas with high ruminant livestock densities are shown in Figure 3B and Additional file 2.

Risk factors

Bivariate logistic regression results (adjusted for age categories) are provided in the supplementary material (Additional file 3). Statistically significant risk factors and confounding variables ('Eating seafood', 'Dining outside home', 'Water supply to home from private bore/spring/creek/or stream', 'Contact with children wearing nappies', and 'Taking antacids') of the final multivariate logistic regression model with imputations are presented in Table 1. The equivalent final model without imputation is provided in the supplementary material (Additional file 4).

Animal and environmental exposures were identified as risk factors for sporadic STEC infections including; 'Other household member having contact with animals other than household pets' for pre-school children aged 0–4, 'Cattle livestock present in meshblock', 'Contact with animal manure', 'Contact with recreational waters', and 'Travelled to areas in New Zealand with interrupted or no main water supply'. Food items such as 'Drinking refrigerated fruit juice from supermarket' and 'Eating raw vegetables' were identified as having a protective effect rather than being risk factors for STEC infections. When the final multivariate logistic regression model was applied to *E. coli* O157:H7 cases only, the strength of associations and significance of variables remained relatively unchanged (data not shown), except for the variable 'Contact with recreational waters', which became non-significant (adjusted odds ratio 2.13, 95% CI 0.84–5.42, $p = 0.112$). This could be explained by a higher proportion of non-O157 cases (30.8%, 4/13) being exposed to this risk factor compared to O157 cases (11.0%, 11/100).

For the multivariate analysis considering cases and the whole population at the meshblock level, the univariate logistic model identified significant associations between STEC and dairy cattle, beef cattle, and sheep, where variables with different functional forms were considered including presence/absence, numbers of animals and densities per km^2. The best fitting variables, in terms of AIC, were presence/absence of cattle and sheep. These included presence of beef cattle (odds ratio 2.45, 95% CI 1.65–3.59, Wald test p-value <0.001, AIC = 1556.9), presence of dairy cattle (OR 2.14, 95% CI 1.27–3.42, $p = 0.003$, AIC = 1567.5), presence of all cattle (OR 2.40, 95% CI 1.62–3.52, p <0.001, AIC = 1557.6), and presence of sheep (OR 1.98, 95% CI 1.29–2.97, $p = 0.001$, AIC = 1565.7). When considered in multivariate models, there was strong confounding and collinearity between these variables but only species combinations of beef cattle with dairy cattle (Likelihood ratio test p-value <0.001,

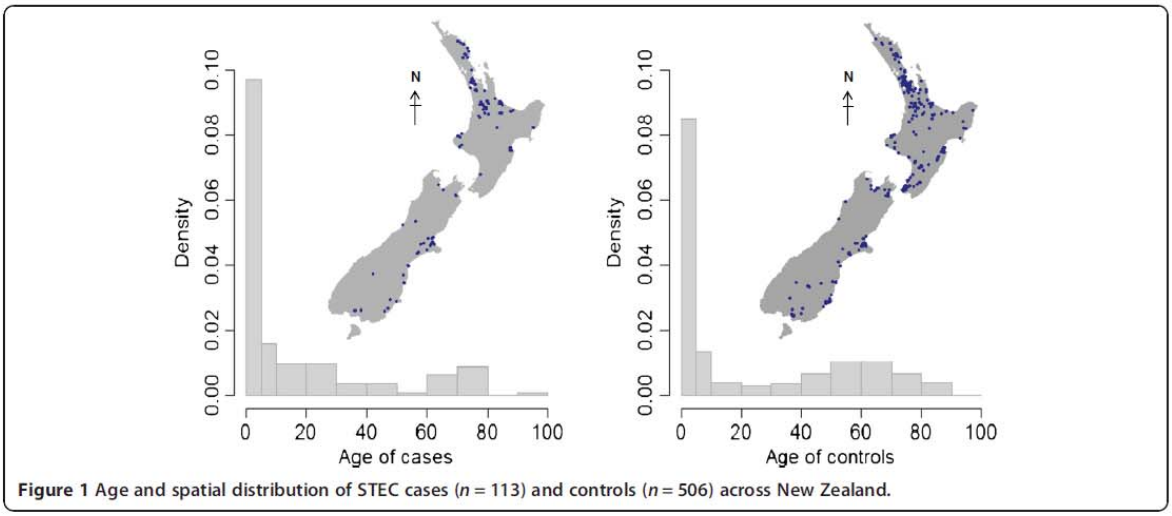


Figure 1 Age and spatial distribution of STEC cases ($n = 113$) and controls ($n = 506$) across New Zealand.

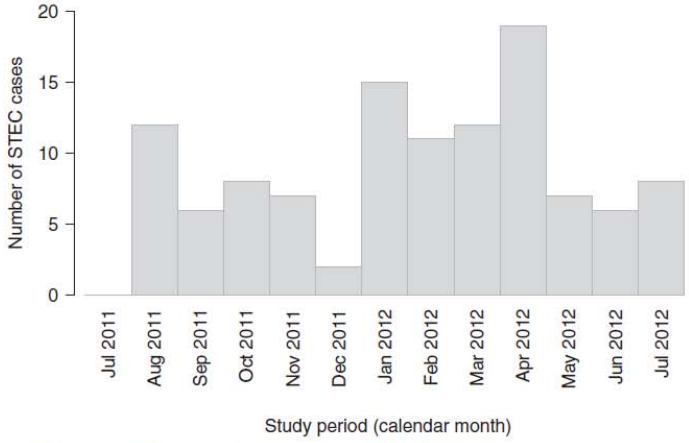


Figure 2 Temporal distribution of sporadic STEC cases ($n = 113$) from July 2011 to July 2012.

AIC = 1558.9), and dairy cattle with sheep ($p = 0.005$, AIC = 1566.6) provided biologically meaningful results. According to lowest AIC, the variable presence of beef cattle fitted the best.

Population attributable fractions (PAF)

PAF of exposure variables associated with increased risk for sporadic STEC infections (Table 1) are summarised in Table 2. The interaction term 'Other household member having contact with animals other than household pets' for 0–4 year-old children, 'Cattle livestock present in meshblock' and 'Contact with animal manure' showed the highest estimated proportions that could be attributed to STEC infections in the study population.

Molecular analysis of *E. coli* O157:H7 isolates

E. coli O157:H7 and non-O157 STECs, as confirmed by isolation, caused 100/113 (88.5%) and 13/113 (11.5%) of the STEC infections, respectively. The non-O157 STECs were of serogroups O26, O84, O103, O123, O176, O180, and ONT (O serogroup not typable). Only 97/100 O157:H7 isolates and their PFGE profiles were available for molecular analysis; PFGE profiles of non-O157 STEC isolates were not available. The most frequent SBI types

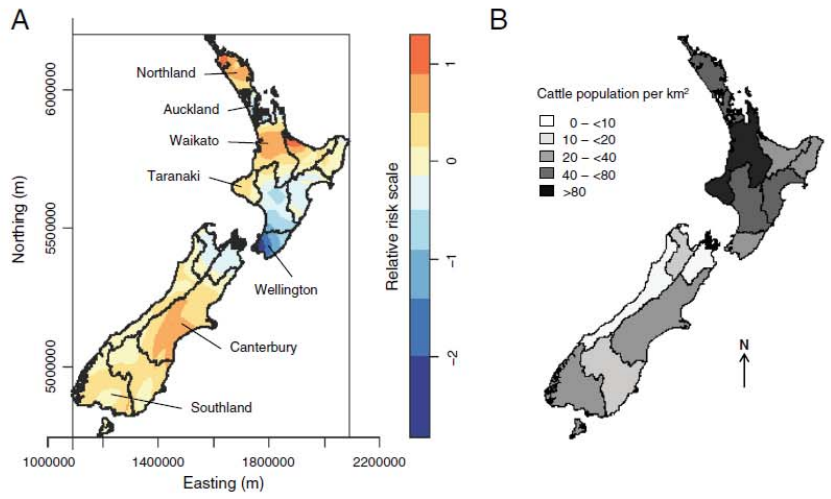


Figure 3 (A) **Relative risk estimates of sporadic STEC infection across New Zealand and (B) cattle density from 2011. (A)** The bivariate kernel density plot shows estimates of relative risks of STEC infection across regions in New Zealand. Values >0 on the relative risk scale indicate increased risk of infection. **(B)** Total cattle density (dairy and beef animals/km^2) from 2011 is shown to indicate areas with high cattle densities. Similar plots of the densities of dairy, beef, sheep, and deer are provided in the supplementary material (Additional file 2).

Table 1 Multivariate logistic regression model showing risk factors for sporadic cases of STEC infections in New Zealand

Variable	Coefficient (SE)[a]	Adjusted odds ratio (95% CI)[b]	*p*-value[c]
Other household member having contact with animals other than household pets[d]*			
for 0–4 year-old	1.39 (0.42)	4.03 (1.78–9.13)	0.001
for 5–19 year-old	−0.77 (0.67)	0.47 (0.13–1.72)	0.251
for >19 year-old	0.30 (0.49)	1.35 (0.51–3.56)	0.541
Cattle livestock present in meshblock	0.64 (0.30)	1.89 (1.04–3.42)	0.037
Contact with animal manure	0.74 (0.32)	2.09 (1.12–3.90)	0.021
Contact with recreational waters	1.08 (0.42)	2.95 (1.30–6.70)	0.010
Travelled to areas in New Zealand with interrupted or no main water supply	0.89 (0.43)	2.43 (1.04–5.65)	0.040
Handling raw offal	−0.94 (0.35)	0.39 (0.20–0.78)	0.008
Drinking refrigerated fruit juice from supermarket	−1.37 (0.32)	0.25 (0.14–0.47)	<0.001
Visiting childcare/kindergarten/or school	−0.92 (0.31)	0.40 (0.22–0.73)	0.003
Eating raw vegetables	−0.65 (0.33)	0.52 (0.27–0.99)	0.046
Eating seafood	−0.52 (0.27)	0.59 (0.35–1.02)	0.057
Dining outside home	−0.44 (0.29)	0.65 (0.37–1.14)	0.133
Water supply to home from private bore/spring/creek/or stream	0.62 (0.39)	1.85 (0.86–4.00)	0.117
Contact with children wearing nappies	−0.33 (0.31)	0.72 (0.39–1.33)	0.298
Taking antacids	−0.84 (0.58)	0.43 (0.14–1.34)	0.147

Likelihood ratio test = 153.70 (df = 18, $p < 0.001$).
[a]Standard error.
[b]95% confidence interval.
[c] *p*-values were computed based on 50 imputations.
[d]This variable was modelled using a multiplicative interaction term comprising the variables 'Other household member having contact with animals other than household pets' and 'Age'.
It can be interpreted as follows: a child 0–4 years of age is at significantly higher risk of being an STEC case, if another household member had contact with animals other than household pets, compared to a child of the same age without this risk factor.
*p-value = 0.064 for the variable 'Other household member having contact with animals other than household pets' without the interaction term.

of *E. coli* O157:H7 isolates were 1 (55/97, 56.7%), 3 (17/97, 17.5%), and 5 (20/97, 20.6%); equivalent to SBI genotypes AY2, WY12, and ASY2c/ASWY2c/SY2c, respectively, according to the recently proposed coding system by Shringi *et al.* [27]. All isolates of SBI type 1 (AY2) carried the *stx*2a gene, while all SBI type 3 (WY12) had both the *stx*2a and *stx*1 genes; all SBI type 5 (ASY2c/SY2c) contained only the *stx*2c gene.

PFGE profiles of the 97 human *E. coli* O157:H7 isolates were compared (Figure 4). The two small clusters of indistinguishable PFGE profiles (two clusters of seven and eight isolates) were not concurrent in space and time and therefore do not present clusters of infections or small outbreaks.

Four statistically significant relationships were observed between SBI types and exposure variables considered in the multivariate logistic regression analysis of the case–control study. These were SBI type vs. 'Age' with SBI type 5 isolates being overrepresented in 0–4 year-old children (Fisher's exact test, $p = 0.009$); SBI type vs. 'Island of residence' with SBI type 5 isolates being associated with the South Island ($p = 0.017$); SBI type vs. 'Season' with SBI type 3 isolates being overrepresented in autumn ($p = 0.034$), and SBI type vs. 'Contact with animal manure' with SBI type 3 isolates being associated with direct exposure to animal manure ($p = 0.047$).

The molecular relatedness between PFGE profiles of *E. coli* O157:H7 isolates considering SBI types, age of cases and island of residence is shown in Figure 5. PFGE profile clusters were strongly associated with SBI types 1, 3, and 5 (Figure 5A). The cluster containing SBI type 5 was more prevalent in pre-school children (0–4 years) (Figure 5B) and in the South Island (Figure 5C), while SBI types 1 and 3 were found more frequently in the North Island (Figure 5C).

PERMANOVA analysis results in Table 3 show the proportional contribution of variables to the molecular variation of PFGE profiles of the *E. coli* O157:H7 isolates. Other than SBI type, only island of residence explained a significant amount of the variation in PFGE profiles in multivariate models.

Discussion

This study was designed to identify risk factors associated with domestically-acquired sporadic STEC infections in humans in New Zealand. The results strongly

Table 2 Population attributable fractions (PAF in %) with 95% credible intervals (CrI in %) of identified risk factors

Variable	Cases (n)	Proportion of cases (p)	Adjusted odds ratio[a]	PAF (95% CrI)[b]
For children 0–4 years old: Other household member having contact with animals other than household pets				
No	28	0.549	Ref	-
Yes	23	0.451	4.03	16.82 (9.0–23.7)
Cattle livestock present in meshblock				
No	74	0.655	Ref	-
Yes	39	0.345	1.89	18.20 (0.6–29.4)
Contact with animal manure				
No	66	0.660	Ref	-
Yes	34	0.340	2.09	17.47 (4.4–27.7)
Contact with recreational waters				
No	97	0.866	Ref	-
Yes	15	0.134	2.95	9.41 (2.7–16.5)
Travelled to areas in NZ with interrupted or no main water supply				
No	96	0.865	Ref	-
Yes	15	0.135	2.43	8.17 (0.7–15.7)
Water supply to home from private bore/spring/creek/or stream				
No	89	0.802	Ref	-
Yes	22	0.198	1.85	9.46 (−2.5–18.8)

[a]Adjusted odds ratios were derived from multivariate logistic regression analysis model using multiple imputations by chained equations (Table 1).
[b]PAF and 95% CrI were computed based on 1000 simulations.
Ref = reference level for comparison.

suggest that direct exposure to animal and/or environmental sources of infection, most likely originating from dairy and beef livestock, is the most important contributor to the burden of sporadic STEC cases observed in New Zealand. No food items were identified as risk factors for sporadic STEC cases in this study.

New Zealand–an agricultural country

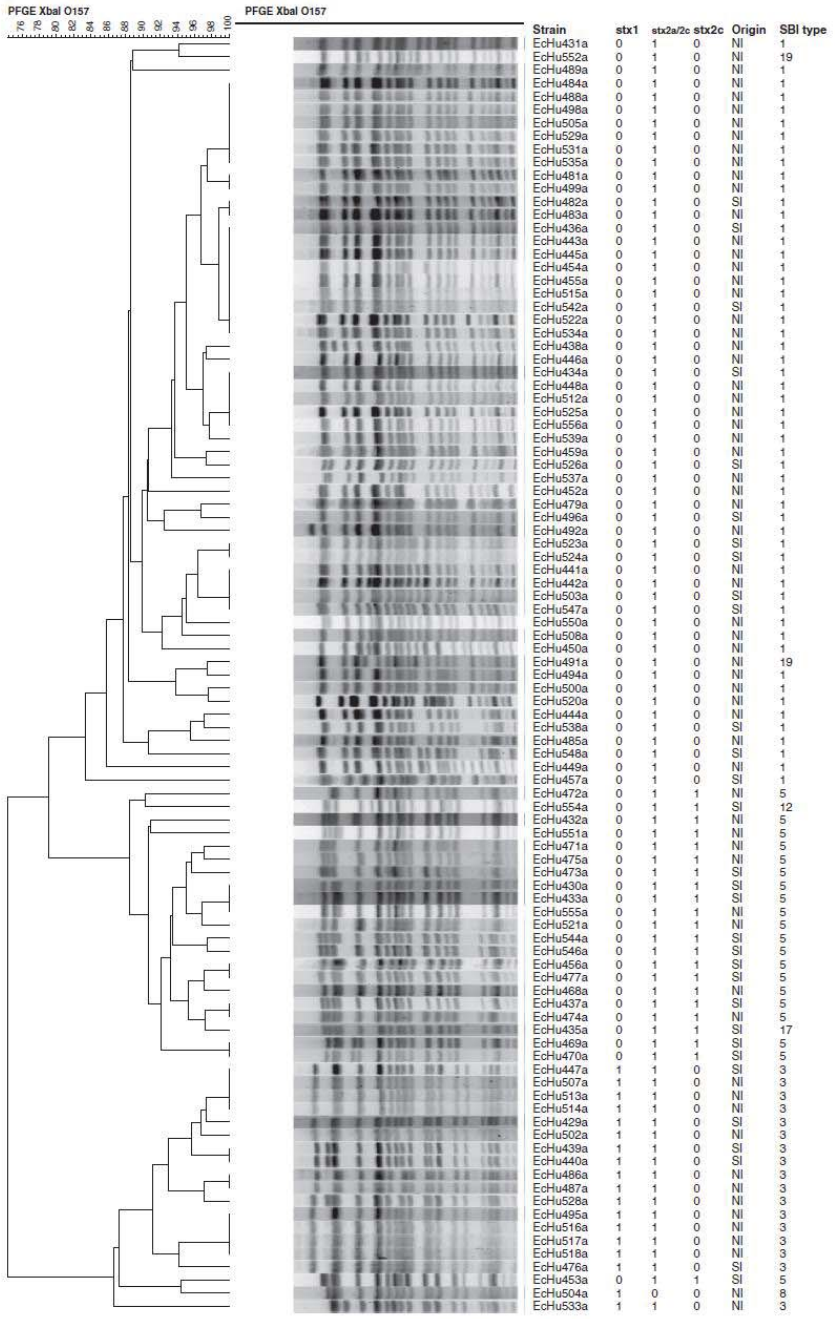
To interpret our findings in context, it is essential to recognise that agriculture is New Zealand's largest primary industry sector contributing to approximately 48% of New Zealand's export earnings in 2009 [43]. In 2011, 6.1 million dairy cattle, 3.8 million beef cattle, 31.1 million sheep, and 1.0 million deer were recorded in New Zealand. By contrast the estimated human population was approximately 4.4 million with 14% living in rural areas and only about 1.2% working in the agricultural industry [33]. Pastoral agriculture is the predominant land use in New Zealand with dairy cattle farming in the flatter and/or wetter areas in Northland, Waikato, Taranaki, and Manawatu in the North Island; and Canterbury, West Coast, Otago, and Southland in the South Island; while sheep and beef cattle farming are practiced in hill and high country areas across both islands.

Spatial and temporal epidemiology

The highest number of reported STEC infections in this study was in the youngest age category (children aged 0–4 years), which is consistent with New Zealand's health surveillance reports [44-46], and the number of cases peaked in summer/autumn (January until April) [47].

The seasonality of cases is likely to be associated with environmental exposure during the warmer season, such as increased outdoor activities in recreational waters potentially contaminated with STEC from ruminant livestock, but could also be related to the seasonal variation in the prevalence of faecal shedding of STEC in cattle. These phenomena were observed in The Netherlands [48], Great Britain [49], and also in New Zealand during a recent two-year cross-sectional study conducted at four slaughter plants across the country [50]. Similarly, a recent multinational systematic review of seasonality in human zoonotic enteric diseases [51] confirmed a strong summer peak for STEC incidence.

The spatial distribution of sporadic STEC cases across New Zealand has suggested that infections might be associated with farming [45,46,52]. We observed increased relative risks of STEC infections in dairy farming regions (Northland, Waikato, Taranaki, Canterbury, and Southland), however, it was not possible to consider dairy and beef farming separately in the analysis of the



Strain	stx1	stx2a/2c	stx2c	Origin	SBI type
EcHu431a	0	1	0	NI	1
EcHu552a	0	1	0	NI	19
EcHu489a	0	1	0	NI	1
EcHu484a	0	1	0	NI	1
EcHu488a	0	1	0	NI	1
EcHu498a	0	1	0	NI	1
EcHu505a	0	1	0	NI	1
EcHu529a	0	1	0	NI	1
EcHu531a	0	1	0	NI	1
EcHu535a	0	1	0	NI	1
EcHu481a	0	1	0	NI	1
EcHu499a	0	1	0	NI	1
EcHu482a	0	1	0	SI	1
EcHu483a	0	1	0	NI	1
EcHu436a	0	1	0	SI	1
EcHu443a	0	1	0	NI	1
EcHu445a	0	1	0	NI	1
EcHu454a	0	1	0	NI	1
EcHu455a	0	1	0	NI	1
EcHu515a	0	1	0	NI	1
EcHu542a	0	1	0	SI	1
EcHu522a	0	1	0	NI	1
EcHu534a	0	1	0	NI	1
EcHu438a	0	1	0	NI	1
EcHu446a	0	1	0	NI	1
EcHu434a	0	1	0	SI	1
EcHu448a	0	1	0	NI	1
EcHu512a	0	1	0	NI	1
EcHu525a	0	1	0	NI	1
EcHu556a	0	1	0	NI	1
EcHu539a	0	1	0	NI	1
EcHu459a	0	1	0	NI	1
EcHu526a	0	1	0	SI	1
EcHu537a	0	1	0	NI	1
EcHu452a	0	1	0	NI	1
EcHu479a	0	1	0	NI	1
EcHu496a	0	1	0	SI	1
EcHu492a	0	1	0	NI	1
EcHu523a	0	1	0	SI	1
EcHu524a	0	1	0	SI	1
EcHu441a	0	1	0	NI	1
EcHu442a	0	1	0	NI	1
EcHu503a	0	1	0	SI	1
EcHu547a	0	1	0	SI	1
EcHu550a	0	1	0	NI	1
EcHu508a	0	1	0	NI	1
EcHu450a	0	1	0	NI	1
EcHu491a	0	1	0	NI	19
EcHu494a	0	1	0	NI	1
EcHu500a	0	1	0	NI	1
EcHu520a	0	1	0	NI	1
EcHu444a	0	1	0	NI	1
EcHu538a	0	1	0	SI	1
EcHu485a	0	1	0	NI	1
EcHu548a	0	1	0	SI	1
EcHu449a	0	1	0	NI	1
EcHu457a	0	1	0	SI	1
EcHu472a	0	1	1	NI	5
EcHu554a	0	1	1	SI	12
EcHu432a	0	1	1	NI	5
EcHu551a	0	1	1	NI	5
EcHu471a	0	1	1	NI	5
EcHu475a	0	1	1	NI	5
EcHu473a	0	1	1	SI	5
EcHu430a	0	1	1	SI	5
EcHu433a	0	1	1	SI	5
EcHu555a	0	1	1	NI	5
EcHu521a	0	1	1	NI	5
EcHu544a	0	1	1	SI	5
EcHu546a	0	1	1	SI	5
EcHu456a	0	1	1	SI	5
EcHu477a	0	1	1	SI	5
EcHu468a	0	1	1	NI	5
EcHu437a	0	1	1	SI	5
EcHu474a	0	1	1	NI	5
EcHu435a	0	1	1	SI	17
EcHu469a	0	1	1	SI	5
EcHu470a	0	1	1	SI	5
EcHu447a	1	1	0	SI	3
EcHu507a	1	1	0	NI	3
EcHu513a	1	1	0	NI	3
EcHu514a	1	1	0	NI	3
EcHu429a	1	1	0	SI	3
EcHu502a	1	1	0	NI	3
EcHu439a	1	1	0	SI	3
EcHu440a	1	1	0	SI	3
EcHu486a	1	1	0	NI	3
EcHu487a	1	1	0	NI	3
EcHu528a	1	1	0	NI	3
EcHu495a	1	1	0	NI	3
EcHu516a	1	1	0	NI	3
EcHu517a	1	1	0	NI	3
EcHu518a	1	1	0	NI	3
EcHu476a	1	1	0	SI	3
EcHu453a	0	1	1	SI	5
EcHu504a	1	0	0	NI	8
EcHu533a	1	1	0	NI	3

Figure 4 Comparison of PFGE profiles from 97 human *E. coli* O157:H7 isolates. PFGE profile comparison performed using UPGMA cluster analysis, Dice similarity coefficient, and 1% band matching tolerance. *stx*1, *stx*2a/c, *stx*2c virulence genes encoding for Shiga toxins present (1) or absent (0). Island of residence (Origin) presented as North Island (NI) or South Island (SI) of New Zealand, and genotypes of isolates as Shiga toxin (Stx)-encoding bacteriophage insertion (SBI) types.

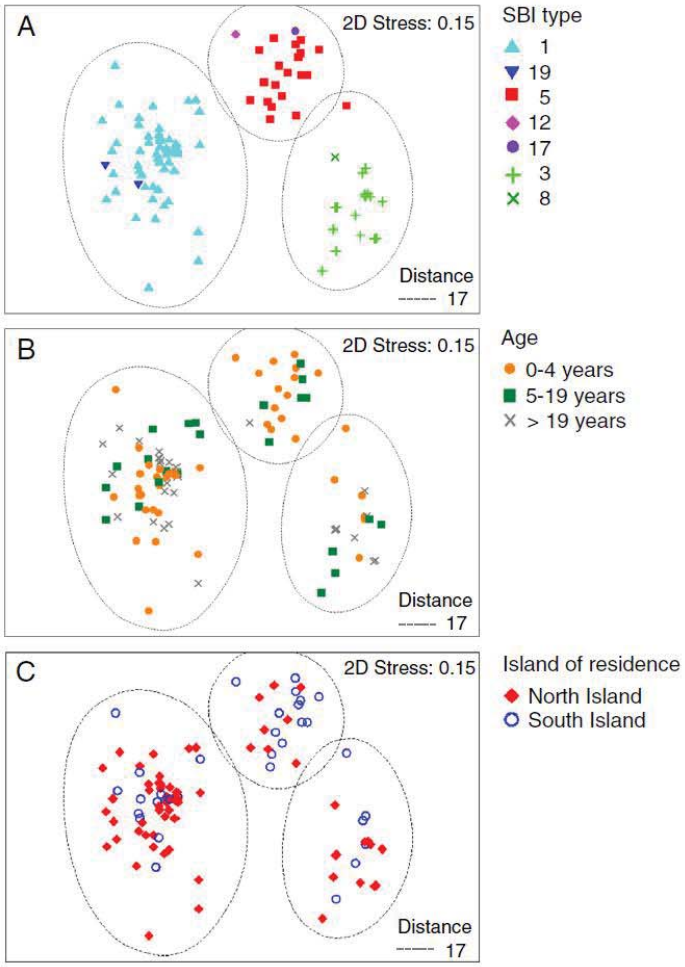


Figure 5 Multidimensional scaling plots (MDS). MDS showing the genotypic clustering of *E. coli* O157:H7 human isolates and **(A)** Shiga toxin-encoding bacteriophage insertion (SBI) types; **(B)** age categories; and **(C)** island of residence. The three clusters of isolates share a genetic difference of 17% (Distance = 17) based on the isolates' PFGE profiles.

case–control data due to the strong collinearity between these two variables. As all dairy farms in the meshblocks occupied by cases and controls also had beef cattle, hence the two variables were combined into a single variable comprising all cattle. The best fitting variable in the second analysis, which considered the entire population at the meshblock level, was the presence of beef cattle in the meshblock. Associations between STEC infections and areas with higher densities of cattle have been observed in previous studies conducted in The Netherlands [48], Finland [53], Scotland [54], Sweden [55], and Canada [56,57], providing evidence of direct or indirect contact with cattle as a likely source of infection.

Table 3 PERMANOVA analysis of *E. coli* O157:H7 isolates

Variable	Df[a]	Mean square	*p*-value	Perms[b]	Estimated component of variation (%)
Island of residence	1	859.0	0.012	999	22.3
Residuals	95	187.5			77.7

[a]Degrees of freedom.
[b]Number of permutations.

Risk factors

Since reporting of STEC commenced in New Zealand in 1997, cases have occurred sporadically or as small clusters throughout the country, suggesting highly dispersed animal and/or environmental exposures rather than STEC-contaminated food as likely sources of infection.

In this study, animal and environmental contacts were identified as significant risk factors for sporadic STEC infections. A child 0–4 years of age was at significantly higher risk, if another household member had contact with animals other than household pets, compared to a child of the same age without this risk factor. This finding is biologically plausible and is more likely to occur in rural than urban settings; for example, when household members working on a farm could be a source of infection. Pre-school children might be exposed to contaminated work clothing and footwear harbouring pathogenic organisms. For example, *Campylobacter* was recovered from loose debris shaken off protective overalls worn on broiler farms [58]. Household occupation contact with farm animals (sheep or lambs) was also the major risk factor for *Salmonella* Brandenburg infection in a previous New Zealand case–control study where the infection also particularly affected rural children [59].

In addition, infants and pre-school children exhibit high frequencies of hand-to-mouth and object-to-mouth behaviour indoors and outdoors and this will inevitably increase the risk of ingesting pathogens from clothing, surfaces, objects, hands, and soil [60,61]. This is particularly apparent when children are raised in a farming environment, where they are more likely to be exposed to zoonotic and soil-associated pathogens than in urban areas. Therefore, practicing good hand-hygiene and supervising activities of infants and toddlers could help to reduce the risk of ingesting pathogens.

Although 'Living in a rural area' was not statistically significant in our model, there was sufficient evidence that risk factors related to agricultural or rural characteristics were associated with sporadic STEC infection, and these would have confounded the strong univariate relationship with rurality. Similar associations between rurality and notification rates of both STEC and cryptosporidiosis in New Zealand have been observed by Thorburn [62], reporting higher rates in rural areas.

Exposures to farming environments were reported as risk factors for sporadic STEC infections in case–control studies conducted in England [63], North America [11], Germany [8], and Argentina [21]. A prospective case–control study by Locking *et al*. [64] identified contact, or likely contact, with animal manure as a strong risk factor for sporadic STEC O157 infection in Scotland, while a retrospective case–control study by Voetsch *et al*. [65] observed direct or indirect contact with cattle manure as a leading source of sporadic STEC O157 infections in North America. We also identified contact with animal manure as a significant risk factor in addition to cattle being present in the meshblock in which the case resided. Both variables were associated with the largest population attributable fractions and, when combined with the spatial analysis and the strong correlation between the presence of cattle, and particularly beef cattle and STEC cases at the meshblock level, it indicates that contact with cattle faeces is the major exposure pathway for infection in New Zealand.

'Travelling to areas of New Zealand with interrupted or no main water supply' and 'Contact with recreational waters' emerged as significant environmental risk factors for sporadic STEC infections; activities which increasingly occur in the summer period. Previous studies conducted in Finland [66] and North America [20,67] reported an association between gastrointestinal illnesses, including STEC, after exposure to recreational waters during summer. Such an association could also explain some of the observed seasonality of STEC cases as discussed above. In addition, an estimated 14% of New Zealand's population is not served by community drinking-water supplies [68] but retrieves drinking water from private springs and bores, streams and creeks, or roof runoffs. This risk applies particularly to residents of rural areas. Considering the large ruminant livestock population in New Zealand, ground water and particularly surface water in rural areas are potentially contaminated with ruminant faeces containing STEC.

We found no evidence to suggest that sporadic STEC cases in New Zealand were associated with exposure to STEC-contaminated food products, while 'Drinking refrigerated fruit juice from supermarket', 'Eating raw vegetables' and 'Eating seafood' were negatively correlated with disease. An inverse effect of fruit and vegetables has also been reported in previous case–control studies conducted in Australia [69] and Scotland [64] and merits further investigation. The association seems biologically plausible compared to other food products, as they are associated with health benefits such as antimicrobial properties against human pathogens in berries [70] and sweet potato leaves [71]. An alternative explanation might be the association between fruit and vegetable consumption and the participants' choice of healthy eating. This apparent protective effect might also be caused by recall bias, as discussed under *sources of bias*.

Molecular epidemiology of *E. coli* O157:H7

The molecular analysis of PFGE profiles from human *E. coli* O157:H7 isolates revealed three distinctive clusters of genotypes, each represented by a specific SBI type. SBI types are defined based on the insertion site of the Stx-associated bacteriophage and the presence or absence of *stx* genes in the bacterial genome, which encode for the Shiga toxin proteins. *stx*2c is a subtype of the *stx*2 gene and characteristic for isolates of SBI type 5. The observed clustering was significantly associated with 'Island of residence', indicating that SBI type 5 was more prevalent in the South Island, whereas SBI types 1 and 3 were more common in the North Island. This distinct geographical difference in genotype distribution was also

observed in a recent molecular study including 28 bovine and 209 human *E. coli* O157:H7 isolates originating from both islands of New Zealand [72]. The distinct between-island distributions of genotypes found among bovine and human isolates indicate localised transmission between cattle and humans. SBI type 5 accounted for 20.6% of human isolates in that study, which is much greater than its frequency in other international studies [27,73]. This is consistent with a limited historical introduction of this strain into New Zealand and subsequent evolution.

A significant relationship between SBI types and age categories of cases was observed, in particular between SBI type 5 and 0–4 year-old children. It can be hypothesised, if this genotype possesses host-adapted characteristics to affect specifically the immature gastrointestinal tract of children, or whether the observed association is due to SBI type 5 being a more persistent environmental contaminant to which very young children are more likely to be exposed than adults. Recent studies investigating differential virulence of STEC O157 strains have suggested that STEC O157 strains carrying *stx*2c alone are likely to be less pathogenic compared to strains carrying combinations of *stx*2c and *stx*1 as shown in a piglet model [74], or less potent on human kidney cell lines and in mouse models [75].

The molecular variation of PFGE profiles of the isolates was explained by only one explanatory variable: 'Island of residence', which was consistent with the observed clustering of isolates. Together with significant associations observed between SBI types and both 'Season' and 'Contact with animal manure', these findings provide further evidence of an animal/environmental-associated pathway of sporadic STEC infection in New Zealand.

Sources of bias

The two week window of exposure might have resulted in some recall bias due to difficulty remembering previous exposures. However, this time period was chosen to cover the likely incubation period for STEC (3–12 days) while lists of possible answers facilitated recall of consumed food items, contact with animal species and environmental exposures. Observational studies of this type can also introduce recall bias due to the cases being more likely to recall events than the non-affected controls [76]. This effect could explain the apparent protective association seen for consumption of a range of foods, where recall was less complete amongst controls, as found in a previous New Zealand case–control study of similar populations [59]. In addition, 31% of cases were interviewed differently to the other cases and controls, which might have introduced some systematic differences between them, though such a bias is unlikely to have had an important effect on the findings.

There was evidence of some selection bias in the control population as a result of using random landline dialling for recruitment. Based on the national census data, the older age group of controls was overrepresented compared to the younger age groups. This might be because younger age groups favouring mobile technology over landlines, or their tendency to reside in relatively fewer households with a larger number of individuals. Nonetheless, little bias was observed in the distribution of ethnicities, gender, and rural/urban living among controls, compared to national census data.

The exclusion of potentially eligible cases had a negligible effect on the findings. Only one case probably acquired their infection overseas so was excluded. There were no apparent outbreaks or clusters of concurrent cases observed during the study period, indicating that secondary infections occur only relatively infrequently in New Zealand suggesting that this study was effectively one of sporadic cases.

The number of confirmed cases reported through the disease surveillance system is likely to be an underestimation of the true incidence of human STEC infections in New Zealand. Scallan *et al.* [77] and Tam *et al.* [78], using different approaches, have estimated under-ascertainment fractions of STEC cases in the USA and the UK. Asymptomatic or mild cases are unlikely to present to medical practitioners and not all stool samples received at diagnostic laboratories are routinely tested for *E. coli* O157:H7 and non-O157 STECs in New Zealand. In addition, the majority of diagnostic laboratories test stool samples of STEC cases for *E. coli* O157:H7 only, which could explain the current predominance of STEC O157. Therefore risks presented could be underestimated, or different measures of association could apply compared to findings in this study.

Conclusions

Our findings strongly indicate that environmental and animal contact, but not food, are important exposure pathways for sporadic cases of human STEC infection in New Zealand. There are strong indications that dairy cattle and beef cattle are the most important sources of STEC and contact with manure from these animals represents an important exposure pathway. Notably, outbreaks of STEC infections are rare in New Zealand and this further suggests that food is not a significant exposure pathway.

Additional files

Additional file 1: Questionnaire. Questions asked in interview of study cases and controls.

Additional file 2: Ruminant livestock densities in New Zealand from 2011. Densities (animals/km^2) of (A) dairy cattle, (B) beef cattle, (C) sheep, and (D) deer in New Zealand from 2011.

Additional file 3: Results of bivariate logistic regression analysis (adjusted for age categories).

Additional file 4: Multivariate logistic regression model without imputations. Results showing identified risk factors after deleting 57 of 619 observations (113 cases and 506 controls) due to missing values. 'No exposure/contact' was chosen as reference level for comparison in each variable (odds ratio = 1.00).

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
PJ designed and coordinated the study, performed the screening for *stx*2c of isolates, performed the statistical analysis and drafted the manuscript; ALC helped with the study design and reviewed the manuscript; DMC conceived of the study, participated in its design, and reviewed the manuscript; TEB and SS performed the SBI genotyping of isolates; GFM helped with study design, coordination of the study, and reviewed the manuscript; EL set up the Survey Gizmo and database, and LL participated in coordination of study and data collection; MD performed the PFGE genotyping of isolates; JCM contributed to the statistical analysis; MGB contributed to the study design and reviewing of the manuscript; SH and DJP contributed to the study design; NPF contributed to the study design, statistical analysis, drafting and reviewing of the manuscript. All authors read and approved the final manuscript.

Acknowledgements
We would like to acknowledge Public Health Units across New Zealand for their collaboration and participation in this study; the Health Intelligence team at ESR (Ali Borman, Ruth Pirie, Kerry Sexton) with study preparations and technical support; UMR for recruiting and interviewing study controls; ERL for providing STEC isolates and PFGE profiles (Brent Gilpin, ESR, Christchurch, New Zealand); Charlotte Bolwell (Hopkirk Research Institute, Massey University, Palmerston North, New Zealand) for providing the meshblock data and livestock numbers; Dr John Holmes (Ministry of Health, Wellington, New Zealand) for advice in study design; Martyn Kirk (National Centre for Epidemiology and Population Health, Australian National University, Canberra, Australia) for advice in questionnaire and study design; and the Ministry for Primary Industries (Wellington, New Zealand) for funding this study.

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Received: 24 April 2013 Accepted: 26 September 2013
Published: 30 September 2013

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doi:10.1186/1471-2334-13-450
Cite this article as: Jaros *et al.*: A prospective case–control and molecular epidemiological study of human cases of Shiga toxin-producing *Escherichia coli* in New Zealand. *BMC Infectious Diseases* 2013 **13**:450.

Appendix 6: Questionnaire. Questions asked in interview of study cases and controls.

VTEC Questionnaire

Demographics

1) *If adult*: Interviewer to enter gender of respondent ( ) Male ( ) Female
If child: What is the gender of [insert child's name]? ( ) Male ( ) Female

2a) Would you mind telling me your / [insert child's name] age please?
Enter age in years. For a child whose age is stated in months, record the age in years at the child's last birthday. i.e. 1 to 11 months is '0' years of age, 12–23 months is '1' year, 24–35 months is '2' years

( ) Age given in years _____ (Go to 3 after age is recorded).

( ) Refused to answer – Go to 2b.

2b) Would you mind telling which one of the following age groups you / [insert child's name] is in?
( ) 0–4 years, ( ) 5–9 years, ( ) 10–14 years, ( ) 15–19 years, ( ) 20–29 years, ( ) 30–39 years, ( ) 40–49 years, ( ) 50–59 years, ( ) 60–69 years, ( ) 70+ years, ( ) Don't know, ( ) Refused to answer.

3) Which of the following ethnic group(s) do you /does [insert child's name] belong to? One or several groups may apply to you.
( ) New Zealand European, ( ) New Zealand Maori, ( ) Samoan, ( ) Cook Island Maori, ( ) Tongan, ( ) Niuean, ( ) Chinese, ( ) Indian, ( ) Other Asian (specify)_____, ( ) Other1 (specify)_____, ( ) Other2 (specify)_____, ( ) Refused to answer.

4) *If adult*: What is your occupation? _____.
If unknown, enter "Unknown". If respondent refuses to provide an answer, enter "Refused to answer".

5) *If child*: Is [insert child's name] a:
( ) Preschooler, ( ) Primary or intermediate student, ( ) Secondary student, ( ) Tertiary student, ( ) Refused to answer.

6) What is the name of the nearest school to where you / [insert name] live and what town or city is this school in? This could be a primary, intermediate or secondary school. If there are a number of schools nearby, just name one.
Reasons for wanting to know the nearest school: have a rough idea of where the case lives. If unknown, enter "Unknown". If refused to answer, enter "Refused to answer".
Name of school: _____, Town/city: _____.

7) Do you / [insert child's name] live at a rural delivery (RD) address?
( ) Yes, ( ) No, ( ) Unknown, ( ) Refused to answer.

Food

8a) Did you / [insert name] drink any treated milk or eat any dairy products made from treated milk in the 2 weeks before getting sick? By treated milk we mean heated, treated or pasteurised milk; milk or milk products bought in a shop.
( ) Yes – Go to 8b, ( ) No – Go to 9a, ( ) Don't know – Go to 9a, ( ) Refused to answer – Go to 9a.

8b) Can you please specify the type, brand and where you had obtained the dairy or dairy products you / [insert child's name] had consumed?
Type:_____, Brand:_____, Source: _____.

9a) Did you / [insert child's name] drink any raw milk or eat any products made from raw milk which you bought directly from a farm in the 2 weeks before getting sick? By raw milk we mean untreated or unpasterurised milk.
( ) Yes – Go to 9b, ( ) No – Go to 10, ( ) Don't know – Go to 10, ( ) Refused to answer – Go to 10.

9b) Can you please specify the type, brand and where you had obtained the raw milk or any products products made from raw milk you / [insert child's name] had consumed?
Type: _____ , Brand: _____, Source: _____.

10) Did you / [insert child's name] eat any meat or poultry, excluding eggs, in the 2 weeks before getting sick?
( ) Yes – Go to 11a, ( ) No – Go to 21, ( ) Don't know – Go to 21, ( ) Refused to answer – Go to 21.

11a) Did you / [insert child's name] eat any beef meat or beef products that needed to be cooked in the 2 weeks before getting sick?
Examples: Do not read - list of examples is for reference/clarification only:
Steak, beef sausages, eye fillet, roast beef, beef stir fry, beef stew, beef casserole, beef schnitzel, prime beef, beef curry, veal burger, veal fillet, any type of offal from calf/cattle.
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

11b) Did you / [insert child's name] eat any minced beef that needed to be cooked?
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

If yes to 11a OR 11b:

11c) Did any of this meat appear undercooked on the inside when you / [insert child's name] ate it?
Undercooked meat: meat products that need to be cooked thoroughly before eating e.g. meat patties, sausages, meat loafs etc. as they are potential sources of E. coli infection.
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

If yes to 11a OR 11b:

11d) Can you please specify the type, brand and where you had obtained the beef or beef products consumed?
Type: _____, Brand: _____, Source: _____.

12a) Did you / [insert child's name] eat any lamb or mutton (meat of adult sheep) or lamb/mutton products that needed to be cooked in the 2 weeks before getting sick?
Examples: Do not read - list of examples is for reference/clarification only:
Lamb shanks, lamb sausages, roast lamb, lamb stew, lamb stir fry, lamb chops, lamb casserole, lamb mince, lamb flaps, lamb patties, lamb curry, roast mutton, any type of offal from lamb/sheep.
( ) Yes – Go to 12b, ( ) No – Go to 13a, ( ) Don't know – Go to 13a, ( ) Refused to answer– Go to 13a.

12b) Did any of the lamb or mutton meat appear undercooked on the inside when you/ [insert child's name] ate it?
Undercooked meat: meat products that need to be cooked thoroughly before eating e.g. meat patties, sausages, meat loafs etc. as they are potential sources of E. coli infection.
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

12c) Can you please specify the type, brand and where you had obtained the lamb/mutton or lamb/mutton products consumed?
Type: _____, Brand: _____, Source: _____.

13a) Did you / [insert child's name] eat any chicken, other poultry (e.g. duck, goose, turkey or ostrich) or poultry products that needed to be cooked in the 2 weeks before getting sick?
Examples: Do not read - list of examples is for reference/clarification only:
Roast chicken, chicken sausages, chicken breasts, chicken drumsticks, chicken stir fry, chicken curry, chicken nuggets, roast turkey, turkey breasts, roast goose, duck breasts, ostrich, any type of offal from poultry.
( ) Yes – Go to 13b, ( ) No – Go to 14, ( ) Don't know – Go to 14, ( ) Refused to answer – Go to 14.

13b) Can you please specify the type, brand and where you had obtained the chicken/poultry or chicken/poultry products consumed?
Type: _____, Brand: _____, Source: _____.

14) Did you / [insert child's name] eat any pork meat or pork products that needed to be cooked in the 2 weeks before getting sick?
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

15a) Did you / [insert child's name] eat any venison meat or venison products that needed to be cooked in the 2 weeks before getting sick?
Examples: Do not read - list of examples is for reference/clarification only:
Venison steak, venison sausages, venison fillet, venison burger, venison mince, any type of offal from deer.
( ) Yes – Go to 15b, ( ) No – Go to 16a, ( ) Don't know – Go to 16a, ( ) Refused to answer – Go to 16a.

15b) Did any of the venison meat or venison products appear undercooked on the inside when you/ [insert child's name] ate it?
Undercooked meat: meat products that need to be cooked thoroughly before eating e.g. meat patties, sausages, meat loafs etc. as they are potential sources of E. coli infection.
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

16a) Did you / [insert child's name] eat any meat products such as meat from the delicatessen counter or cold cuts of meat that do not need to be cooked in the 2 weeks before getting sick?
Examples: Do not read - list of examples is for reference/clarification only:
Any type of meat pies, any type of sandwiches containing sliced meat, any type of kebabs containing meat, bacon, ham, luncheon, salami or other cured meats (pastrami, prosciutto, parma ham, pancetta), any type of sliced cold meat, corned beef/silverside, cheerios, frankfurters, saveloys, meat paté = meat paste, Mettwurst.
( ) Yes – Go to 16b, ( ) No – Go to 17a, ( ) Don't know – Go to 17a, ( ) Refused to answer – Go to 17a.

16b) Can you please specify the type, brand and where you had obtained the meat products (processed meats) such as deli meat or cold cuts consumed?
Type: _____, Brand: _____, Source: _____.

17a) Did you / [insert child's name] eat any home kill meat, excluding wild game, in the 2 weeks before getting sick?
Examples: Do not read - list of examples is for reference/clarification only:
Meat of animals raised and slaughtered on farm, such as pork, beef, veal, lamb, mutton, venison, chicken, turkey.
( ) Yes – Go to 17b, ( ) No – Go to 18a, ( ) Don't know – Go to 18a, ( ) Refused to answer – Go to 18a.

17b) Did any of the home kill meat appear undercooked on the inside when you / [insert child's name] ate it?
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

18a) Did you / [insert child's name] eat any wild game meat in the 2 weeks before getting sick?
( ) Yes – Go to 18b, ( ) No – Go to 20, ( ) Don't know– Go to 20, ( ) Refused to answer– Go to 20.

18b) Did any of the wild game meat appear undercooked on the inside when you / [insert child's name] ate it?
Undercooked meat: meat products that need to be cooked thoroughly before eating e.g. meat patties, sausages, meat loafs etc. as they are potential sources of E. coli infection.
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

If Yes to 17a OR 18a:

19) Can you please specify the type of and where you had obtained the home kill or wild game meat consumed?
Type: _____, Source: _____.

20) Did you / [insert child's name] eat any offal, from any species of animal, in the 2 weeks before getting sick?
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

21) Did you / [insert child's name] handle or touch any raw meat or offal at home or work, including raw meat or offal given to pets, in the 2 weeks before getting sick? By handled, we do not mean you / [insert child's name] ate it.
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

22) Did you / [insert child's name] eat any fish or shellfish, excluding seafood from a can, in the 2 weeks before getting sick?
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

23a) Did you / [insert child's name] eat any raw fruit, either as part of a meal or by themselves, in the 2 weeks before getting sick? (this excludes canned fruit)
Examples: Do not read - list of examples is for reference/clarification only:
Fresh fruit salad, fruit platters, apples, pears/nashi pears, bananas, berries, citrus fruits (mandarins, oranges, grapefruits, tangelos), kiwi fruit, peaches, nectarines, apricots, cherries, strawberries, blueberries, any other berries, pineapple, grapes, plums, watermelon, rockmelon, passion fruit, feijoas.
( ) Yes – Go to 23b, ( ) No – Go to 24a, ( ) Don't know – Go to 24a, ( ) Refused to answer – Go to 24a.

23b) Were any of the fruits you/ [insert child's name] ate home grown, either by yourself or someone else?
( ) Yes - Go to 23c, ( ) No – Go to 24a, ( ) Don't know – Go to 24a, ( ) Refused to answer – Go to 24a.

23c) Was animal manure, fertilizer or compost containing animal manure used to grow the home grown fruits?
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

24a) Did you / [insert child's name] eat any raw (not cooked) vegetables or herbs, for example salad vegetables such as lettuce, carrots, tomatoes, spinach etc, either as part of a meal or by themselves, in the 2 weeks before getting sick?
Examples: Do not read - list of examples is for reference/clarification only:
Bean sprouts or similar, watercress, lettuce or other salad leaves, silver beet, spinach, fresh herbs, puha, carrots, beetroot, coleslaw, celery, tomatoes, cucumber, courgettes, capsicum, fennel, eggplant, sweet corn, broccoli, cauliflower, spring onions, peas / snow peas or similar, parsley, chives, other herbs, mushrooms.
( ) Yes – Go to 24b, ( ) No – Go to 25a, ( ) Don't know – Go to 25a, ( ) Refused to answer – Go to 25a.

24b) Were any of the vegetables you/ [insert child's name] ate home grown, either by yourself or someone else?
( ) Yes – Go to 24c, ( ) No – Go to 25a, ( ) Don't know – Go to 25a, ( ) Refused to answer – Go to 25a.

24c) Was animal manure, fertilizer or compost containing animal manure used to grow the home grown vegetables or herbs?
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

If Yes to 23b OR 24b:

24d) Please specify type of raw fruit/vegetables consumed that were homegrown:
_____.

If Yes to 23a OR 24a:

24e) Can you please specify the type, brand and where you had obtained the any raw fruit or vegetables consumed that were not homegrown?
Type: _____, Brand: _____, Source: _____.

25a) Did you / [insert child's name] drink any fruit or vegetable juice purchased from the refrigerated section of a supermarket or shop in the 2 weeks before getting sick?
Note: Does NOT include fruit/vegetable juice bought from dry goods shelves (shelf stable juice)
( ) Yes – Go to 25b, ( ) No – Go to 26a, ( ) Don't know – Go to 26a, ( ) Refused to answer – Go to 26a.

25b) Can you please specify type and brand of fruit/vegetable juice bought from the refrigerated section of the supermarket or shop?
Note: Does NOT include fruit/vegetable juice bought from dry goods shelves (shelf stable juice)
Type: _____, Brand: _____.

26a) Did you / [insert child's name] drink any fruit or vegetable juice prepared fresh from a café/restaurant/juice bar or at home in the 2 weeks before getting sick? This includes grasses.
( ) Yes – Go to 26b, ( ) No – Go to 27a, ( ) Don't know – Go to 27a, ( ) Refused to answer – Go to 27a.

26b) Please specify type and source of juice prepared fresh:
Type: _____, Source: _____.

Dining locations

27a) Did you / [insert child's name] eat any food prepared and/or cooked outside of your home kitchen in the 2 weeks before getting sick?
Examples: Do not read – list of examples is for reference/clarification only:
National hamburger chain (McDonalds, Burger King, KFC, Wendy's), national pizza chain (Hells Pizza, Dominos, Pizza Hut), Subway, other takeaway outlets, fish and chips shop, bakery, café/restaurant/pub, food bought at petrol stations, other location with professional caterers (e.g. wedding), other location without professional caterers (e.g. BBQ at friends, birthday/family party, work party, social event at sports club, Xmas/New Year's party, school function, school/sports camp, hangi, umu), at an agricultural show (e.g. Field days)/petting zoo/wildlife park, BBQ at home.
( ) Yes – Go to 27b, ( ) No – Go to 28a, ( ) Don't know – Go to 28a, ( ) Refused to answer – Go to 28a.

27b) Did any of the meat you / [insert child's name] ate, prepared and/ or cooked outside of your home kitchen, appear undercooked on the inside?
Undercooked meat: meat products that need to be cooked thoroughly before eating e.g. meat patties, sausages, meat loafs etc. as they are potential sources of E. coli infection.
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

Drinking water

28a) Which of the following is the main source of water supply to your/ [insert child's name] home/ house?
( ) Usual town supply – Go to 29a, ( ) Private bore/spring water – Go to 28b, ( ) Tanker truck water – Go to 28b, ( ) Roof/rain water – Go to 28b, ( ) Creek/stream water – Go to 28b, ( ) Don't know – Go to 29a.

28b) If not town supply, is the water treated or filtered?
( ) Yes – Go to 28c, ( ) No – Go to 29a, ( ) Don't know – Go to 29a, ( ) Refused to answer – Go to 29a.

28c) How is the water treated or filtered? *Select as many as apply.*

	Yes	No	Don't know
Chlorinated	( )	( )	( )
Boiled	( )	( )	( )
Filtered	( )	( )	( )
Treated with UV	( )	( )	( )
Don't know	( )	( )	( )

29a) *If adult*: Which of the following is the main source of water supply at your workplace?
( ) Usual town supply – Go to 30, ( ) Private bore/spring water – Go to 29b, ( ) Tanker truck water – Go to 29b, ( ) Roof/rain water – Go to 29b, ( ) Creek/stream water – Go to 29b, ( ) Don't know – Go to 30.

If child: Which of the following is the main source of water supply at [insert child's name's] school?
( ) Usual town supply – Go to 30, ( ) Private bore/spring water – Go to 29b, ( ) Tanker truck water – Go to 29b, ( ) Roof/rain water – Go to 29b, ( ) Creek/stream water – Go to 29b, ( ) Don't know – Go to 30.

29b) If not town supply, is the water treated or filtered?
( ) Yes – Go to 29c, ( ) No – Go to 30, ( ) Don't know – Go to 30, ( ) Refused to answer – Go to 30.

29c) How is the water treated or filtered? *Select as many as apply.*

	Yes	No	Don't know
Chlorinated	( )	( )	( )
Boiled	( )	( )	( )
Filtered	( )	( )	( )
Treated with UV	( )	( )	( )
Don't know	( )	( )	( )

30) Were there any problems or changes in the regular water supply to your home/house or workplace or [insert child's name]'s school in the 2 weeks before getting sick?
For example, changes due to natural disasters (e.g. earthquakes or flooding), technical issues and therefore forced to use other water sources such as untreated water on a farm, bore water, tanker/ truck water, roof/rain water, untreated creek/ stream water.
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

31a) In the 2 weeks before getting sick, did you / [insert child's name] drink any water from supplies that you would not normally drink from? We mean outside of your home, work, school, etc.
( ) Yes – Go to 31b, ( ) No – Go to 32a, ( ) Don't know – Go to 32a, ( ) Refused to answer – Go to 32a.

31b) Specify the type(s) of water supply. *Select all that apply.*
( ) Town supply, ( ) Private bore/spring water, ( ) Tanker truck water, ( ) Roof/rain water, ( ) Creek/stream water, ( ) Don't know.

Contact with recreational waters

32a) Did you / [insert child's name] swim in a public swimming pool in the 2 weeks before getting sick?
( ) Yes – Go to 32b, ( ) No – Go to 33a, ( ) Don't know – Go to 33a, ( ) Refused to answer – Go to 33a.

32b) Can you please specify the name and location of the public swimming pool(s)?
_____.

33a) Did you / [insert child's name] swim in any other private swimming pools in the 2 weeks before getting sick?
( ) Yes – Go to 33b, ( ) No – Go to 34a, ( ) Don't know – Go to 34a, ( ) Refused to answer – Go to 34a.

33b) Would you mind telling me the name and/or location of the private pool(s)?
_____.

34a) Did you / [insert child's name] use a spa or paddling pool in the 2 weeks before getting sick?
( ) Yes – Go to 34b, ( ) No – Go to 35a, ( ) Don't know – Go to 35a, ( ) Refused to answer – Go to 35a.

34b) Was that a spa pool or a paddling pool, or both? If spa pool, please specify name and/or location of pool(s). *Select all that apply.*
( ) Paddling pool, ( ) Refused to answer, ( ) Spa pool (specify name and location): _____.

35a) Did you / [insert child's name] take part in recreational activities in fresh water such as in a stream, river, lake, dam or pond in the 2 weeks before getting sick? This does not include salt or sea water.
( ) Yes – Go to 35b, ( ) No – Go to 36a, ( ) Don't know – Go to 36a, ( ) Refused to answer – Go to 36a.

35b) Please specify location of recreational activities in fresh water such as stream, river, lake, etc.:

_____.

36a) Did you / [insert child's name] have any contact with sewage, stagnant water or flood waters in the 2 weeks before getting sick?
( ) Yes – Go to 36b, ( ) No – Go to 37a, ( ) Don't know – Go to 37a, ( ) Refused to answer – Go to 37a.

36b) Which of the following did you / [insert child's name] have contact with? *Select all that apply*:
( ) Sewage, ( ) Stagnant waters, ( ) Flood waters, ( ) Refused to answer.

Hunting activities

37a) Did you / [insert child's name] go hunting in the 2 weeks before getting sick?
( ) Yes – Go to 37b, ( ) No – Go to 38a, ( ) Don't know – Go to 38a, ( ) Refused to answer – Go to 38a.

37b) Did you / [insert child's name] handle any of the following animals or birds as a result of the hunting trip? *Select as many as apply*:
( ) Pig, ( ) Boar, ( ) Deer, ( ) Goat, ( ) Thar, ( ) Chamois, ( ) Possum, ( ) Rabbit, ( ) Duck, ( ) Pheasant, ( ) Other (specify) _____, ( ) Refused to answer.

Animal contact

38a) Did you / [insert child's name] have any physical contact with your own or other people's household pets in the 2 weeks before getting sick? If no contact, please just say so.
( ) Yes – Go to 38b, ( ) No – Go to 39, ( ) Don't know – Go to 39, ( ) Refused to answer – Go to 39.

38b) What household pets did you / [insert child's name] have physical contact with in the 2 weeks before getting sick? *Select as many as apply*:
( ) Dog, ( ) Cat, ( ) Rabbits, ( ) Guinea pig/hamster/gerbil, ( ) Mouse/rat, ( ) Aviary birds, ( ) Chickens/other poultry, ( ) Horse/pony/donkey, ( ) Fish tank or plants, ( ) Turtle, ( ) Other reptile (e.g. snake), ( ) Other (specify) _____, ( ) Refused to answer.

39) Do you / [insert child's name] live on a farm/lifestyle block with animals?
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

40a) Did you / [insert child's name] have any physical contact with animals other than household pets in the 2 weeks before getting sick? If no contact, please just say so.
( ) Yes – Go to 40b, ( ) No – Go to 41, ( ) Don't know – Go to 41, ( ) Refused to answer – Go to 41.

40b) Which of the following animals did you / [insert child's name] have contact with in the 2 weeks before getting sick? *Select as many as apply*:
( ) Horse/pony/donkey, ( ) Chickens/poultry, ( ) Cattle (including dairy cows/calves), ( ) Pigs/piglets, ( ) Sheep/lamb, ( ) Deer, ( ) Llamas/alpacas, ( ) Working dog, ( ) Farm cat, ( ) Aviary birds, ( ) Other animals in a zoo or wildlife park, ( ) Other (specify) _____, ( ) Don't know, ( ) Refused to answer.

If Yes to 38a OR 40a:

41) What kind of contact did you / [insert child's name] have with the animals, was it:
Select as many as apply:
( ) General farm work with animals, ( ) Job related (e.g. slaughter house worker, veterinarian, animal trainer, milker, shepherd, stock agent), ( ) Riding/walking/exercising, ( ) Feeding, ( ) Petting/stroking, ( ) Cleaning/bathing the animal(s), grooming, ( ) Cleaning cages/kennels/stables etc., ( ) Picking up faeces/manure, ( ) Giving medication, ( ) Other (specify) _____, ( ) Don't know, ( ) Refused to answer.

42a) Did you / [insert child's name] have any intentional or unintentional contact with animal manure / faeces or compost containing animal manure/faeces in the 2 weeks before getting sick?
( ) Yes – Go to 42b, ( ) No – Go to 43a, ( ) Don't know – Go to 43a, ( ) Refused to answer – Go to 43a.

42b) What type of animal manure / faeces was this? *Read list - select all that apply*:
( ) Bird droppings, ( ) Cattle/calf, ( ) Sheep/lamb, ( ) Horse/pony/donkey, ( ) Chicken/poultry, ( ) Pigs, ( ) Dog, ( ) Cat, ( ) Rabbit, ( ) Mice/rat, ( ) Guinea pig/hamster/gerbil, ( ) Compost from garden centre, ( ) Fertilizer or compost made from animal manure, ( ) Don't know, ( ) Refused to answer.

43a) Did anyone else in your household other than you / [insert child's name] have contact with animals other than household pets in the 2 weeks before getting sick?
( ) Yes – Go to 43b, ( ) No – Go to 44, ( ) Don't know – Go to 44, ( ) Refused to answer – Go to 44.

43b) What kind of contact did this household member have with the animals, was it:
Select as many as apply:
( ) General farm work with animals, ( ) Job related (e.g. slaughter house worker, veterinarian, animal trainer, milker, shepherd, stock agent), ( ) Feeding, ( ) Petting/stroking, ( ) Cleaning/bathing the animal(s), grooming, ( ) Cleaning cages/kennels/stables etc., ( ) Picking up faeces/manure, ( ) Giving medication, ( ) Other (specify) _____, ( ) Don't know, ( ) Refused to answer.

Human contact

44) Did you / [insert child's name] visit a school, pre-school or childcare facility in the 2 weeks before getting sick?
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

45) Did you / [insert child's name] have any contact with children in nappies in the 2 weeks before getting sick?
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

46a) Did you / [insert child's name] have any contact with a **person with vomiting or any symptoms of diarrhoea or (gastrointestinal) disease** in the 2 weeks before getting sick? For example, at a rest or nursing home, at a care/health centre in town, at the doctor/clinic, at a hospital.
( ) Yes – Go to 46b, ( ) No – Go to 47a, ( ) Don't know – Go to 47a, ( ) Refused to answer – Go to 47a.

46b) Could you please tell me the nature of contact you had with them and when they had gotten sick?
Nature of contact: _____.
Date of onset of illness in other case (dd/mm/yyyy): _____.

47a) Did you / [insert child's name] attend any social functions in the 2 weeks before getting sick?
( ) Yes – Go to 47b, ( ) No – Go to 48, ( ) Don't know – Go to 48, ( ) Refused to answer – Go to 48.

47b) Can you please specify details of the function(s)?
Specify type, size, and location of function (e.g. children's party at home of case – 30 people attended). *Do not record any names (or identifiable information) here.*
_____
_____

Travel

48) In the 2 weeks before getting sick, did you / [insert child's name] visit a town/area in New Zealand apart from where you usually live and work, where there was no main water supply or an interruption in the usual water supply (e.g. Christchurch/recent flooding)?
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

49a) In the 2 weeks before getting sick, did you / [insert child's name] travel within New Zealand?
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

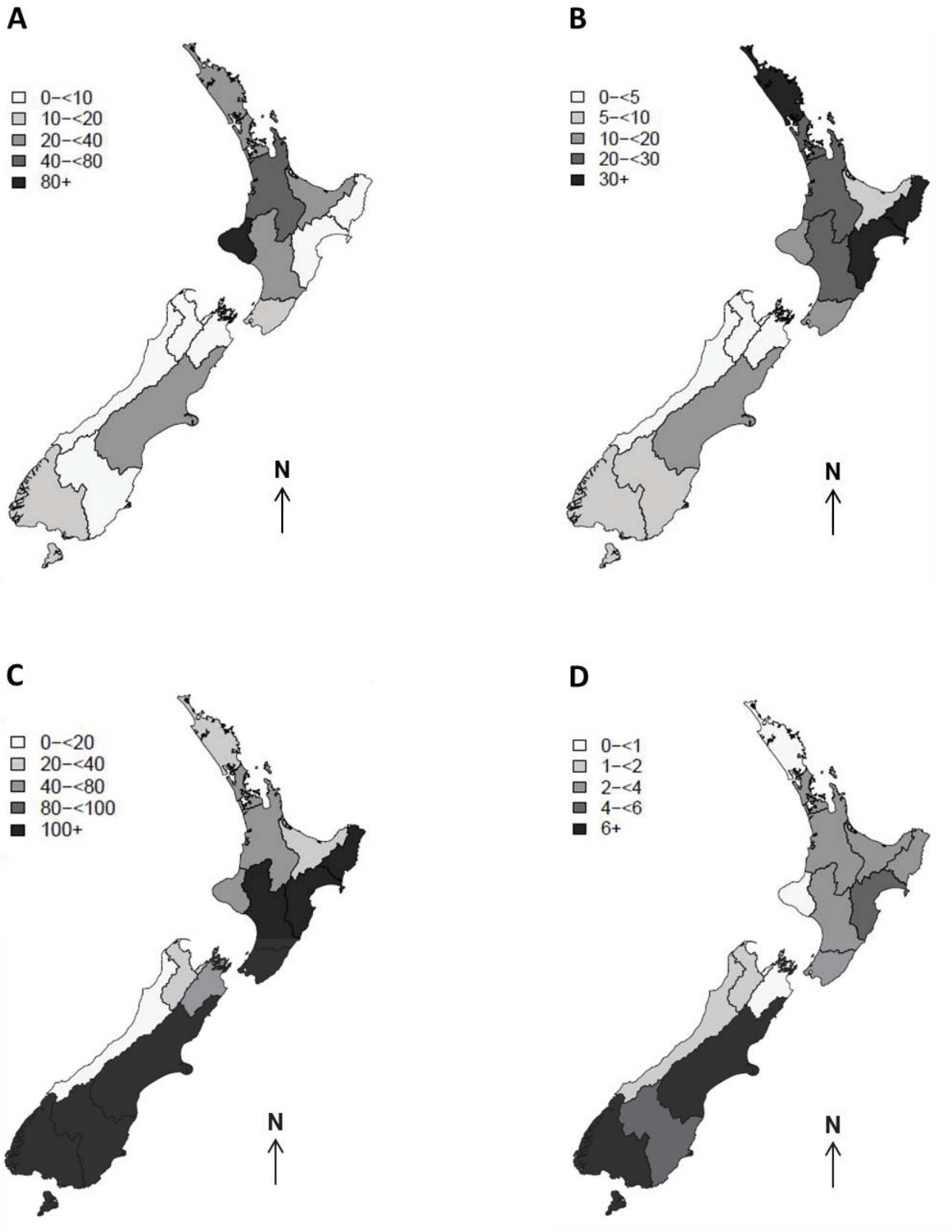
49b) Please specify where in New Zealand you / [insert child's name] had travelled to?
_____.

Antibiotic/Antacid use

50) Did you / [insert child's name] take any antibiotics in the 2 weeks before getting sick?
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

51) Did you / [insert child's name] take any antacids/pills to reduce stomach acid in the 2 weeks before getting sick?
( ) Yes, ( ) No, ( ) Don't know, ( ) Refused to answer.

Appendix 7: Ruminant livestock densities in New Zealand from 2011.



Densities (animals/km^2) of (A) dairy cattle, (B) beef cattle, (C) sheep, and (D) deer in New Zealand from 2011.

Appendix 8: Results of univariate logistic regression analysis.

Variable	Cases	Controls	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Demographic data					
Age					0.009*
0–4 years	52	204	0.48 (0.23)	1.62 (1.02 –2.56)	0.040
5–19 years	23	61	0.87 (0.30)	2.39 (1.33–4.31)	0.004
>19 years	38	241	Ref	-	-
Gender					
Male	59	217	Ref	-	-
Female	54	289	–0.38 (0.21)	0.69 (0.46–1.03)	0.072
Season					0.214*
Spring (Sep–Nov)	21	128	Ref	-	-
Summer (Dec–Feb)	28	126	0.30 (0.32)	1.35 (0.73–2.51)	0.335
Autumn (Mar–May)	38	126	0.61 (0.30)	1.84 (1.02–3.31)	0.042
Winter (Jun–Aug)	26	126	0.23 (0.32)	1.26 (0.67–2.35)	0.472
Ethnicity					0.049*
New Zealand European	98	415	Ref	-	-
Maori	8	23	0.39 (0.43)	1.47 (0.64–3.39)	0.363
Others	7	68	–0.83 (0.41)	0.44 (0.19–0.98)	0.044
Occupation					0.368*
Baby/Preschooler	52	206	Ref	-	-
Student (primary school to Uni)	17	62	0.08 (0.32)	1.09 (0.59–2.01)	0.793
Professional/Management	13	83	–0.48 (0.34)	0.62 (0.32–1.20)	0.156
Trade/Agricultural/Unemployed	18	77	–0.08 (0.30)	0.93 (0.51–1.68)	0.801
Retired	12	78	–0.50 (0.35)	0.61 (0.31–1.20)	0.153
NA	1	-			
Location within New Zealand					0.005*
Upper half of North Island	62	243	Ref	-	-
Lower half of North Island	12	127	–0.99 (0.33)	0.37 (0.19–0.71)	0.003
Upper half of South Island	24	86	0.09 (0.27)	1.09 (0.64–1.86)	0.741
Lower half of South Island	15	50	0.16 (0.33)	1.18 (0.62–2.23)	0.620
Living in rural area					
No	71	401	Ref	-	-
Yes	42	102	0.84 (0.22)	2.33 (1.50–3.61)	<0.001
NA	-	3			
Food associated risk factors					
Drinking treated milk					
No	12	60	Ref	-	-
Yes	101	446	0.12 (0.34)	1.13 (0.59–2.18)	0.711
Drinking raw milk					
No	108	490	Ref	-	-
Yes	5	16	0.35 (0.52)	1.42 (0.51–3.95)	0.505
Eating meat					
No	11	48	Ref	-	-
Yes	101	458	–0.04 (0.35)	0.96 (0.48–1.92)	0.913
NA	1	-			
Handling raw offal					
No	95	350	Ref	-	-
Yes	16	156	–0.97 (0.29)	0.38 (0.22–0.66)	0.001
NA	2	-			

Appendix 8 continued

Variable	Cases	Controls	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Eating seafood					
No	67	215	Ref	-	-
Yes	41	291	–0.79 (0.22)	0.45 (0.30–0.69)	<0.001
NA	5	-			
Eating raw fruit					
No	14	43	Ref	-	-
Yes	99	462	–0.42 (0.33)	0.66 (0.35–1.25)	0.201
NA	-	1			
Eating raw vegetables					
No	37	76	Ref	-	-
Yes	73	430	–1.05 (0.24)	0.35 (0.22–0.56)	<0.001
NA	3	-			
Drinking refrigerated fruit juice from supermarket					
No	94	280	Ref	-	-
Yes	18	222	–1.42 (0.27)	0.24 (0.14–0.41)	<0.001
NA	1	4			
Drinking fresh fruit juice from café					
No	101	405	Ref	-	-
Yes	8	97	–1.11 (0.38)	0.33 (0.16–0.70)	0.004
NA	4	4			
Dining outside home					
No	40	100	Ref	-	-
Yes	70	405	–0.84 (0.23)	0.43 (0.28–0.67)	<0.001
NA	3	1			
Water associated risk factors					
Water supply to home from					
Town supply/tanker/roof run off	89	468	Ref	-	-
Private bore/spring/creek/stream	22	35	1.20 (0.30)	3.31 (1.85–5.90)	<0.001
NA	2	3			
Visiting areas of New Zealand without main water supply or recently interrupted main water supplies					
No	96	473	Ref	-	-
Yes	15	28	0.97 (0.34)	2.64 (1.36–5.13)	0.004
NA	2	5			
Swimming in public pool					
No	94	407	Ref	-	-
Yes	19	98	–0.18 (0.28)	0.84 (0.49–1.44)	0.525
NA	-	1			
Swimming in private pool					
No	105	460	Ref	-	-
Yes	8	46	–0.27 (0.40)	0.76 (0.35–1.66)	0.494
Swimming in spa pool					
No	106	442	Ref	-	-
Yes	7	63	–0.77 (0.41)	0.46 (0.21–1.04)	0.062
NA	-	1			

Appendix 8 continued

Variable	Cases	Controls	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Recreational activities in fresh water					
No	97	474	Ref	-	-
Yes	15	32	0.83 (0.33)	2.29 (1.19–4.39)	0.013
NA	1	-			
Contact with sewage/standing water/flood waters					
No	105	486	Ref	-	-
Yes	6	19	0.38 (0.48)	1.46 (0.57–3.75)	0.430
NA	2	1			
Animal associated risk factors					
Beef livestock on meshblock					
No	74	422	Ref	-	-
Yes	39	84	0.97 (0.23)	2.65 (1.68–4.16)	<0.001
Dairy livestock on meshblock					
No	94	477	Ref	-	-
Yes	19	29	1.20 (0.32)	3.32 (1.79–6.18)	<0.001
Sheep livestock on meshblock					
No	82	434	Ref	-	-
Yes	31	72	0.82 (0.25)	2.28 (1.41–3.69)	0.001
Deer livestock on meshblock					
No	105	488	Ref	-	-
Yes	8	18	0.73 (0.44)	2.07 (0.87–4.88)	0.098
Living on farm/lifestyle block with animals					
No	72	423	Ref	-	-
Yes	41	83	1.07 (0.23)	2.90 (1.85–4.55)	<0.001
Contact with household pets					
No	28	140	Ref	-	-
Yes	82	366	0.11 (0.24)	1.12 (0.70–1.79)	0.637
NA	3	-			
Contact with animals other than household pets					
No	74	415	Ref	-	-
Yes	37	90	0.84 (0.23)	2.31 (1.46–3.64)	<0.001
NA	2	1			
Exposure to animal manure or compost containing animal manure					
No	66	413	Ref	-	-
Yes	34	86	0.91 (0.24)	2.47 (1.54–3.98)	<0.001
NA	13	7			
Hunting activities					
No	112	500	Ref	-	-
Yes	1	6	–0.30 (1.09)	0.74 (0.09–6.24)	0.785
Other household member having contact with animals other than household pets					
No	71	407	Ref	-	-
Yes	41	95	0.91 (0.23)	2.47 (1.59–3.86)	<0.001
NA	1	4			

Appendix 8 continued

Variable	Cases	Controls	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Human associated risk factors					
Visiting childcare/pre-school/school facilities					
No	72	250	Ref	-	-
Yes	41	256	–0.59 (0.22)	0.56 (0.36–0.85)	0.006
Contact with children in nappies					
No	66	253	Ref	-	-
Yes	41	249	–0.46 (0.22)	0.63 (0.41–0.97)	0.035
NA	6	4			
Contact with person vomiting or having gastrointestinal disease					
No	88	414	Ref	-	-
Yes	15	76	–0.07 (0.31)	0.93 (0.51–1.69)	0.808
NA	10	16			
Host associated risk factors					
Taking antibiotics					
No	99	456	Ref	-	-
Yes	11	48	0.05 (0.35)	1.06 (0.53–2.11)	0.878
NA	3	2			
Taking antacids					
No	106	448	Ref	-	-
Yes	4	57	–1.22 (0.53)	0.30 (0.11–0.84)	0.021
NA	3	1			

[a] SE = Standard error.

[b] CI = 95% confidence interval.

* p-value of variable as whole using Likelihood ratio test.

Ref = reference level for comparison.

NA = observations with either 'unknown' or missing values.

Appendix 9: Results of bivariate logistic regression analysis (adjusted for age categories).

Variable	Cases	Controls	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Demographic data					
Gender					
Male	59	217	Ref	-	-
Female	54	289	–0.30 (0.21)	0.74 (0.49–1.13)	0.165
Season					0.021*
Spring (Sep–Nov)	21	128	Ref	-	-
Summer (Dec–Feb)	28	126	0.33 (0.32)	1.39 (0.75–2.60)	0.294
Autumn (Mar–May)	38	126	0.59 (0.30)	1.80 (1.00–3.25)	0.051
Winter (Jun–Aug)	26	126	0.24 (0.32)	1.27 (0.68–2.38)	0.458
Ethnicity					0.005*
New Zealand European	98	415	Ref	-	-
Maori	8	23	0.27 (0.43)	1.30 (0.56–3.04)	0.538
Others	7	68	–0.84 (0.42)	0.43 (0.19–0.97)	0.042
Occupation					0.004*
Baby/Preschooler	52	206	Ref	-	-
Student (primary school to Uni)	17	62	0.77 (1.37)	2.16 (0.15–31.69)	0.574
Professional/Management	13	83	3.58 (1.80)	35.90 (1.07–1207.32)	0.046
Trade/Agricultural/Unemployed	18	77	3.67 (1.75)	39.07 (1.28–1195.34)	0.036
Retired	12	78	3.56 (1.80)	35.26 (1.04–1190.56)	0.047
NA	1	-			
Location within New Zealand					<0.001*
Upper half of North Island	62	243	Ref	-	-
Lower half of North Island	12	127	–0.98 (0.34)	0.37 (0.19–0.72)	0.003
Upper half of South Island	24	86	0.06(0.27)	1.06 (0.62–1.81)	0.829
Lower half of South Island	15	50	0.17 (0.33)	1.19 (0.62–2.26)	0.606
Living in rural area					
No	71	401	Ref	-	-
Yes	42	102	0.82 (0.23)	2.26 (1.45–3.52)	<0.001
NA	-	3			
Food associated risk factors					
Drinking treated milk					
No	12	60	Ref	-	-
Yes	101	446	0.16 (0.34)	1.17 (0.60–2.29)	0.638
Drinking raw milk					
No	108	490	Ref	-	-
Yes	5	16	0.30 (0.53)	1.35 (0.48–3.79)	0.574
Eating meat					
No	11	48	Ref	-	-
Yes	101	458	0.02 (0.36)	1.02 (0.50–2.06)	0.961
NA	1	-			
Handling raw offal					
No	95	350	Ref	-	-
Yes	16	156	–0.91 (0.31)	0.40 (0.22–0.74)	0.003
NA	2	-			
Eating seafood					
No	67	215	Ref	-	-
Yes	41	291	–0.72 (0.22)	0.48 (0.31–0.75)	0.001
NA	5	-			

Appendix 9 continued

Variable	Cases	Controls	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Eating raw fruit					
No	14	43	Ref	-	-
Yes	99	462	–0.41 (0.33)	0.67 (0.35–1.28)	0.223
NA	-	1			
Eating raw vegetables					
No	37	76	Ref	-	-
Yes	73	430	–1.07 (0.25)	0.34 (0.21–0.56)	<0.001
NA	3	-			
Drinking refrigerated fruit juice from supermarket					
No	94	280	Ref	-	-
Yes	18	222	–1.49 (0.28)	0.23 (0.13–0.39)	<0.001
NA	1	4			
Drinking fresh fruit juice from café					
No	101	405	Ref	-	-
Yes	8	97	–1.20 (0.39)	0.30 (0.14–0.65)	0.002
NA	4	4			
Dining outside home					
No	40	100	Ref	-	-
Yes	70	405	–0.88 (0.23)	0.41 (0.26–0.65)	<0.001
NA	3	1			
Water associated risk factors					
Water supply to home from					
Town supply/tanker/roof run off	89	468	Ref	-	-
Private bore/spring/creek/ stream	22	35	1.16 (0.30)	3.19 (1.77–5.72)	<0.001
NA	2	3			
Visiting areas of New Zealand without main water supply or recently interrupted main water supplies					
No	96	473	Ref	-	-
Yes	15	28	0.94 (0.35)	2.56 (1.29–5.05)	0.007
NA	2	5			
Swimming in public pool					
No	94	407	Ref	-	-
Yes	19	98	–0.51 (0.29)	0.60 (0.34–1.07)	0.083
NA	-	1			
Swimming in private pool					
No	105	460	Ref	-	-
Yes	8	46	–0.52 (0.41)	0.60 (0.27–1.33)	0.209
Swimming in spa pool					
No	106	442	Ref	-	-
Yes	7	63	–0.97 (0.42)	0.38 (0.17–0.87)	0.021
NA	-	1			
Recreational activities in fresh water					
No	97	474	Ref	-	-
Yes	15	32	0.69 (0.34)	1.99 (1.02–3.86)	0.043
NA	1	-			

Appendix 9 continued

Variable	Cases	Controls	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Contact with sewage/standing water/flood waters					
No	105	486	Ref	-	-
Yes	6	19	0.39 (0.48)	1.47 (0.57–3.8)	0.426
NA	2	1			
Animal associated risk factors					
Beef livestock present in meshblock					
No	74	422	Ref	-	-
Yes	39	84	0.96 (0.23)	2.61 (1.65–4.13)	<0.001
Dairy livestock present in meshblock					
No	94	477	Ref	-	-
Yes	19	29	1.18 (0.32)	3.26 (1.74–6.13)	<0.001
Sheep livestock present in meshblock					
No	82	434	Ref	-	-
Yes	31	72	0.78 (0.25)	2.19 (1.35–3.57)	0.002
Deer livestock present in meshblock					
No	105	488	Ref	-	-
Yes	8	18	0.72 (0.44)	2.05 (0.86–4.88)	0.106
Living on farm/lifestyle block with animals					
No	72	423	Ref	-	-
Yes	41	83	1.01 (0.23)	2.74 (1.74–4.31)	<0.001
Contact with household pets					
No	28	140	Ref	-	-
Yes	82	366	0.03 (0.24)	1.03 (0.64–1.67)	0.897
NA	3	-			
Contact with animals other than household pets					
No	74	415	Ref	-	-
Yes	37	90	0.76 (0.24)	2.14 (1.35–3.39)	0.001
NA	2	1			
Exposure to animal manure or compost containing animal manure					
No	66	413	Ref	-	-
Yes	34	86	0.96 (0.25)	2.60 (1.61–4.22)	<0.001
NA	13	7			
Hunting activities					
No	112	500	Ref	-	-
Yes	1	6	–0.58 (1.10)	0.56 (0.06–4.86)	0.600
Other household member having contact with animals other than household pets					
No	71	407	Ref	-	-
Yes	41	95	0.84 (0.23)	2.31 (1.47–3.63)	<0.001
NA	1	4			

Appendix 9 continued

Variable	Cases	Controls	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Human associated risk factors					
Visiting childcare/pre-school/school facilities					
No	72	250	Ref	-	-
Yes	41	256	–1.07 (0.25)	0.34 (0.21–0.56)	<0.001
Contact with children in nappies					
No	66	253	Ref	-	-
Yes	41	249	–0.75 (0.26)	0.47 (0.29–0.78)	0.004
NA	6	4			
Contact with person vomiting or having gastrointestinal disease					
No	88	414	Ref	-	-
Yes	15	76	–0.08 (0.31)	0.92 (0.50–1.68)	0.788
NA	10	16			
Host associated risk factors					
Taking antibiotics					
No	99	456	Ref	-	-
Yes	11	48	0.11 (0.36)	1.11 (0.56–2.24)	0.760
NA	3	2			
Taking antacids					
No	106	448	Ref	-	-
Yes	4	57	–0.94 (0.55)	0.39 (0.13–1.15)	0.087
NA	3	1			

[a] SE = Standard error.
[b] CI = 95% confidence interval.
* p-value of variable as whole using Likelihood ratio test.
Ref = reference level for comparison.
NA = observations with either 'unknown' or missing values.

Appendix 10: Multivariate logistic regression model without imputations. Results showing identified risk factors after deleting 57 of 619 observations (113 cases and 506 controls) due to missing values. 'No exposure/contact' was chosen as reference level for comparison in each variable (odds ratio = 1.00).

Variable	Coefficient (SE)[a]	Odds ratio (95% CI)[b]	Wald test p-value
Other household member having contact with animals other than household pets[c]			
for 0–4 year-old	2.30 (0.55)	10.01 (3.38–29.71)	<0.001
for 5–19 year-old	–0.15 (0.74)	0.86 (0.20–3.64)	0.838
for >19 year-old	0.07 (0.58)	1.08 (0.35–3.35)	0.899
Cattle livestock present in meshblock	1.12 (0.35)	3.07 (1.53–6.15)	0.002
Exposure to animal manure or compost containing animal manure	0.82 (0.36)	2.27 (1.11–4.63)	0.025
Recreational activities in fresh water	1.41 (0.50)	4.10 (1.55–10.85)	0.004
Visiting areas of New Zealand without main water supply or recently interrupted main water supplies	1.42 (0.47)	4.15 (1.64–10.49)	0.003
Water supply to home from private bore/ spring/ creek/ or stream	0.61 (0.47)	1.85 (0.73–4.68)	0.195
Handling raw offal	–1.26 (0.42)	0.29 (0.12–0.65)	0.003
Eating seafood	–0.58 (0.32)	0.56 (0.30–1.06)	0.074
Eating raw vegetables	–0.58 (0.41)	0.56 (0.25–1.25)	0.156
Drinking refrigerated fruit juice from supermarket	–1.35 (0.39)	0.26 (0.12–0.56)	0.001
Dining outside home	–0.62 (0.34)	0.54 (0.28–1.06)	0.073
Contact with children in nappies	–0.21 (0.37)	0.81 (0.39–1.68)	0.576
Visiting childcare/pre-school/school facilities	–1.68 (0.41)	0.19 (0.08–0.42)	<0.001
Taking antacids	–1.03 (0.67)	0.36 (0.10–1.33)	0.124

Likelihood ratio test = 152.94 (df = 18, p <0.001)

[a] SE = Standard error

[b] CI = 95% confidence interval

[c] This variable was modelled using a multiplicative interaction term comprising the variables 'Other household member having contact with animals other than household pets' and 'Age'.
It can be interpreted as follows: a child 0-4 years of age is at significantly higher risk of being an STEC case, if another household member had contact with animals other than household pets, compared to a child of the same age without this risk factor.

Appendix 11: E-published article ahead of print.

RESEARCH

Geographic Divergence of Bovine and Human Shiga Toxin–Producing *Escherichia coli* O157:H7 Genotypes, New Zealand[1]

Patricia Jaros, Adrian L. Cookson, Donald M. Campbell, Gail E. Duncan, Deborah Prattley, Philip Carter, Thomas E. Besser, Smriti Shringi, Steve Hathaway, Jonathan C. Marshall, and Nigel P. French

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 is a zoonotic pathogen of public health concern worldwide. To compare the local and large-scale geographic distributions of genotypes of STEC O157:H7 isolates obtained from various bovine and human sources during 2008–2011, we used pulsed-field gel electrophoresis and Shiga toxin–encoding bacteriophage insertion (SBI) typing. Using multivariate methods, we compared isolates from the North and South Islands of New Zealand with isolates from Australia and the United States. The STEC O157:H7 population structure differed substantially between the 2 islands and showed evidence of finer scale spatial structuring, which is consistent with highly localized transmission rather than disseminated foodborne outbreaks. The distribution of SBI types differed markedly among isolates from New Zealand, Australia, and the United States. Our findings also provide evidence for the historic introduction into New Zealand of a subset of globally circulating STEC O157:H7 strains that have continued to evolve and be transmitted locally between cattle and humans.

Shiga toxin–producing *Escherichia coli* (STEC) O157:H7 and related non-O157 STEC strains are zoonotic pathogens that can cause severe gastrointestinal illness in humans; clinical signs and symptoms of disease range from diarrhea and hemorrhagic colitis to life-threatening hemolytic uremic syndrome (*1*,*2*). Ruminants, asymptomatic carriers of STEC, shed the pathogen in their feces, and are considered a primary source of foodborne and environmental outbreaks of STEC infection in humans (*3*).

The incidence of STEC infections in New Zealand has been among the highest in the world. In 2012, a total of 147 clinical STEC cases (3.3 cases/100,000 population) were notified, of which 142 were confirmed (*4*). Consistent with observations in previous years, the predominant serotype among the confirmed cases was O157:H7 (83.8%; 119/142). STEC became a notifiable disease in New Zealand in 1997, and since then, the annual number of notifications has increased steadily (*4*). Although the spatial distribution of STEC cases in New Zealand suggests an association with farming and other rural activities, limited epidemiologic data are available on the transmission pathways of STEC from cattle to humans.

The objectives of this research were to 1) compare the population structure and geographic distribution of different genotypes of STEC O157:H7 isolates from bovine and human sources in New Zealand; 2) assess evidence for localized transmission of STEC from cattle to humans in New Zealand; and 3) compare the genotype distribution of isolates from New Zealand with those from Australia, the predominant historic source of imported New Zealand cattle (*5*), and the United States. To investigate the molecular divergence of isolates, we used 2 molecular typing methods: Shiga toxin–encoding bacteriophage insertion (SBI) typing and pulsed-field gel electrophoresis (PFGE) profiling. Although PFGE can provide an indication of genomic similarities, it cannot provide a reliable measure of

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DOI: http://dx.doi.org/10.3201/eid2012.140281

[1]Preliminary results from this study were presented at the New Zealand Veterinary Association Conference; June 16–20, 2014, Hamilton, New Zealand.

genetic relatedness of isolates, and the visual assessment of bands on an agarose gel to create PFGE profiles can result in misclassification bias (*6*). By using 2 methods and by examining the concordance between them, we could use the combined genotyping datasets to assess structuring and patterns of diversity among STEC O157:H7 isolates of bovine and human origin in New Zealand.

Methods

Human Isolates and Data

For the study, we obtained a total of 363 human-derived STEC O157:H7 isolates from the national Enteric Reference Laboratory (Institute of Environmental Science and Research Ltd, Upper Hutt, New Zealand) along with the associated PFGE profiles (restriction enzyme *Xba*I) and geographic data (North or South Island, New Zealand, and region on each island). Of the 363 isolates, 278 (76.6%) originated from the North Island. The isolates were from patients with clinical STEC infections that occurred in New Zealand during 2008–2011 and represent 71.3% (363/509) of the STEC O157 cases notified and confirmed during 2008–2011 (*7*). The cases were reported as sporadic cases or household clusters (i.e., 2 STEC infections in the same home) and were not associated with confirmed foodborne outbreaks.

Bovine Fecal Isolates and Data

Fecal STEC O157:H7 isolates (n = 40) used in the study had been collected from cattle in previous studies conducted at beef slaughter plants in New Zealand during 2008 (*8*) and 2009–2011 (*9*). Data regarding the origin (North or South Island, region, farm location) of the cattle and the virulence profiles of the isolates (virulence genes *ehx*A, *eae*, *stx*1, *stx*2, and subtype *stx*2c) were available. The isolates were retrieved from feces samples collected from 26 calves and 14 adult cattle, most (80.0%, 32/40) of which were from the North Island; the animals originated from 35 farms.

Bovine Meat Isolates and Data

Bovine meat isolates (n = 235) used in the study were from test samples used in routine mandatory testing at beef-processing plants across New Zealand during 2008–2011. Only PFGE profiles (*Xba*I) of STEC O157:H7 isolates were available for this study; the profiles were obtained from the national Enteric Reference Laboratory. Geographic data associated with meat-sample location (regions in North and South Islands) were obtained from the Ministry for Primary Industries (Wellington, New Zealand). Most isolates (85.5%, 201/235) originated from beef-slaughtering plants in the North Island. Virulence profiles of the isolates were not available.

PCRs for Detection of Virulence Genes

All human isolates were regrown on Columbia Horse Blood Agar (Fort Richard Laboratories, Auckland, New Zealand). Bacterial DNA was extracted from 5 colonies by using 2% Chelex beads solution (Chelex 100 Resin; Bio-Rad, Richmond, CA, USA) and analyzed in 2 PCR assays by using an automated real-time thermocycler (Rotor Gene 6200HRM; Corbett Research, Mortlake, NSW, Australia).

A multiplex PCR assay was performed using previously published primer sequences to detect the presence of virulence genes encoding for enterohemolysin (*ehx*A) (*10*), intimin (*eae*) (*10*), and Shiga toxins (*stx*1 and *stx*2) (*11*). Primers for detection of genes *stx*1 and *stx*2 did not differentiate between subtypes of toxins. The final 25-mL PCR reaction volume contained 2× PCR buffer (Express qPCR SuperMix; Invitrogen, Carlsbad, CA, USA), 2 μmol/L of each primer, 2.0 μL of DNA, and 2.5 μL of sterile water. The amplification program included an initial enzyme-activation step of 5 min at 94°C, which was followed by 40 cycles of, 20 s at 94°C, 20 s at 64°C, and 20 s at 72°C, followed in turn by a final extension of 5 min at 72°C. The PCR products were detected by electrophoresis using a 2% (wt/vol) agarose gel (Agarose low EEO; AppliChem, Darmstadt, Germany) and then stained with ethidium bromide and visualized under ultraviolet illumination.

*stx*2-positive isolates were further tested to determine whether the *stx*2 gene that was present was the genetic subtype *stx*2c. The *stx*2c gene was detected by using previously published primer sequences (*12*,*13*). The final 20-μL PCR reaction volume contained 2× PCR buffer (Express qPCR SuperMix; Invitrogen), 2 μmol/L of each primer, 2.0 μL of DNA, and 6.0 mL of sterile water. The PCR included an initial enzyme-activation step of 5 min at 94°C, followed by 35 cycles of 20 s at 94°C and 20 s at 55°C; no extensions were used. The amplified PCR product was detected as described above.

Molecular Typing Methods

All human and bovine fecal isolates were genotyped by using SBI typing (*12*,*13*); SBI typing is a multiplex PCR method for screening specific *stx*-associated bacteriophage insertion sites and *stx* genes (*stx*1 and genetic subtypes *stx*2a and *stx*2c of *stx*2). The characters A, W, Y, S and 1, 2a, 2c represent bacteriophage insertion sites *argW*, *wrbA*, *yehV*, *sbcB*, and Shiga toxin genes *stx*1, *stx*2a, *stx*2c (2 subtypes of *stx*2), respectively (*12*,*14*). All bovine fecal isolates were subtyped by using PFGE (*Xba*I) according to the standardized laboratory protocol published by PulseNet International (*15*). The SBI typing was completed at Washington State University, Pullman, Washington, USA.

Location of Work and Ethical Approval

This work was completed at the Molecular Epidemiology and Public Health Laboratory, Infectious Disease

Research Centre, Hopkirk Research Institute, Massey University, Palmerston North, New Zealand. The use of STEC isolates from clinical case-patients in New Zealand was approved by the Multi-region Ethics Committee, Wellington, New Zealand, on March 19, 2012; reference number MEC/11/04/043.

Data Management and Statistical Analysis

For initial analysis, SBI types were grouped into 4 categories of 3 predominant SBI types (AY2a, WY12a, and ASY2c/SY2c) and other, less common, SBI types (AS12c, AS2c, ASWY2c, ASY12c, ASY2a2c, AWY12a, AWY2a, SWY2c, and Y2c). SBI types SY2c and ASY2c were grouped together because both were relatively common and shared the same virulence gene profile.

Although bovine meat isolates were not SBI-typed, a close correlation between PFGE profile and SBI type was observed for the human samples (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/20/14/14-0281-Techapp1.pdf) and the bovine fecal samples (online Technical Appendix Figure 2). On the basis of the PFGE/SBI clusters, the most likely SBI type was inferred from the PFGE profiles for the meat isolates by taking the following approach. First, BioNumerics software version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium) was used to compare PFGE profiles of human and bovine fecal isolates by conducting an UPGMA (unweighted pair group method with arithmetic mean) cluster analysis using the Dice similarity coefficient, with a band matching tolerance of 1%. Second, the UPGMA cluster analysis was applied on PFGE profiles of bovine meat isolates. The dominant SBI types in human and bovine fecal isolates were used to assign SBI-like types (AY2a, WY12a, and ASY2c/SY2c) to clusters with similar PFGE band patterns in bovine meat isolates.

χ^2 and Fisher exact test for count data were used to evaluate associations between island and SBI type (AY2a, WY12a, ASY2c/SY2c, and other SBI types) for bovine fecal, bovine meat, and human isolates; R software (http://www.r-project.org/) was used for statistical computing. p values for associations between SBI types and region and between SBI types and year for human and bovine meat isolates were computed by simulating 10^8 tables from the null hypothesis (independence) and comparing the results with the test statistic from the observed data.

Population differentiation among human and bovine meat isolates was assessed by using analysis of molecular variance (AMOVA) applied to haplotypes of isolates' PFGE profiles (generated in BioNumerics) using Arlequin software version 3.5.1.2 (http://cmpg.unibe.ch/software/arlequin3/). A multilevel hierarchy was used for the AMOVA model to assess population differentiation between island, between regions within island, and within regions. Regions with <5 isolates were excluded from the analysis. A matrix of pairwise F_{ST} values was computed by comparing the PFGE haplotype frequency distributions for each pair of regions (using Arlequin, version 3.5.1.2). F_{ST} is an index of population differentiation, measuring the variance between subpopulations relative to the total variance, and ranges from 0 (no divergence) to 1 (complete divergence). The computed pairwise F_{ST} matrix, representing genetic distances between the regional populations of STEC O157:H7, was illustrated graphically as a NeighborNet tree by using SplitsTree software version 4.12.6 (*16*).

To illustrate the molecular relatedness and genotypic clustering of isolates, we used Primer 6 software (http://www.primer-e.com/primer.htm) to link distance matrices of PFGE profiles of human and bovine meat isolates (generated in BioNumerics) with explanatory variables (SBI type and region) to create multidimensional scaling plots. Regions with <5 isolates were excluded from the analysis.

To assess the population structure of New Zealand isolates, we compared published frequency distributions of SBI types in 205 cattle and 79 human STEC O157:H7 isolates sourced from Australia and in 143 cattle and 179 human STEC O157:H7 isolates sourced from the United States (*17*) with frequency distributions of SBI types among bovine and human STEC O157:H7 isolates from New Zealand. To evaluate genetic similarities of human and bovine fecal isolates, we computed proportional similarity indices (PSI) based on the frequency distributions of SBI types in humans and cattle from all 3 countries. PSI is a similarity measure that estimates the area of congruence between 2 frequency distributions (*18*); measurements range from 0 (distributions with no common SBI types) to 1 (highest possible similarity between distributions). Bootstrapped 95% confidence intervals for PSI values were calculated according to the percentile method described by Efron and Tibshirani (*19*), using 2,000 iterations. No grouping of SBI types was applied for PSI calculations. To illustrate the international geographic divergence of isolates, we used differences in PSI values (1 – PSI) to construct a NeighborNet tree with SplitsTree software version 4.12.6.

Results

Genotype Diversity

All 403 human and bovine fecal isolates were positive for *ehx*A, *eae*, and *stx*2 (except 1 *ehx*A-negative human isolate); of these, 61 (15.1%) were also positive for *stx*1. The different virulence profiles of isolates, each represented by a dominant SBI type, are shown in Table 1. The predominant SBI types AY2a, WY12a, and ASY2c/SY2c accounted for 55.0% (22/40), 15.0% (6/40), and 22.5% (9/40) of the studied bovine fecal isolates, respectively. Similarly, in human isolates, SBI types AY2a, WY12a, and ASY2c/SY2c were detected in

Table 1. Virulence profiles and SBI types of Shiga toxin–producing *Escherichia coli* O157:H7 isolates obtained from humans and fecal samples from slaughterhouse cattle, New Zealand, 2008–2011*

Species,			Virulence genes†					SBI type	
no. isolates	NI	SI	*ehx*A	*eae*	*stx*2	*stx*2c	*stx*1	Dominant (no., %)	Other (no., %)
Bovine									
6	6	0	+	+	+	–	+	WY12a (6, 100.0)	–
10	2	8	+	+	+	+	–	ASY2c (7, 70.0), SY2c (2, 20.0)	AS2c (1, 10.0)
24	24	0	+	+	+	–	–	AY2a (22, 91.7)	AWY2a (2, 8.3)
Human									
51	43	9	+	+	+	–	+	WY12a (49, 96.2)	AWY12a (2, 3.8)
1	0	1	+	+	+	–	+	WY12a (1, 100.0)	–
94	54	40	+	+	+	+	–	ASY2c (69, 73.4), SY2c (15, 16.0)	SWY2c (3, 3.2), ASWY2c (2, 2.1), AS2c (2, 2.1), Y2c (2, 2.1), ASY2a2c (1, 1.1)
214	179	35	+	+	+	–	–	AY2a (210, 98.1)	AWY2a (4, 1.9)
3	2	1	+	+	+	+	+	ASY12c (2, 66.7)	AS12c (1, 33.3)

*NI, North Island of New Zealand; SBI, Shiga toxin–encoding bacteriophage insertion; SI, South Island of New Zealand; +, gene present; –, gene absent. †*ehx*A gene encodes for enterohemolysin; *eae* gene encodes for intimin; *stx*2, primers for detection of this gene did not differentiate between subtypes of Shiga toxin type 2; *stx*2c gene encodes for Shiga toxin subtype 2c; *stx*1, primers for detection of this gene did not differentiate between subtypes of Shiga toxin type 1.

57.9% (210/363), 13.8% (50/363), and 23.1% (84/363) of the isolates, respectively. The distributions of AY2a, WY12a, ASY2c/SY2c, and other SBI types varied by year ($p = 0.037$) (Figure 1). On the basis of the genotype calibration of PFGE profiles of bovine meat isolates, SBI-like types AY2a, WY12a, and ASY2c/SY2c were prevalent in 64.7% (152/235), 23.4% (55/235), and 11.9% (28/235) of the isolates, respectively. Association between SBI-like type and year was marginally nonsignificant ($p = 0.052$).

Between-Island Comparisons

The distribution of SBI types observed differed between North and South Islands in bovine fecal and human isolates; SBI types AY2a and WY12a were more common in the North Island, and ASY2c/SY2c was more common in the South Island (Table 2). Similarly, a significant difference in the prevalence of SBI-like types between islands was observed in bovine meat isolates (Table 2).

Within-Island Comparisons

By using a 3-level hierarchy of island, region within island, and within region for the AMOVA model, we found that most of the molecular variation (>98%) resided between isolates within regions (on the basis of PFGE haplotypes). However, for the human isolates, a small but highly significant proportion of the molecular variation was estimated to be between regions within islands (1.03% variation, $p<0.001$); this finding provided evidence for highly localized geographic structuring. After we allowed for between region variation in the model, island was no longer

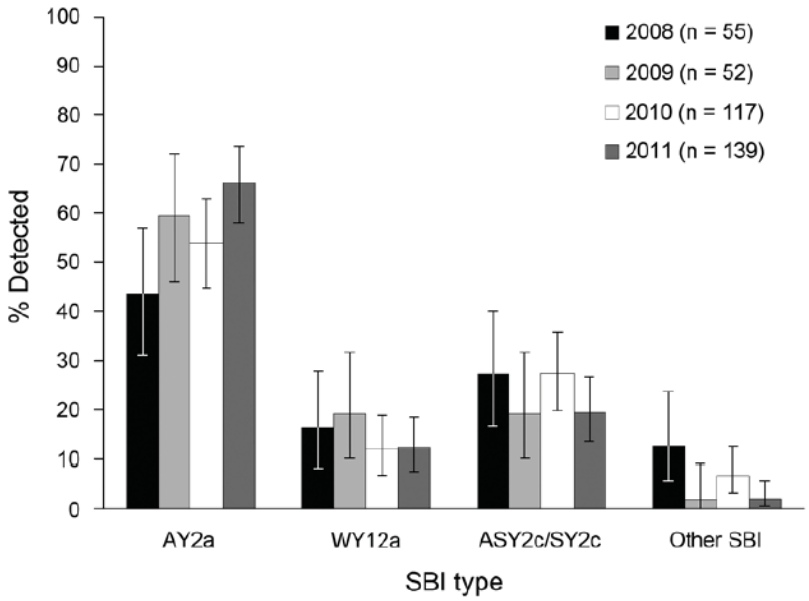

Figure 1. Proportional distributions, stratified by year, of Shiga toxin–encoding bacteriophage insertion (SBI) types AY2a, WY12a, and ASY2c/SY2c of 363 human Shiga toxin–producing *Escherichia coli* O157:H7 isolates from clinical case-patients in New Zealand, 2008–2011. Error bars indicate 95% CIs.

Table 2. Frequency distribution of predominant SBI genotypes of Shiga toxin–producing *Escherichia coli* O157:H7 isolates obtained from humans, bovine fecal samples, and bovine meat samples, New Zealand, 2008–2011*

Isolate type, SBI type	No. with SBI type/no. total (%) North Island	No. with SBI type/no. total (%) South Island	p value†
Human			
AY2a	175/278 (62.9)	35/85 (41.2)	<0.001
WY12a	41/278 (14.7)	9/85 (10.6)	
ASY2c/SY2c	49/278 (17.6)	35/85 (41.2)	
Other	13/278 (4.7)	6/85 (7.1)	
Bovine fecal			
AY2a	22/32 (68.8)	0/8	<0.001
WY12a	6/32 (18.8)	0/8	
ASY2c/SY2c	1/32 (3.1)	8/8 (100.0)	
Bovine meat			
AY2a-like	137/201 (68.2)	15/34 (44.1)	<0.001
WY12a-like	49/201 (24.4)	6/34 (17.6)	
ASY2c/SY2c-like	15/201 (7.5)	13/34 (38.2)	

*SBI, Shiga toxin–encoding bacteriophage insertion.
†Values refer to differences between frequency distributions of SBI types and North and South Islands (χ^2 and Fisher exact test).

a significant source of variation for the human isolates (p = 0.212). In contrast, a very small but significant amount of molecular variation was apparent between islands among the bovine meat isolates (0.38% variation, p = 0.017), but the proportion of variation between regions within islands was nonsignificant (0.34% variation, p = 0.121).

The population differentiation and geographic clustering of genotypes of STEC O157:H7 isolates from human cases and bovine meat samples from regions of both islands of New Zealand are illustrated in Figure 2. Consistent with the AMOVA results, we found evidence of within-island clustering of human isolates. Two main clusters were observed representing North and South Island regions, with the exception of Canterbury, which clustered with North Island regions, and Wellington, Taranaki, and Gisborne, which were North Island outliers. Among human cases, the highest population differentiation of genotypes of STEC O157:H7 isolates was observed between the regions of Wellington (15 isolates) and Gisborne (12 isolates) on the North Island (pairwise F_{ST} value of 0.071), followed by Wellington and Otago (10 isolates) (F_{ST} = 0.060); the isolates from Gisborne included 2 household clusters (2 human cases each). For bovine meat isolates, no obvious structuring was apparent; however, Auckland region (5 isolates) appeared as a strong North Island outlier. Consequently, the most distinct difference in genotypes was observed between the regions of Auckland and Otago (9 isolates) (F_{ST} = 0.060), followed by Auckland and Northland (26 isolates) (F_{ST} = 0.057).

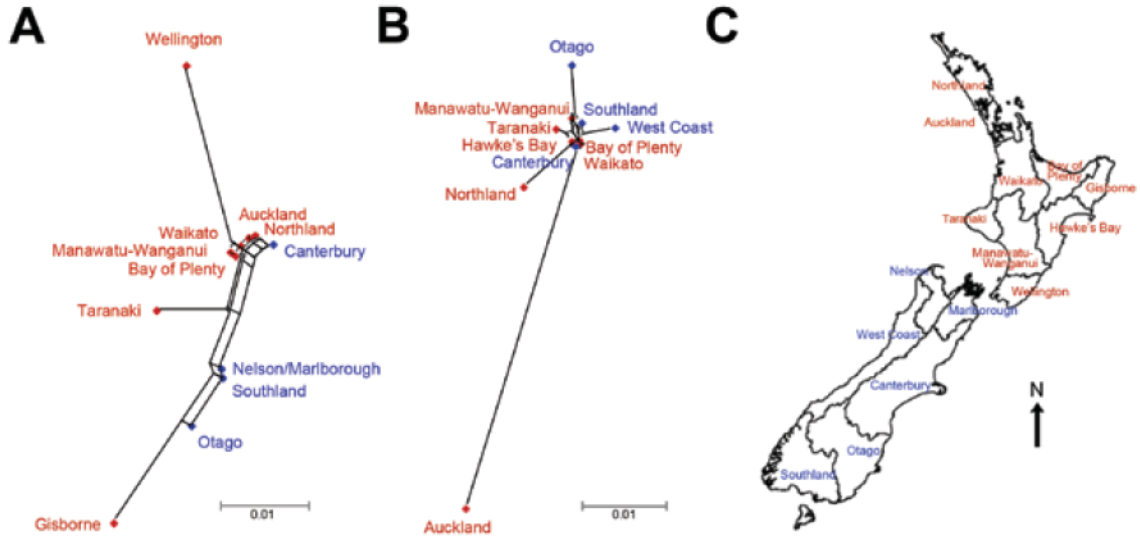

Figure 2. NeighborNet (*16*) trees showing population differentiation of Shiga toxin–producing *Escherichia coli* O157:H7 isolates from humans and cattle from different regions in the North Island (red) and the South Island (blue), New Zealand. A) Isolates from human case-patients (n = 355, 8 isolates excluded). B) Isolates from bovine meat samples (n = 233, 2 isolates excluded). C) Map of New Zealand showing different regions from which samples were collected. The distances indicate population differentiation measured as pairwise F_{ST} values.

The molecular relatedness between PFGE profiles of human isolates, considering SBI type and region of origin as explanatory variables, is shown in Figure 3. PFGE profiles showed genotypic clustering that was strongly associated with SBI types AY2a, WY12a, and ASY2c/SY2c, even after stratifying by island of origin (Figure 3, panels A, B). Clusters containing SBI type AY2a and ASY2c/SY2c were the predominant genotypes in the Taranaki and Gisborne regions, respectively, on the North Island (Figure 3, panel C); the association between SBI type and region of origin was statistically significant ($p<0.001$). A similar genotypic clustering of regions was observed in bovine meat isolates from the North and South Islands (online Technical Appendix Figure 3).

International Comparison

Within each country, similar frequencies of SBI types were observed in cattle and human cases, but there were distinct differences in the population structure of SBI types between countries (Figure 4). Bovine and human genotypes in New Zealand shared the highest similarity (PSI value 0.92, 95% CI 0.74–0.93), followed by those in Australia (PSI 0.69, 95% CI 0.57–0.79) and the United States (PSI 0.61, 95% CI 0.51–0.69) (online Technical Appendix Figure 4). The observed differences in proportional similarities of SBI types among isolates from cattle and humans in all 3 countries are shown in Figure 5.

Discussion

We assessed the molecular epidemiologic evidence for transmission of STEC from cattle to humans in New Zealand and the relationship between population structure and geography at multiple spatial scales. The molecular analysis of bovine and human STEC O157:H7 isolates showed a concordant geographic variation of genotypes (SBI types)

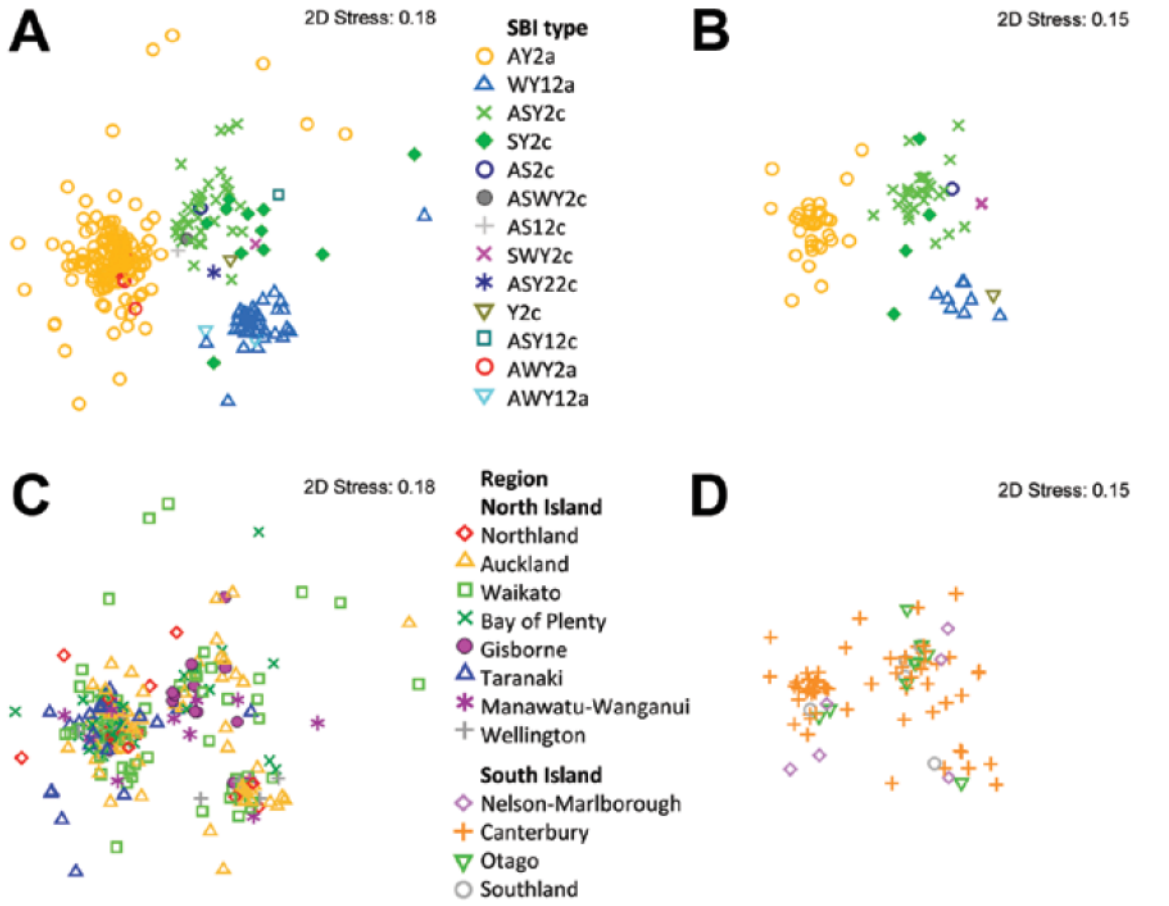

Figure 3. Multidimensional scaling plots showing the genotypic clustering of human Shiga toxin–producing *Escherichia coli* O157:H7 isolates originating from the North Island (n = 274, 4 isolates excluded) and the South Island (n = 81, 4 isolates excluded), New Zealand. The plots were determined on the basis of the isolates' pulsed-field gel electrophoresis profiles. Clusters associated with Shiga toxin–encoding bacteriophage insertion (SBI) types (A) and regions (C) for isolates from the North Island. Clusters associated with SBI types (B) and regions (D) for isolates from the South Island. 2D, 2 dimensional.

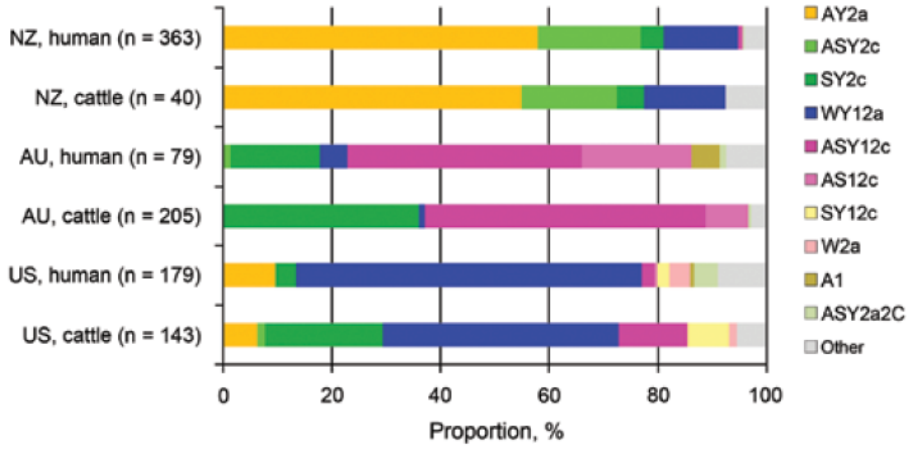

Figure 4. Proportional distributions of Shiga toxin–encoding bacteriophage insertion types of Shiga toxin–producing *Escherichia coli* O157:H7 isolates sourced from cattle and humans in New Zealand (NZ), Australia (AU), and the United States (US).

in both populations. In addition, there were marked differences between isolates from New Zealand's North and South Islands, a finding that is consistent with localized transmission of STEC between cattle and humans.

The evidence of localized transmission of STEC between cattle and humans in New Zealand has advanced our understanding of the epidemiology of sporadic STEC infections in the country and is consistent with environmental- or animal-associated sources of infection rather than more disseminated foodborne outbreaks (*20*). Measures to prevent direct contact with animal fecal material in the environment include the wearing of protective clothing, increased hand washing, and targeted education of the population at risk regarding possible sources of STEC infection.

The North and South Islands of New Zealand are separated by the Cook Strait, a geographic barrier of >20 km. This barrier might contribute to the island-associated differences in distribution of genotypes observed in this study, by restricting the movement of carrier animals between islands. Cattle populations on each island are large: ≈6.6 million on the North Island and ≈3.5 million on the South Island (*21*). Despite the islands' large cattle populations, the number of livestock moved between the islands (i.e., from farm to farm or farm to slaughter) is relatively low: ≈42,400 cattle from North to South Island, and ≈64,600 cattle from South to North Island per year (*22*). Thus, the movement of cattle probably has a limited influence on the distinct distribution of genotypes across both islands.

Although none of the bovine meat isolates were SBI typed, the PFGE data showed a strong island-associated distribution of bovine STEC O157:H7 genotypes, which was equivalent to the patterns observed in fecal isolates from cattle and humans. Bovine meat isolates were retrieved from carcass swab samples and bulk meat samples collected at beef-processing plants, so it could be hypothesized that fresh beef meat might be an exposure pathway for humans. However, although various food sources (including beef) were considered as potential risk factors during a nationwide prospective case-control study on sporadic STEC infections in humans, food was not identified as a major exposure pathway of infections in New Zealand (*20*).

Significant genetic variation was observed among human isolates at the regional level, indicating a more localized spatial clustering of STEC O157:H7 genotypes. Strong regional variation in the prevalence of zoonotic diseases has been observed previously in New Zealand. For example, there is marked regional variation in the distribution of serotypes in human cases of salmonellosis: *Salmonella enterica* ser. Brandenburg was associated with sheep and human infections in the southern regions of the South Island (*23*), whereas the wild bird–associated *S. enterica* ser. Typhimurium DT160 was distributed more evenly across the whole country (*24*). *S. enterica* ser. Brandenburg has not been found to be endemic in any other regions in New Zealand, and it is likely that the spatial pattern of disease is influenced by environmental factors, such as the presence and density of local maintenance hosts.

Cattle are considered the most likely maintenance host of STEC O157:H7, and the association between human cases and cattle density suggests that spillover from cattle to humans is the main pathway (*20*); however, overseas, the pathogen has frequently been isolated from sheep (*25*–*27*) and deer (*28*,*29*). Cookson et al. (*30*,*31*) identified STEC serotypes of public health concern in sheep from the lower North Island of New Zealand but did not isolate STEC O157:H7. No nationwide studies of sheep or deer have been undertaken in New Zealand, hence sheep cannot be ruled out as potential maintenance hosts for region-specific populations of STEC O157:H7.

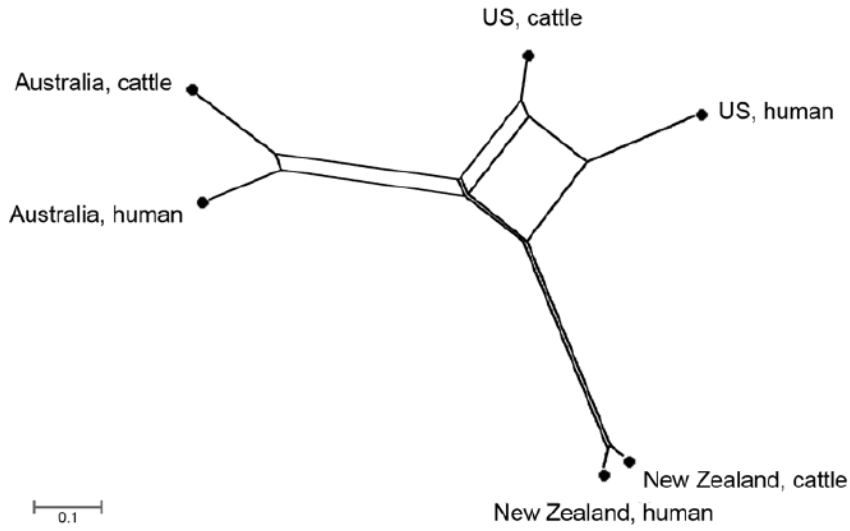

Figure 5. NeighborNet (*16*) tree showing geographic divergence of bovine and human Shiga toxin–producing *Escherichia coli* O157:H7 isolates sourced from New Zealand (40 cattle, 363 human), Australia (205 cattle, 79 human), and the United States (US) (143 cattle, 179 human). The distance indicates the difference in proportional similarity of Shiga toxin–encoding bacteriophage insertion types among the isolates.

The observed regional clustering of genotypes among human STEC O157:H7 isolates leads to another hypothesis: other, yet unidentified, hosts could be reservoir/maintenance hosts in the epidemiology of STEC, and cattle are possibly only serving as "bridging hosts" at the human–animal interface (*32*), transmitting STEC to humans. For example, starlings have been implicated as biologic vectors in the dissemination of STEC among dairy farms in Ohio, United States (*33*,*34*), indicating that wildlife might play a key role in the epidemiology and ecology of STEC.

A relatively high prevalence of SBI types AY2a and ASY2c was observed in human and bovine fecal isolates from New Zealand. These findings are in contrast to those from the Australian study by Mellor et al. (*17*), in which SBI type AY2a was not identified (0/284 isolates; $p<0.001$) and accounted for only 8.1% (26/322) of the isolates from the United States (human and cattle combined); SBI type ASY2c was prevalent in <1.0% of combined isolates in both countries. These differences in frequency distributions of SBI types indicate marked differences in the population structure of SBI types between countries. Australia and New Zealand are neighboring countries but separated by the Tasman Sea, a distance of ≈1,250 km. On the basis of historic data, Australia has been the predominant source of imported New Zealand cattle, mainly in the 19th century (*5*). Hence, the distinct geographic divergence of STEC O157:H7 genotypes between the 2 countries is somewhat puzzling and would suggest a limited historic introduction of STEC O157:H7 from Australia or elsewhere into New Zealand and a subsequent evolution in the New Zealand host population. Alternatively, the observed divergence of genotypes between Australia and New Zealand could be the result of genetic drift and/or selection driven by different environmental factors, such as climate, types of feed, husbandry systems, or animal genetics.

In this study, the highest PSI was observed between cattle and human isolates from New Zealand, followed by that between isolates from Australia and the United States. These findings provide evidence for a close association between populations of isolates from cattle and humans, which is consistent with the transmission of STEC from cattle to humans. This finding is in agreement with the national case–control study on clinical STEC cases in New Zealand, which identified variables related to beef and dairy cattle as major risk factors (*20*).

The molecular analysis of STEC O157:H7 isolates from cattle and persons with STEC infection revealed that prevalences of bovine and human isolates in the North Island were distinctly different from those of the South Island, suggesting localized transmission of STEC between cattle and humans. Furthermore, a distribution of STEC O157:H7 genotypes different from that observed overseas suggests a historic introduction of a subset of the globally circulating STEC O157:H7 strains into New Zealand.

Acknowledgments

We thank Muriel Dufour, Brent Gilpin, Kari Gobius, and Glen Mellor for their contributions and Charlotte Bolwell for very helpful comments on this manuscript.

This work was supported by the Meat Industry Association of New Zealand and the Ministry for Primary Industries.

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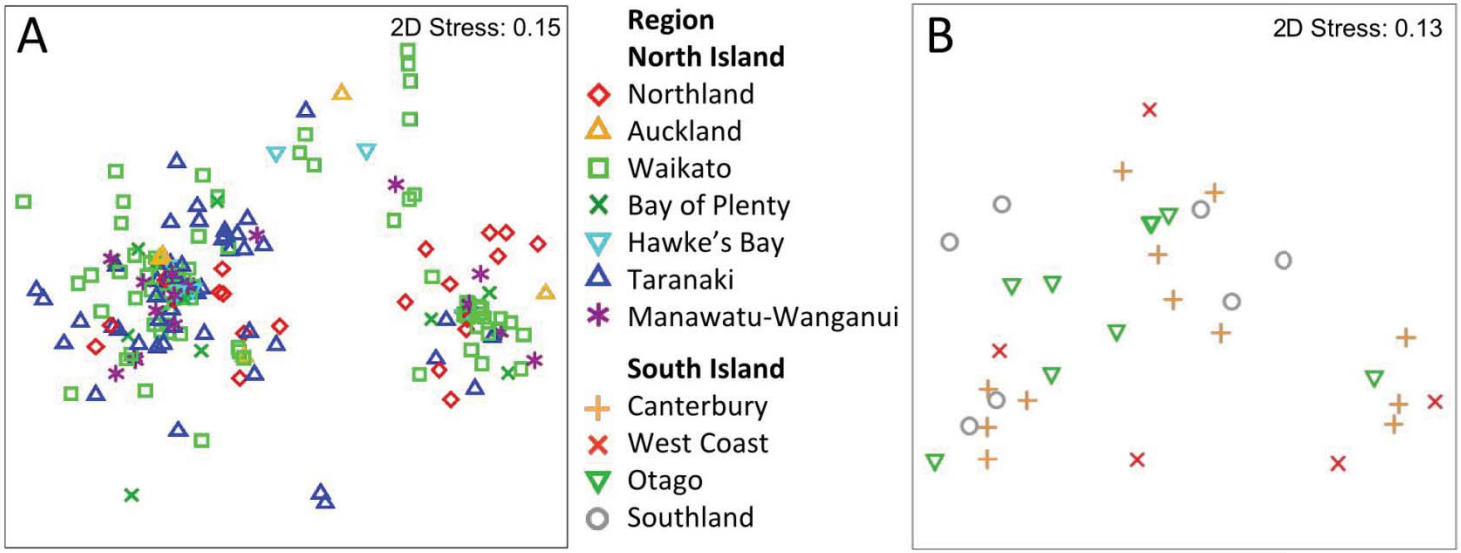
Appendix 12: Multi-dimensional scaling plots showing genotypic clustering of bovine meat isolates.



Multi-dimensional scaling plots showing the genotypic clustering of bovine meat Shiga toxin-producing *Escherichia coli* O157:H7 isolates originating from the North Island (n = 200, 1 isolate excluded) and South Island (n = 33, 1 isolate excluded) of New Zealand, based on the isolates' pulsed-field gel electrophoresis profiles. Clusters associated with regions are presented for isolates from the North Island (A) and the South Island (B), respectively.

Appendix 13: Proportional similarity index (PSI) values.

	NZ Cattle	NZ Human	AU Cattle	AU Human	US Cattle	US Human
NZ Cattle (n = 40)		0.74 – 0.93	0.00 – 0.13	0.04 – 0.20	0.16 – 0.41	0.18 – 0.41
NZ Human (n = 363)	0.92		0.04 – 0.09	0.06 – 0.18	0.21 – 0.33	0.23 – 0.34
AU Cattle (n = 205)	0.06	0.06		0.57 – 0.79	0.27 – 0.43	0.05 – 0.13
AU Human (n = 79)	0.11	0.12	0.69		0.24 – 0.44	0.08 – 0.21
US Cattle (n = 143)	0.28	0.27	0.35	0.35		0.51 – 0.69
US Human (n = 179)	0.30	0.29	0.09	0.15	0.61	

Proportional similarity index (PSI) values assessing the similarity of frequency distributions of Shiga toxin-encoding bacteriophage insertion (SBI) types among human and bovine Shiga toxin-producing *Escherichia coli* O157:H7 isolates originating from New Zealand (NZ), Australia (AU), and the United States (US). PSI values are presented in the lower half of the matrix with corresponding bootstrapped 95% confidence intervals in the upper half. Increased font size of PSI value shows higher degree of similarity.

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We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Patricia Jaros

Name/Title of Principal Supervisor: Prof. Nigel P. French

Name of Published Research Output and full reference:

A prospective case-control and molecular epidemiological study of human cases of Shiga toxin-producing Escherichia coli in New Zealand. BMC Infect Dis. 2013;13(1):450.

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  PJ is first author on this paper. She designed and coordinated the study, performed the screening for stx2c of isolates, performed the statistical analysis and drafted the manuscript.

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