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H Irshad a, AL Cookson b, G Hotter a, TE Besser c, SLW On d & NP French a

a Hopkirk Research Institute, Massey University, Private Bag 11222, Palmerston North, 4442, New Zealand
b Agri-Foods & Health Section, AgResearch Ltd, Grasslands Research Centre, Private Bag 11008, Palmerston North, 4442, New Zealand
c Department of Veterinary Microbiology and Pathology, Washington State University, PO Box 647040, Pullman Washington, USA
d Food Programme, Institute of Environmental Science and Research (ESR) Limited, PO Box 29-181, Christchurch, New Zealand

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Epidemiology of Shiga toxin-producing Escherichia coli O157 in very young calves in the North Island of New Zealand

H Irshad*†, AL Cookson‡, G Hotter*, TE Besser#, SLW On¥ and NP French*

Abstract

Aims: To study the occurrence and spatial distribution of Shiga toxin-producing Escherichia coli (STEC) O157 in calves less than 1-week-old (bobby calves) born on dairy farms in the North Island of New Zealand, and to determine the association of concentration of IgG in serum, carcass weight, gender and breed with occurrence of E. coli O157 in these calves.

Methods: In total, 309 recto-anal mucosal swabs and blood samples were collected from bobby calves at two slaughter plants in the North Island of New Zealand. The address of the farm, tag number, carcass weight, gender and breed of the sampled animals were recorded. Swabs were tested for the presence of E. coli O157 using real time PCR (RT-PCR). All the farms were mapped geographically to determine the spatial distribution of farms positive for E. coli O157. K function analysis was used to test for clustering of these farms. Multiplex PCR was used for the detection of Shiga toxin 1 (stx1), Shiga toxin 2 (stx2), E. coli attaching and effacing (eae) and Enterohaemolysin (ehxA) genes in E. coli O157 isolates. Genotypes of isolates from this study (n=10) along with human (n=18) and bovine isolates (n=4) obtained elsewhere were determined using bacteriophage insertion typing for stx encoding.

Results: Of the 309 samples, 55 (17.7%) were positive for E. coli O157 by RT-PCR and originated from 47/197 (23.8%) sampled animals were recorded. Swabs were tested for the presence of E. coli O157 using real time PCR (RT-PCR). All the farms were mapped geographically to determine the spatial distribution of farms positive for E. coli O157. K function analysis was used to test for clustering of these farms. Multiplex PCR was used for the detection of Shiga toxin 1 (stx1), Shiga toxin 2 (stx2), E. coli attaching and effacing (eae) and Enterohaemolysin (ehxA) genes in E. coli O157 isolates. Genotypes of isolates from this study (n=10) along with human (n=18) and bovine isolates (n=4) obtained elsewhere were determined using bacteriophage insertion typing for stx encoding.

Conclusions: Healthy bobby calves are an asymptomatic reservoir of E. coli O157 in New Zealand and may represent an important source of infection for humans. Carriage was not associated with concentration of IgG in serum, carcass weight or gender.

Key Words: STEC, stx, eae, calf, IgG, public health

Introduction

Shiga toxin-producing Escherichia coli (STEC) have emerged as important foodborne zoonoses associated with outbreaks and sporadic cases of diarrhoea, haemorrhagic colitis and haemolytic uremic syndrome in humans (Griffin et al. 1988). There are many different STEC serotypes that have been associated with disease, but the most important is STEC O157, or E. coli O157, due to its association with large outbreaks and many of the cases of haemolytic uremic syndrome and haemorrhagic colitis in humans. The association of E. coli O157 with outbreaks of haemorrhagic colitis in humans was observed for the first time in the United States of America in 1982 (Riley et al. 1983). Since then outbreaks of disease associated with E. coli O157 have been reported from all over the world (Gyles 2007). In New Zealand, the first case of E. coli O157 was reported in 1993 in an 11-month-old boy (Wright et al. 1993). Subsequently many cases of E. coli O157 have been recorded in New Zealand; in 2009 137 cases (3.6 per 100,000 population) of E. coli O157 were recorded in humans (Anonymous 2009). It is likely that infection is under reported as many faecal samples from asymptomatic or mild cases may not be assessed for the presence of E. coli O157 (Baker et al. 1999).

Cattle and sheep are the main reservoirs of E. coli O157 (Hancock et al. 1998; Paiba et al. 2003; Synge et al. 2003). Faecal excretion of E. coli O157 may cause infection in humans due to direct contact with these animals or contact with food or water contaminated with their faeces during slaughter or grazing, respectively (Renwick et al. 1993; Armstrong et al. 1996).

CT-SMAC Sorbitol MacConkey agar supplemented with cefixime and potassium tellurite
eae Escherichia coli attaching and effacing gene
ehxA Enterohaemolysin gene
RT-PCR Real time polymerase chain reaction
STEC Shiga toxin-producing Escherichia coli
stx Shiga toxin gene
Therefore, cattle play an important role in the epidemiology of
*E. coli* O157.

Cattle and sheep are believed to be the major reservoir of *E. coli* O157 in New Zealand (Buncic and Avery 1997; Cookson et al. 2006a). However, very little information is available about the epidemiology of carriage of *E. coli* O157 in New Zealand, and to test the hypothesis that the carriage of *E. coli* O157 was associated with concentration of IgG in serum. Calves that are culled from a dairy herd at less than a week of age are known asobby calves. In New Zealand, approximately 1.4 millionobby calves are slaughtered annually for local and export markets (Anonymous 2010). A better understanding of the epidemiology of *E. coli* O157 is likely to assist in reducing the increasing number of human cases in New Zealand and also in designing interventions to avoid possible losses to red meat and dairy export industries that are economically important in New Zealand (exports equivalent to NZ$10.8 billion) (Cavanagh 2003).

### Materials and methods

#### Collection of samples

Two abattoirs that slaughtered and exported products derived fromobby calves (A and B) were selected from those in the North Island of New Zealand. Abattoirs A and B were visited seven and six times, respectively, during the 4-month study period between July and October 2008. Twenty-five calves were selected systematically (every 10th calf on the chain) at each visit, for collection of recto-anal mucosal swabs and blood. Calves from different farms were transported together to the abattoirs.

Free draining blood samples were collected after slaughter into plain vacuum tubes (Becton Dickinson, Auckland, NZ). Recto-anal mucosal swabs were taken using sterile cotton swabs (Copan, Brescia, Italy) at the abattoir after the slaughter of the animal. These swab samples were then placed in the transport media provided by the manufacturer and maintained at approximately 4°C until processed in the laboratory. The details of the sampled animals, address of the farm, tag number, carcass weight, gender and breed of the animal were recorded.

#### Laboratory methods

Recto-anal mucosal swabs were enriched inbuffered peptone water for 24 h at 37°C. After enrichment, a 1-mL aliquot of enriched broth was taken for DNA extraction. This aliquot was centrifuged at 12,000 g for 2 minutes. After centrifugation, the supernatant was discarded, and the pellet was re-suspended in 500 μL of 2% chelex (Bio-Rad, Auckland, NZ) solution and heated at 95°C for 10 minutes. The lysed bacterial cell suspension was then cooled at 4°C for 2 minutes and re-centrifuged at 12,000 g for 2 minutes. The supernatant containing the DNA was transferred to another tube. It was then tested using real time PCR (RT-PCR) for the presence of *rbe* (O157) gene (Perelle et al. 2004). The *rbe* gene expresses an enzyme necessary for synthesis of O-antigen biosynthesis and is specifically expressed in *E. coli* O157 serotypes (Bilge et al. 1996). Each PCR reaction contained 1× Reaction buffer (Invitrogen, NZ), 0.2 μM of each primer, 0.1 mM of each dNTP (Fermentas, Auckland, NZ), 0.15 mM MgCl2 (Invitrogen, NZ), 0.03 mM SYTO-9 (Invitrogen, NZ), 1 unit of Taq DNA Polymerase (Invitrogen, NZ), 2 μL of DNA and made to final volume of 20 μL with sterile water. The amplification was carried out in a Rotor Gene 6000 series thermal cycler (Bio Strategy, Auckland, NZ) which was programmed for 5 minutes at 96°C, 40 cycles of 15 seconds at 96°C, 10 seconds at 62°C and 10 seconds at 72°C, after which the PCR product was detected by thermal melt from 75°C to 90°C at a rate of 0.05°C per second. Positive and negative controls were included in each reaction. The performance of the RT-PCR incorporating *rbe* (O157) primers was assessed in three different ways. Firstly, faecal samples negative for O26, O103, O111, O145 and O157 were spiked with O26, O103, O111, O145 and O157 isolates. The spiked samples were enriched inbuffered peptone water at 37°C for 24 h. DNA from each sample was isolated and tested with *rbe* (O157) primers. Positive RT-PCR amplicons using the *rbe* (O157) primers were only obtained from samples spiked with *E. coli* O157 while samples spiked with O26, O103, O111 and O145 serotypes were negative with *rbe* (O157) primers. Secondly, the DNA from pure O26, O103, O111, O145 and O157 colonies was extracted and tested with *rbe* (O157) primers. An amplicon of correct size using the *rbe* (O157) primers was only obtained from DNA extracted from an *E. coli* O157 colony. Thirdly, DNA from five samples that were positive for *E. coli* O157 but did not yield isolates were sequenced. The sequences of these five samples were identical to the *rbe* (O157) sequence.

The samples positive for *E. coli* O157 by RT-PCR were further processed for the isolation of *E. coli* O157 using immunomagnetic separation where beads coated with antibodies against *E. coli* O157 (Invitrogen, NZ) were plated onto Sorbitol MacConkey Agar supplemented with ceftaxime and potassium tellurite (CT-SMAC) (Fort Richard, Auckland, NZ). Grey non-sorbitol fermenting colonies suspected to be *E. coli* O157 were identified using a latex agglutination kit (Oxoid, Auckland, NZ). *E. coli* O157 colonies were also analysed using multiplex PCR to detect the presence of Shiga toxin 1 (*stx1*), Shiga toxin 2 (*stx2*), *E. coli* attaching and effacing (*eae*) and enterohaemolysin (*ehx*) genes. Each PCR reaction contained 1× Reaction buffer (Invitrogen, NZ), 0.2 μM of each primer, 0.1 mM of each dNTP (Fermentas), 0.15 mM MgCl2 (Invitrogen, NZ), 1 unit of *Taq* DNA Polymerase (Invitrogen, NZ), 2 μL of DNA and made to a final volume of 25 μL with sterile water. The amplification was carried out in a Gene Amp PCR system 9700 (Applied Biosystems, Melbourne, Australia) which was programmed for 5 minutes at 96°C, 40 cycles of 30 seconds at 96°C, 30 seconds at 60°C, 30 seconds at 72°C, with final extension of 5 minutes at 72°C, after which the PCR products were electrophoresed through an agarose (2% w/v) gel (Invitrogen, NZ) and visualised using ethidium bromide under ultra violet illumination. DNA was extracted from these bovine isolates (n=10) and from a further 22 isolates (18 human and 4 bovine) supplied by Dr Stephen On (Institute of Environmental Science and Research Ltd, Christchurch, NZ). All bovine *E. coli* O157 isolates analysed in this study were from different animals and almost all of the human isolates were from sporadic cases of diarrhoeal disease and not associated with any specific source. These isolates were assessed using the *stx* encoding bacteriophage insertion typing system. Briefly, multiplex PCR was used to determine the *stx* encoding bacteriophage insertion sites. Multiplex 1 included *stx1*, the right *wrbA* bacteriophage junction and the left *rbe* bacteriophage junction while multiplex 2 included *stx2*, the left *wrbA* bacteriophage junction and the right *rbe* bacteriophage junction as described previously (Whitworth et al. 2008). Each PCR reaction contained
2.5 U/μL Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 2 mM MgCl₂ (Invitrogen, USA), and 0.4 mM dNTP (Invitrogen, USA), 1 μM primers, 5 μL 10× buffer and 2 μL of DNA to make to a final volume of 50 μL with sterile water. The amplification was carried out in an iCycler (Bio-Rad, Richmond, CA, USA) which was programmed for 5 minutes at 95°C, 35 cycles of 15 seconds at 94°C, 45 seconds at 58°C and 90 seconds at 72°C followed by a final extension step of 10 minutes at 72°C.

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

Statistical analysis
Calves were sampled at two abattoirs and the addresses of all the farms from which calves had originated were recorded along with farm coordinates (latitude and longitude) using Agribase™ (Sanson and Pearson 1997) data and Google Earth (www.google.com/earth). The latitude and longitude of each farm were then converted to XY coordinates. The latitude and longitude of 17 farms were missing. Negative farms were defined as those from which animals were sampled but did not give rise to a positive sample by RT-PCR. Assumptions on whether a farm was positive or negative could only be made on the basis of the animals sampled from it, inevitably leading to some misclassification at the farm-level. Further, the study was undertaken over a small time period and the status of farms was likely to change as other animal cohorts were sent for processing. Hence, most analyses were conducted at the calf-level, with the exception of the test for spatial clustering, which was carried out at both farm and calf level.

The farms (180) were mapped using R v2.8.1 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria) and evidence for localised clustering, also known as second-order spatial clustering, of positive (cases) compared with negative (controls) farms was investigated using K function analysis (Ripley 1976). This was done by calculating the difference between two K functions at a range of distances from 1 km to 10 km. This technique is a standard approach to comparing the spatial properties of one spatial point pattern with another that does not require the underlying process to be stationary (for a description of this method see Bailey and Gatrell 1995). In this case we compared the local spatial properties of positive farms compared with negative farms, and repeated the analysis at the calf level.

The difference between the two K functions was plotted against distance along with upper and lower 95% simulation envelopes which are computed by repeatedly simulating the difference using the same farm or animal locations and randomly relabelling positive and negative farms. If the difference between the K functions fell above the upper envelope this indicated the positive farms compared with negative farms, and repeated the analysis for E. coli O157. There were 10 values missing for the variable gender. The spatial distribution of the farms that were positive and negative for E. coli O157 is shown in Figure 1.

K function analysis provided evidence of localised clustering of farms positive for E. coli O157 compared with negative farms, and this was significant between 3 and 4 km (Figure 2). A very similar pattern was observed when the analysis was carried out at the individual animal level (data not shown).

Immuno-magnetic separation with selective plating on CT-SMAC of samples positive for E. coli O157 using RT-PCR (n=55) resulted in 10 isolates of E. coli O157, seven of which were positive for stx2, eae, ehxA; the remaining three were positive for stx1, stx2, eae and ehxA. The results of stx insertion typing

Results

Recto-anal mucosal swabs and serum samples were obtained from 309 bobby calves (Jersey n=180, Friesian n=85 and Hereford n=44) originating from 197 farms. Fifty-five (17.7%) of the swabs, originating from 47/197 (23.8%) farms were positive for E. coli O157 by RT-PCR. On average there were four samples positive for E. coli O157 on each sampling occasion. There were 35/211 (16.5%) male and 16/88 (18%) female calves positive for E. coli O157. There were 10 values missing for the variable gender. The spatial distribution of the farms that were positive

Figure 1. Map showing the distribution of the farm of origin of 309 bobby calves sampled for Escherichia coli O157 in the North Island of New Zealand. The data are aggregated to 5 x 5 km grid cells. Only cells containing farms that submitted calves that were subsequently sampled are displayed; cells containing >1 animal that was positive are grey, cells with no positive animals are black.
revealed 12/18 (67%) human isolates belonged to genotypes 1 and 3, 4/18 (22%) human isolates to genotype 5 and one each to genotype 11 and 17. In contrast, of the 14 bovine isolates, 13 (93%) belonged to genotypes 1 and 3 and one isolate (7%) belonged to genotype 19 (Figure 3).

Results of linear mixed-effects models fitted consecutively for each variable (IgG, carcass weight, gender and breed) are shown in Table 1. No significant relationship was observed between samples positive for *E. coli* O157 by RT-PCR and concentration of IgG or carcass weight of the calves (Table 1). However, Jersey calves tended to be less likely to be positive for *E. coli* O157 compared with Friesian calves (p = 0.094). Mean concentration of IgG in serum for calves that were positive or negative for *E. coli* O157 was 21.8 (SE 17) mg/mL and 21.4 (SE 17.6) mg/mL, respectively. Mean carcass weight was 17.4 (SE 0.39) kg and 16.9 (SE 0.39) kg, respectively. Results of the model in which all variables were fitted together are also shown in Table 1. Again, Jersey calves tended to be less likely to be positive for *E. coli* O157 by RT-PCR compared with Friesian calves (p = 0.055). There were 18 values missing for the variable carcass weight.

**Discussion**

Despite the importance of the beef and dairy industries to the economy of New Zealand, there are limited data on the prevalence of carriage of *E. coli* O157 in cattle. The first isolation of *E. coli* O157 from cattle in New Zealand was described by Buncic and Avery (1997), where two *E. coli* O157 isolates were obtained from 371 animals using immuno-magnetic separation. Non-O157 STEC serogroups including O5, O26, O84 and O128 have also been previously isolated from cattle (n = 187) and sheep (n = 132) in New Zealand (Cookson et al. 2006b) but unlike this study no specific methods, such as RT-PCR or immuno-magnetic separation, were included for the specific selection of O157.

RT-PCR has been used successfully for detection of *E. coli* O157 in faeces, food and water samples by others (Witham et al. 1996; Ibekwe et al. 2002; Sharma and Dean-Nystrom 2003) and is a
fast and sensitive method for detection of E. coli O157 (Stefan et al. 2007). This may be the reason that the prevalence of carriage of E. coli O157 in this study was higher compared with other studies conducted in dairy calves in which conventional culture of E. coli O157 was used as the diagnostic test. For example, a prevalence of 6% for carriage of E. coli O157 was reported for dairy calves in Denmark (Rugbjerg et al. 2003), and of 0.5% and 10% in veal calves and adult cattle, respectively, in the Netherlands (Heuvelink et al. 1998). However, this difference in prevalence with other studies may also be due to differences in sample size, management practices and environmental factors.

The low prevalence of isolation of E. coli O157 (10/55) from samples positive by RT-PCR may be due to the higher sensitivity of RT-PCR compared with isolation. It may also be due to the presence of dead E. coli O157 in the samples which are detectable by RT-PCR but cannot be isolated. All 10 E. coli O157 isolates in this study were positive for stx2, eae and ebhA while three isolates were also positive for stx1 in addition to stx2, eae and ebhA. These genes are commonly found in the human isolates of E. coli O157 (Pradel et al. 2008). The results of six insertion typing showed that 78% (25/32) of the E. coli O157 isolates belonged to the genotypes 1 and 3. There was more diversity among human isolates (5 genotypes) compared with bovine isolates (3 genotypes) and the most common genotype in human (50%) and bovine isolates (71%) was genotype 1. These results differ from those of an American study in which 95% (268/282) of the human isolates and 51% (41/80) of bovine isolates belonged to genotypes 1, 2 and 3, indicating that there was more diversity in bovine compared with human isolates (Besser et al. 2007). In that study, the most common genotype in human and bovine isolates was genotype 3 while in this study genotype 1 was the most commonly found genotype in human and bovine isolates. However, these differences may be due to the small number of human (n=18) and bovine isolates (n=14) tested in this study. Furthermore, human strains used in this study had been chosen on the basis of prior knowledge of their genetic diversity based, in part, on macrorestriction profiles (S. Brandt, pers. comm.). It is also possible that the association of specific E. coli O157 genotypes differs internationally. Therefore, analysis of additional E. coli O157 isolates (human and bovine) is required to provide a better understanding of genetic diversity.

Some spatial clustering of farms positive for E. coli O157 was observed in this study using K function analysis. This showed evidence that positive farms were locally clustered at a distance of around 3–4 km when compared with negative farms, suggesting that positive farms may form localised clusters on this spatial scale. There is a possibility that this clustering may be a statistical artefact due to low numbers of farms located within shorter distances, and the differences between K functions only crossed the simulation envelopes at 3–4 km and was not sustained at greater distances. However, there were 18, 71, 118, 152, 161, 165 and 173 farms in the study which have another farm within 1 km, 2 km, 3 km, 4 km, 5 km, 6 km and 10 km, respectively, and the confidence bounds are estimated to represent uncertainty around the null value (zero) that may arise given the distribution of farms at each distance. Further, any apparent clustering at farm-level may be due to cross contamination and infection of calves of E. coli O157 during transportation or lairage (Arthur et al. 2007). Those authors collected samples from the 286 animals at feedlot (before transporting to slaughter plant) and again after slaughter at the slaughter plant on three different occasions. The increase in prevalence of E. coli O157 on hides from 50.3% to 90.4% suggests the possible role of transportation and lairage at the slaughter plant. Spatial clustering of badgers (Meles meles) excreting Salmonella enterica serovar Agama was also reported in the United Kingdom (Wilson et al. 2003), and of Salmonella positive pig farms in the counties of Nordjylland, Ringkøbing and Sønderjylland in Denmark (Benschop et al. 2009).

The likelihood of shedding E. coli O157 has been reported to be lower in calves-fed colostrum compared with calves that were not fed colostrum (Rugbjerg et al. 2003). In this study, the concentration of IgG in serum was between <0.50 and 62.28 mg/mL. A concentration of 10 mg/mL or more, indicative of colostrum having been fed (Beam et al. 2009), was observed in 72% of the calves that were fed colostrum. However no significant difference was observed in concentration of IgG between calves that were RT-PCR positive for E. coli O157 and those that were negative. Several other studies have also reported that the higher concentration of immunoglobulins in serum may not be protective against intestinal colonisation of E. coli O157 (Snodgrass et al. 1980; van Diemen et al. 2007). Therefore, the effectiveness of colostrum feeding to be protective against E. coli O157 colonisation remains equivocal.

The results of this study showed that the Jersey calves were marginally less prone to infection (p=0.055) compared with Friesian calves. An effect of breed on shedding of E. coli O157 has been reported previously, with Romosiniuano cows less likely (p<0.01) to shed E. coli O157 compared with Angus or Brahman cows (Riley et al. 2003). However, no difference in prevalence of E. coli O157 between various breeds was found in another study (Hancock et al. 1994). Therefore, the result of this study should be interpreted with caution. The effect of breed may be due to small sample size or differences in management practices, as breed was reported as being a confounding factor with geographic region (Meyer-Broseta et al. 2001).

In conclusion, this study showed that there was no association between maternally derived immunity and calves positive for E. coli O157 by RT-PCR. However, Jersey calves were less likely (p=0.01) to shed E. coli O157 compared with Friesian calves. Improved colostrum feeding may not be a factor in reducing the colonisation and shedding of E. coli O157 in calves. Nevertheless, the prevalence data indicate a need for such strategies in New Zealand agricultural practice.

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1 S. Brandt, Institute of Environmental Science and Research, Christchurch, New Zealand

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