Molecular epidemiology of Shiga toxin-producing *Escherichia coli* (STEC) on New Zealand dairy farms: application of a culture-independent assay and whole genome sequencing

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**Running Head:** ‘Top 7’ STEC Prevalence on New Zealand Dairy Farms

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Abstract

New Zealand has a relatively high incidence of human cases of Shiga toxin-producing *Escherichia coli* (STEC), with 8.9 STEC cases per 100,000 people reported in 2016. Previous research showed living near cattle and contact with cattle feces as significant risk factors for STEC infections in humans in New Zealand, but infection was not linked to food-associated factors. During the 2014 spring calving season, a random stratified cross-sectional study of dairy farms (n=102) in six regions across New Zealand assessed the prevalence of the ‘Top 7’ STEC (serogroups O157, O26, O45, O103, O111, O121, O145) in young calves (n=1,508) using a culture-independent diagnostic test (PCR/MALDI-TOF). Twenty percent (306/1,508) of calves on 75% (76/102) of dairy farms were positive for at least one of the ‘Top 7’ STEC. STEC carriage by calves was associated with environmental factors, increased calf age, region, and increased number of calves in a shared calf pen. Intraclass correlation coefficient (ρ) indicated strong clustering of ‘Top 7’ STEC positive calves for O157, O26, and O45 serogroups within the same pens and farms, indicating that if one calf was positive, others in the same environment were likely to be positive as well. This finding was further evaluated with whole genome sequencing that indicated a single *E. coli* O26 clonal strain could be found in calves in the same pen or farm, but different strains existed on different farms. This study provides evidence that would be useful for designing on-farm interventions to reduce direct and indirect human exposure to STEC.

Importance

Cattle are asymptomatic carriers of Shiga toxin-producing *E. coli* (STEC) that can cause bloody diarrhea and kidney failure in humans if ingested. New Zealand has relatively high numbers of
STEC cases, and contact with cattle feces and living near cattle are risk factors for human infection. This study assessed the national prevalence of STEC in young dairy cattle by randomly selecting 102 farms all over New Zealand. The study used a molecular laboratory method that has relatively high sensitivity and specificity compared to other methods, providing a higher test accuracy compared to traditional methods. Top 7 STEC were found in 20% of calves on 75% of the farms studied, indicating widespread prevalence across the country. By examining the risk factors associated with calf carriage, potential interventions that could decrease the prevalence of Top 7 STEC at the farm level were identified, which could benefit both public health and food safety.

**Introduction**

Worldwide, Shiga toxin-producing *Escherichia coli* (STEC) are a growing public health concern. Large scale outbreaks in Europe (1) and the United States (2) have continued to occur. Furthermore, human STEC cases in Argentina have a high rate of serious clinical complications (3). Although STEC may have a lower prevalence than other notifiable zoonotic diseases (4), the pathogen’s propensity to affect very young children, leading to potential long term kidney and brain damage (5), is a concerning public health issue. STEC are primarily transmitted via the fecal-oral route. Ruminant animals, particularly cattle, have been identified as the most important reservoir (6).

New Zealand has a relatively high incidence of notified STEC infection in humans, with 8.9 STEC cases per 100,000 population reported in 2016 (7), compared to 2.85 in the USA in 2016 (8), and 12.92 in Ireland, 5.08 in the Netherlands, and 2.05 in the United Kingdom in 2015 (9). Since it became a notifiable disease in New Zealand, there has been a general increase of STEC cases annually, with both STEC O157:H7 and non-O157 STEC causing human disease (10).
New Zealand case-control study identified contact with animal manure and the presence of cattle in the local area, along with contact with recreational waters, as significant risk factors for human STEC infection (11). Interestingly, the same study did not identify food as a statistically significant exposure pathway in New Zealand (11). Previous research findings overseas have highlighted beef food products and raw produce as the main sources of human infection (6, 12, 13), but findings in the United Kingdom also identified an important contribution from environmental sources (14). Determining the carriage of STEC in ruminant hosts, through targeted national studies, will help our understanding of the epidemiology of this important pathogen.

Since the 1993 outbreak of STEC O157:H7 in the United States (15), monitoring and regulatory requirements regarding this pathogen have increased. Having found STEC O157:H7 in raw ground beef, plus outbreaks associated with consumption of undercooked beef patties, the US declared STEC O157:H7 an adulterant of beef in 1994, followed by declaration of six additional serogroups (O26, O45, O103, O111, O121, O145) as adulterants in 2011 (16). These six additional serogroups and STEC O157 are known as the ‘Top 7’ STEC. In 2015-2016, 50% of New Zealand beef exports were sent to the United States (17). Given the importance of agricultural exports for the New Zealand economy, STEC is an economic as well as a public health concern.

Previous research in New Zealand identified a higher STEC O157 and STEC O26 prevalence in young calves compared to adult cattle (18), and this finding has been supported by ‘Top 7’ STEC research in other countries (19–22). New Zealand dairy farms follow a seasonal calving strategy, where surplus dairy calves, known as bobby calves, may be slaughtered at a very young age (four to ten days old). The higher prevalence of STEC among very young calves means that
preventing inadvertent contamination of veal during dressing of carcasses at primary processing is an important risk management goal. Similarly, reducing children’s contact with calves may lessen the risk to human health.

This study examined the prevalence of young calves shedding ‘Top 7’ STEC (O157, O26, O45, O103, O111, O121 and O145) on dairy farms in New Zealand. We estimated the spatial distribution of STEC-positive farms, clonal relationships of STEC bacteria in calves by pen and farm, and determined risk factors for STEC carriage by calves, which could potentially be targeted for control. By understanding and reducing STEC from its source, we hope to decrease the risk of both veal meat contamination and human exposure to STEC on farms.

Results

Prevalence of ‘Top 7’ serogroups by latent class analysis of real-time PCR and NeoSEEK

Our in-house real-time PCR assay was only able to detect the presence of the O-serogroup in a sample, whereas the NeoSEEK assay claims to be able to discriminate between stx-positive and stx-negative E. coli of a ‘Top 7’ STEC serogroup (e.g. STEC O26 versus non-toxigenic O26). By using latent class modelling techniques, the prevalence of these serogroups was determined using both assays to give a more robust estimation of serogroup prevalence (Figure 1, Figure 2). This modelling technique required the calf population to be divided into groups for comparison; we therefore stratified by region, North and South Island, and age: young (two to nine days) and old (10 to 21 days).

There were notable differences in estimated serogroup prevalence between groups. Northland, Manawatu-Wellington, and Waikato had a high prevalence of several serogroups compared to other regions, particularly serogroups O26 and O45 E. coli (Figure 1). Prevalence between older
and younger calves was similar, but older calves had a higher prevalence of O145 and O26 serogroups (Figure 2). Finally, the North Island had a higher prevalence of most serogroups, with the exception of O26, which was similar between the two islands (Figure 2).

‘Top 7’ STEC detection via culture-independent methods

NeoSEEK detected 20.3% (95% CI 16.1-24.5) of the calves on 75% (76/102) of the dairy farms as positive for at least one of the ‘Top 7’ STEC (Table 1). NeoSEEK identifies both the presence of a ‘Top 7’ serogroup, as well as the presence of eae and stx genes, within the same serogroup, using PCR/MALDI-TOF. All ‘Top 7’ STEC, except for STEC O121, were detected in samples taken from the New Zealand dairy farms tested. The highest estimated STEC prevalence at the farm and calf level was STEC O145 and STEC O26, while STEC O111 was only detected in recto-anal mucosal swabs (RAMS) from three calves located on one farm in the Northland region. Prevalence maps illustrate the regional variability of prevalence of ‘Top 7’ STEC in New Zealand (Figure 3). ‘Top 7’ STEC prevalence varied between serogroups, with STEC O26 more commonly detected in the South Island (Canterbury and Southland), and a much higher prevalence of STEC O45 detected in Northland compared to other regions.

The virulence genes stx and eae were common in calf samples; NeoSEEK detected stx in 70.5% of the calf samples, and eae in 57.6% of calf samples. Both eae and stx genes were detected in 45.4% of calf samples, however it is important to note that this did not necessarily indicate a ‘Top 7’ STEC was present. The stx gene was detected in at least one calf sample from all farms in the study, while eae was detected in at least one calf sample from 101 of the 102 farms.

Estimation of intraclass correlation coefficient (ρ) was calculated based on the presence or absence of a ‘Top 7’ STEC positive calf in a particular pen or farm. Intraclass correlation coefficient (ρ) revealed strong clustering of ‘Top 7’ STEC positive calves within pens, and some
strong clustering of calves on farms, most notably with the STEC O26, STEC O157, and STEC O45 serogroups (Table 2).

Calf and farm level risk factors were evaluated for the three most prevalent STEC serogroups (STEC O26, STEC O103, and STEC O145) and the presence of any ‘Top 7’ STEC. Due to the low calf-level prevalence of STEC O157 (n=29 calves), STEC O45 (n=44 calves), and STEC O111 (n=3 calves), it was not possible to create a final model using the same statistical technique for these serogroups, therefore significant risk factors were not identified. Region, higher humidity measured inside the calf pen compared to outside the calf housing area, older calf age, and increased number of calves in a pen were all identified as significant risk factors for the presence of any ‘Top 7’ STEC (Table 3). Individual STEC serogroup analysis revealed increased number of calves in a pen (STEC O26; Table S2), increased pen humidity and a high ammonia presence (determined subjectively) in a pen (STEC O103; Table S3), and region, increased age, and increased pen humidity (STEC O145; Table S4) as significant risk factors.

**Bacterial isolation of E. coli serogroup O26 and O157**

A total of 31 STEC O157 isolates, 123 STEC O26 isolates, and 69 non-toxigenic O26 isolates were retrieved from 138 calf fecal enrichment broths. The results of bacterial isolation of O157 and O26 E. coli serogroups from calf fecal enrichment broths (samples) are shown in Table 4, where results are based on the successful recovery or failure of recovery of at least one isolate from a calf fecal enrichment broth.

**Whole genome sequencing (WGS) of serogroup O26 bacterial isolates**

WGS data of serogroup O26 isolates (n=66, 45/66 STEC O26) from 24 sheds on 18 farms in five regions of New Zealand were processed using the Nullarbor pipeline and the Center for Genomic Epidemiology output, to evaluate the core genome, accessory genome, virulence genes,
and antibiotic resistance genes (23, 24). The core genome (Figure 4) and accessory genome (Figure 5) were annotated with region, antimicrobial resistance gene class (n=1), and virulence gene (n=26) presence or absence. Clear clustering of STEC O26 isolates (n=45) distinct from non-toxigenic isolates (n=21) was visible in both Figure 4 and Figure 5, but no obvious clustering by region was seen. The heatmap of virulence genes detected (n=26) indicated that STEC O26 and non-toxigenic serogroup O26 E. coli had distinct virulence gene profiles (Figure 4, Figure 5). Antimicrobial resistance gene detection was rare, with only aminoglycoside resistance class genes detected (strA, strB, aph(3')-IIa-like) in eight isolates from the Manawatu-Wellington and Canterbury regions. All genomes sequenced from O26 bacterial isolates retrieved from calves in this study were identified as multilocus sequence type 21 (ST-21), and serotype O26:H11.

SNP analysis between serogroup O26 isolates indicated that the same clonal strain existed in calves in the same pen and the same farm, while strains between farms were different (Figure S1, total SNPs 11,167). For analysis of isolates from calves (n=42) on the same farm (n=14), as well as in the same pens (n=20), 0 to 29 SNPs separated all isolates. A subset of calves (n=5) had multiple isolates (n=19 total, range 3 to 4 isolates from the same animal) sequenced from the same animal; only 0 to 17 SNPs separated isolates retrieved from the same animal sample. Two exceptions were noted in the analysis, where two calves had markedly different (214 SNPs, 223 SNPs) O26 strains compared to other calves in the same farm and pen, indicating multiple serogroup O26 strains were present in the farm environment at the same time.

PERMANOVA analysis was used to compare region and farm with the variability of the core genome (SNP distance), accessory genome (presence or absence of accessory genes), and virulence genes (presence or absence of virulence genes) (Table 5). Farm was a significant
predictor of variability (69.7-88.5%), indicating that the majority of the genetic variability at the core, accessory, and virulence gene level could be associated with each calf’s presence in a specific farm environment. The importance of farm was further evaluated in hierarchical cluster plots (Figure 6), where a clear differentiation based on farm is visible, with the exception of farms which contain both stx positive and stx negative isolates. The hierarchical cluster analysis of core, accessory and virulence gene profiles also separated stx positive and stx negative isolates into different clonal groups, despite all being the same multilocus sequence type (ST-21).

Discussion

This study utilized an established molecular method that distinguishes STEC and non-STEC variants, along with random stratified sampling, to estimate the national prevalence of the ‘Top 7’ STEC in young calves on dairy farms throughout New Zealand. Statistical analyses evaluated risk factors for positive prevalence in calves, while WGS and further statistical analysis determined the similarity of ‘Top 7’ STEC isolates between calves in a shared environment. Systematic review and meta-analysis estimated an 8.7% prevalence of STEC (both eae and stx present in a single bacterium) in calves from 19 countries (25). A comprehensive national prevalence study of cattle and calves at 31 Australian processing plants showed a 6.3% prevalence of STEC O157, with a 1.7% prevalence for the other ‘Top 7’ STEC using culture methods (19). This Australian study also found that veal calves had the highest potential STEC prevalence (12.7%) using PCR methods, compared to young beef, young dairy, and adult cattle, with 51% of all samples testing positive for both eae and stx virulence markers (19). Our results
indicated a higher ‘Top 7’ STEC prevalence of 20.3% in young calves; our use of a culture
independent diagnostic test may have increased the sensitivity of detection of STEC.

Several results in our analysis suggested that STEC transmission occurs between calves or
within the immediate calf pen environment: high intraclass correlation coefficient (ρ) indicated
strong clustering of ‘Top 7’ STEC positive calves in pens for STEC O26, STEC O157, and
STEC O45; increased risk of ‘Top 7’ STEC prevalence with increasing numbers of calves in a
single pen; and clonal strains of serogroup O26 *E. coli* observed in specific farms and pens. In a
controlled transmission study, a calf infected with a low dose of STEC O157 began shedding the
bacteria within six days, and STEC O157 subsequently colonized all other calves in the same pen
within four to 11 days after the initial calf began shedding (26). A separate comparison study of
calves housed in individual pens versus an open group pen showed that a single calf inoculated
with a control STEC strain in a group pen infected all other calves in that group over 10 days
(27). Modelling studies have deduced a R₀ of 4.3 to 7.3 for STEC O157 in young calves from
both natural and induced infection, suggesting calves in shared environments infect numerous
other individuals when shedding (28, 29). The observed clustering was most likely due to
transmission of STEC in the immediate environment, but other factors at the pen and farm level
may explain these findings.

Our WGS analysis indicated that *stx* positive and *stx* negative *E. coli* O26 form distinct clones
with divergent core, accessory, and virulence gene profiles. Further epidemiological analysis also
demonstrated that unique *E. coli* O26 clones disseminate among calves in a farm environment.
PERMANOVA results indicated that farm, but not region, was a significant predictor of genetic
variability (Table 5). The lack of similarity among strains in the same region, as well as the
similarity between isolates on farms, suggests relatively low transmission between farms in the
same region. Only a minority of farms sampled in this study brought animals from outside the farm onto their property in the past two calving seasons: 7/102 farms brought in calves, while 15/102 farms had brought in adult cows. It is likely that once established, specific strains proliferate in farms, leading to transmission between animals on the same farm. This finding has been reflected in other studies, where STEC strains isolated from calves from the same pen showed low variability, indicating high within-pen transmission (30). Unique STEC O157 lineages also proliferated among cattle on US dairy farms with a high STEC O157 prevalence (31). SNP analysis indicated that STEC O157 populations were dominated by a single clonal type on farm, but differences occurred between farms, and some clonal types were still present during resampling 11 months later (31). Pulsed field gel electrophoresis (PFGE) analysis of O26:H11 isolates (n=11) on three Australian farms also found unique strains at each farm (32).

Increased relative humidity inside the pen environment compared to outside the calf housing area was associated with increased ‘Top 7’ STEC prevalence. Higher humidity has been associated with increased risk of shedding STEC O157 (33), but it is unclear whether this is due to environmental factors that would benefit bacterial growth, or high humidity causing stress of the animal. The increase in STEC prevalence with calf age may be associated with the duration of STEC exposure within the pen. The longer the calf is present with other infected animals and in a STEC contaminated environment, the increased likelihood of STEC ingestion and colonization.

The calf pen environment is an important potential intervention point. Decreasing the number of young calves in pens is a practical intervention that may decrease STEC carriage. This may also have animal welfare benefits. Recent legislation in New Zealand has focused on young calf welfare, and mandatory management changes could lead to opportunities for interventions at the
farm level (34). Individual outdoor calf hutches, though used in other countries, are not widely
used in New Zealand and may not be a realistic intervention for dairy farmers from either a time
management or an economic viewpoint.

Limitations of this study included the cross-sectional study design that estimated STEC
prevalence based on a single sampling event. It is well documented that calves may shed STEC
intermittently, showing daily or even hourly variations (35, 36). By sampling many calves from
multiple pens on each farm, we estimated the farm level prevalence, as well as the proportion of
calves shedding any ‘Top 7’ STEC on a dairy farm at a single point in time.
The utilization of culture independent diagnostic tests (CIDTs) to detect bacterial zoonoses is
rapidly increasing, with lower costs and increased speed of detection compared to traditional
culture methods; CIDTs for detecting STEC are widely employed (37). However, our use of a
culture-independent diagnostic test (NeoSEEK) for this epidemiological study may have led to
false positives due to a lower than 100% specificity compared to culture. We evaluated the assay
on New Zealand ‘Top 7’ STEC, and several other USDA studies in the United States have
shown successful bacterial isolation of 84% (61/73) ‘Top 7’ STEC (22), and 55.7% (305/548) of
non-STEC (38) following ‘Top 7’ STEC detection using the NeoSEEK assay. The New Zealand
Ministry of Primary Industries has approved and utilized NeoSEEK as part of the regulatory
testing and holding program for veal beef exports to the United States, and the use of the
NeoSEEK assay in a research context was beneficial for this epidemiological research.

Conclusion
This cross-sectional study of young calves on New Zealand dairy farms identified the
widespread presence of ‘Top 7’ STEC. Future work using similar molecular confirmation
methods, along with WGS, will permit the evaluation of the transmission dynamics of the ‘Top
STEC on New Zealand dairy farms by sampling calves, cows, and their immediate environment throughout the calving season. Data from this research will provide further information as to the importance of specific environmental sources of infection for calves, as well as the persistence and spread of STEC throughout the dairy farm environment.

Practical and economic factors are often key drivers influencing the uptake and adoption of on-farm interventions by dairy farmers. While the use of vaccines or dietary supplements may decrease STEC O157 shedding in cattle (39), there is currently limited economic incentive for New Zealand dairy farmers to allocate time and money to prevent a bacterium colonizing what are considered ‘surplus’ relatively low-value animals with no clinical signs. STEC and other *E. coli* are considered part of the normal bovine microbiota, therefore elimination of STEC from a herd and farm environment may be an unrealistic goal. Previously validated on-farm intervention strategies that are easily adopted, cost-effective, and that target mutual critical control points for several pathogens (e.g. STEC, *Campylobacter*, *Salmonella*, and *Cryptosporidium*), could form the basis of multiple agent control methods to reduce the overall level of zoonoses. This could impact overall STEC prevalence in animals and reduce the likelihood of human infection. Given STEC are found in cattle throughout the world, focusing on methods to decrease human exposure by minimizing the presence of STEC in food and minimizing environmental exposure is likely to be more beneficial than attempting to eliminate the presence of STEC in ruminant reservoirs.

The findings of this study provide important baseline data regarding the national prevalence of a zoonotic pathogen on New Zealand dairy farms. Future goals for STEC research should be multi-modal, addressing issues that could benefit the meat industry and protect public health using social science, epidemiology, and molecular biology.
Materials and Methods

The Animal Ethics Committee of Massey University, Palmerston North, New Zealand approved this study on April 17th, 2014, under protocol number 14/29.

Sample size calculations

We performed sample size calculations using a cluster-sample calculation with a design effect (rho) of 3.6, based on a previous repeated cross-sectional study of STEC O26 and STEC O157 at cattle processing plants in New Zealand (18). Table S5 contains sample size calculations for the number of farms and calves required to be 95% certain that the prevalence estimate is within +/- 20% of the true prevalence. Given previous estimates of STEC O26 and STEC O157 prevalence in calves in New Zealand, we used a conservative estimate of 20% farm prevalence of the ‘Top 7’ STEC, and aimed to recruit a minimum of 93 farms and sample a maximum of 15 calves per farm. The critical probability for all statistical analyses was p<0.05.

Random stratified farm selection

We selected farms using a stratified random sampling scheme based on regionally proportioned sampling of the number of farms in each region. We targeted the six largest dairy regions, which account for approximately 75% of the dairy farms in New Zealand: Northland, Waikato, Taranaki, Manawatu-Wellington, Canterbury, and Southland (40). Given a 60-day calving period, only farms with a documented herd size of more than 150 milking cows were eligible, to ensure enough calves would be present on the day of sampling. Potential farms were selected randomly from a national farm database (Agribase™, AsureQuality Limited, Auckland, New Zealand) and contacted by telephone, leading to the recruitment of 102 dairy farms (Table S6).

Random animal selection and sampling within calf pens on farms

We categorized calves into two groups: young calves from two to nine days of age, and older calves from 10 to 21 days of age. Given the focus on ‘Top 7’ STEC prevalence in very young
calves, where possible ten calves were sampled in the young age group, and five in the older age group.

**Sampling** of calves occurred during a single farm visit from July 28th to September 24th during the 2014 spring calving season. For this study, ‘pen’ was defined as an enclosed area where calves had direct contact with each other and shared water and feeding resources. After identifying calf ages, up to three pens were selected that allowed for the maximum number of animals in the two age groups to be sampled, with equal numbers per pen where possible. A random number generator was used to select pens if more than three suitable pens were available for sampling. If more than five animals were present in a pen, a spin-pointer mobile phone application was used to randomly select the first calf to be sampled, after which animals were chosen in an alternating manner in the clockwise direction, in proportion to the total calves in the pen. Calves were marked for selection and then again following sampling to maintain the random selection and prevent resampling.

**Animals** were excluded from sampling if they appeared injured or sick, based on visual clinical assessment by the sampler (A.S. Browne: a registered veterinarian). In total, 1,508 young calves from 267 pens were sampled by collecting recto-anal mucosal swabs (RAMS) from each calf using Amies transport swabs (Copan Diagnostics Inc., Brescia, Italy). All RAMS were kept on ice in an insulated container immediately after sampling, until they were shipped for processing the same day as they were collected.

**Initial laboratory processing**

All RAMS were shipped on ice overnight to "EpiLab, Massey University, Palmerston North, and enriched in modified Tryptone Soya broth (mTSB, Oxoid Limited, Hampshire, United Kingdom) at 42°C for 15-21 hours. Genomic DNA was extracted from 1mL of enrichment broth using a
double-wash boil preparation method, according to the GeneSeek laboratory’s instructions, and frozen at -80°C. The DNA samples were shipped to GeneSeek Operations (Lincoln, Nebraska, USA) on dry ice. All samples were analyzed using the PCR/MALDI-TOF assay, NeoSEEK (NeoSEEK STEC Confirmation, Neogen Corporation, Lansing, MI, USA), for presence of the ‘Top 7’ STEC.

**Evaluation of NeoSEEK for New Zealand ‘Top 7’ STEC detection**

NeoSEEK uses PCR amplification to generate allele-specific DNA products of different masses, and chip-based mass spectrometry to analyze the extension products. The assay is based on the presence of single nucleotide polymorphisms (SNPs) in the O-antigen gene cluster that can differentiate between STEC and non-STEC bacterial strains of the same serogroup (41), as well as other targets (i.e. virulence genes). NeoSEEK uses over 89 gene targets via PCR/MALDI-TOF to detect the presence of the ‘Top 7’ STEC without the need for agar-based culture isolation (E. Hosking, pers. comm.). This assay has a “Letter of No Objection” from the USDA-FSIS, and is used commercially as a confirmation method for detection of STEC in ground beef and beef trim. As far as we are aware, the evaluation and application of this technology in this study to detect fecal carriage of STEC in calves is unique.

A technical report, including summary data from the study conducted for NeoSEEK to receive a Letter of No Objection, is available online (42). Prior to field collection of samples for this study, 100 characterized New Zealand STEC and non-STEC isolates from the six serogroups (O26, O45, O103, O121, O145, and O157; n=88) as well as non-‘Top 7’ serogroups (n=12) from the Ministry of Primary Industries (n=64) and the Hopkirk Institute (n=36) culture collections were obtained and used by the Institute of Environmental Science and Research to evaluate the detection efficacy of the NeoSEEK assay. One Australian STEC O111 isolate was also tested as
no STEC O111 had been isolated in New Zealand. All 101 isolates had undergone serological analysis and previously been characterized by PCR for the presence of stx1, stx2, and eae virulence markers; there was 100% concordance with the NeoSEEK assay.

All DNA samples derived from the calf fecal samples, in addition to being submitted for NeoSEEK analysis, were tested for the ‘Top 7’ serogroups at EpiLab using a real-time PCR (RT-PCR) assay (43). All DNA samples were run with positive, negative, and blank template controls using PerfeCTa® Multiplex qPCR ToughMix® (Quanta Biosciences, Beverly, Massachusetts, USA) on a Rotor-Gene Q 5plex HRM Platform (Qiagen, Hilden, Germany). In-house validation of the RT-PCR method revealed a limit of detection (LOD) of 10^2 colony forming units (CFU) per mL for all serogroups evaluated, except for O157 and O103 where the LOD was 10^1. The LOD of the NeoSEEK assay was approximately 10^3 CFU/mL (E. Hosking, pers. comm.).

Latent class modelling (44) was used to estimate the sensitivity and specificity of serogroup detection of the ‘Top 7’ STEC serogroups. This modelling technique is used to compare two diagnostic tests, when neither is considered a “gold standard”. Latent class analyses were performed (https://github.com/jmarshallnz/lcar) to calculate a 95% CI for the sensitivity and specificity of the NeoSEEK and RT-PCR methods for detection of all seven serogroups for the 1,508 DNA samples (Table S7, Table S8, Figure 7). Latent class analyses also produced prevalence estimates of all seven serogroups by three factors: region (n=6), age of calf (young and old), and location in the North or South Island.

All 1,508 calf RAMS samples collected were enriched and stored in a glycerol (4:1 ratio) suspension in a -80°C freezer. The isolation of individual STEC from frozen enrichment broth was important for confirming the STEC detection using molecular methods (NeoSEEK, RT-
PCR), as well as analysis of the bacteria using whole genome sequencing. Recovery of bacteria
from frozen enrichment broth samples was attempted based on the NeoSEEK assay results for
‘Top 7’ STEC positive samples.

Due to the large number of STEC detected by analysis of all 1,508 samples using the NeoSEEK
assay (n=408), and the costs and labor required for testing and isolation of bacteria from
enrichment broth samples, isolation was prioritized based on serogroup. Due to their public
health importance, recovery was attempted on all STEC O157 (n=29) and STEC O26-positive
(n=109) samples using a modification of USDA-FSIS methods (45, 46). STEC recovery was
attempted on samples using sorbitol MacConkey agar supplemented with cefixime and tellurite
(CT-SMAC) and rhamnose MacConkey agar supplemented with cefixime and tellurite (CT-
RMAC) (Fort Richard Laboratories, Auckland, New Zealand) for STEC O157 and STEC O26
respectively, using immuno-magnetic separation beads (IMS) (Abraxis, Warminster,
Pennsylvania, USA). The methods used were adapted from the USDA-FSIS methods (45, 46) to
include an initial “direct” culture screen, where frozen glycerol enrichment culture was plated
directly onto selective agar (CT-SMAC for O157 and CT-RMAC for O26). If target STEC
serogroups were not identified, frozen glycerol enrichment broth was re-enriched in mTSB broth
and immunomagnetic separation (IMS) was attempted according to manufacturer’s instructions.
Up to ten colonies were tested for the specific serogroups on a plate using latex agglutination,
and up to four positive individual isolates were subcultured and stored frozen with glycerol.
Subcultured isolates were confirmed for serogroup and tested for virulence-associated genes
using an in-house RT-PCR (43).
Whole genome sequencing, assembly, and analysis of *E. coli* serogroup O26 isolates retrieved from calf fecal samples

We used random stratified selection by region, farm, and calf pen to select 66 serogroup O26 bacterial isolates (45/66 STEC O26, 21/66 non-toxigenic O26) for whole genome sequencing. Multiple isolates were selected from four calves to evaluate within-animal diversity. We performed DNA extraction from a single colony picked from Columbia Horse Blood Agar (Fort Richard Laboratories, Auckland, New Zealand) using the QIAamp® DNA MiniKit (Qiagen, Hilden, Germany), and prepared the libraries using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, United States). Prepared libraries were submitted to New Zealand Genomics Limited (University of Otago, Dunedin, New Zealand), who performed sequencing using Illumina MiSeq 2x250 PE and Illumina HiSeq 2x125bp PE v4. Processed reads are publicly available on the NCBI Sequence Read Archive (SRA) under BioProject ID PRJNA396667, and Table S9 lists the metadata and accession numbers of the sequences.

Raw sequences were evaluated, assembled, annotated, and analyzed using the Nullarbor pipeline in the “accurate” mode (23). RaxML maximum-likelihood trees were generated from Roary data for core genes via single nucleotide polymorphism (SNP) analysis of core genes, and accessory genes via a presence/absence matrix without an external reference (47). Assembled genomes were batch uploaded to the Center for Genomic Epidemiology server for identification of virulence factors, multilocus sequence type (ST), antimicrobial resistance genes and somatic (O) and flagellar (H) type (24). A distance matrix was created from the SNP distances between isolates, and a dissimilarity matrix was created from the presence/absence matrix of the accessory genome from Roary, as well as the 26 virulence genes predicted by the Center for Genomic Epidemiology output, and all three were evaluated in PERMANOVA and CLUSTER.
(PRIMER-E, Quest Research Limited, Auckland, New Zealand) with region and farm as independent factors.

**Figures** depicting the phylogenetic relationships and associated variables were created using iTOL (Interactive Tree of Life) software (48), and further amended using Inkscape open source software version 0.92.2 (https://inkscape.org).

**Data Retrieval and Statistical Analysis**

At the time of the visit, written consent to participate in the study was obtained from a manager on every farm. Animal and farm level data, including management and environmental factors, were collected from each farm through observation, electronic devices, and interviewing a manager on every farm (Table S10).

All statistical analyses were performed using R Version 3.2.1 (49). Eight outcome variables were considered: the presence or absence of each of the ‘Top 7’ STEC, and an additional variable specifying the presence or absence of any of the ‘Top 7’ STEC. All factors were first assessed using machine learning techniques from the “randomForest” package (50). The most important 10% of factors identified in the “randomForest” analysis were considered as explanatory fixed effects in a linear mixed-effects model, with “pen” within “farm” included as random effects variables. A preliminary model was generated by stepwise backward elimination of the least significant variables, and eliminated variables were assessed for confounding. Confounding variables, determined by a change of >30% in the main variable coefficient, were kept in the model even if they were non-significant. Intraclass correlation (ρ) was calculated using the “iccbin” function in the “aod” package with a Monte Carlo 1-way generalized linear mixed model (51). A description of the strength of correlation is as follows: 0.00-0.19: “very weak”; 0.20-0.39: “weak”; 0.40-0.59: “moderate”; 0.60-0.79: “strong”; and 0.80-1.0: “very strong”.

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Figure 1: ‘Top 7’ serogroup prevalence (with 95% CI), including both STEC and non-STEC, detected in calves (n=1508) by region, using latent class analysis of NeoSEEK and real-time PCR results.

Figure 2: ‘Top 7’ serogroup prevalence (with 95% CI), including both STEC and non-STEC, detected in calves (n=1508) by island and age (young, two to nine days; old, 10 to 21 days), using latent class analysis of NeoSEEK and real-time PCR results.

Figure 3: Calf (n=1,508) and farm (n=102) level prevalence of the ‘Top 7’ STEC on New Zealand dairy farms by region (n=6).

Figure 4: Maximum-likelihood core genome tree of serogroup O26 calf isolates (n=66), annotated with region (n=6), antibiotic resistance gene class (n=1), and virulence genes (n=26).

Figure 5: Maximum-likelihood accessory genome tree of serogroup O26 calf isolates (n=66), annotated with region (n=6), antibiotic resistance gene class (n=1), and virulence genes (n=26).

Figure 6: Hierarchical cluster trees of core, accessory, and virulence genes by farm (n=18).

Figure 7: Sensitivity and specificity of NeoSEEK and real-time PCR assays for detection of the ‘Top 7’ serogroups in calf fecal enrichment samples (n=1,508).
Table 1: Farm (n=102) and calf (n=1,508) level prevalence of the ‘Top 7’ STEC on New Zealand dairy farms

| STEC  | STEC | STEC | STEC | STEC | STEC | Any ‘Top 7’ STEC (%) |
|-------|------|------|------|------|------|----------------------|
| O103  | O121 | O111 | O145 | O157 | O26  | O45                  |
| Calves (number +ve) | 75 | 0 | 3 | 148 | 29 | 109 | 44 | 306 |
| % +ve | 5.0% | 0% | 0.2% | 9.8% | 1.9% | 7.2% | 2.9% | 20.3% |
| [95% CI] | [2.7-7.2] | [0.0-0.6] | [6.7-12.9] | [0.5-3.3] | [4.5-9.9] | [1.2-4.7] | [16.1-24.5] |
| Farms (number +ve) | 36 | 0 | 1 | 44 | 15 | 23 | 18 | 76 |
| % +ve | 35% | 0% | 1% | 43% | 15% | 23% | 18% | 75% |

The detection of at least one of the ‘Top 7’ STEC in an individual calf. 408 instances of ‘Top 7’ STEC were detected, but some calves shed multiple STEC serogroups: 1 serogroup (n=217), 2 serogroups (n=76), 3 serogroups (n=13) (Table S1).
Table 2: Intraclass correlation coefficient (ρ) values of STEC using farm (n=102) and calf pen (n=267) as a random factor

|       | STEC O103 | STEC O145 | STEC O157 | STEC O26 | STEC O45 | STEC Any ‘Top 7’ |
|-------|-----------|-----------|-----------|----------|----------|------------------|
| Farm  | 0.13      | 0.29      | 0.61*     | 0.68*    | 0.62*    | 0.24             |
| Calf pen | 0.57   | 0.60*     | 0.71*     | 0.79*    | 0.77*    | 0.34             |

*Strong clustering observed
Table 3: Logistic mixed effects regression model of factors associated with prevalence of any ‘Top 7’ STEC

| Factor                                      | OR   | 95% CI       | p-value |
|---------------------------------------------|------|--------------|---------|
| **Humidity**: Difference between inside pen vs. outside the calf housing area (increase in 1% relative humidity) |      |              |         |
| Humidity                                   | 1.09 | 1.02, 1.16   | 0.006*  |
| **Region** (compared to Northland)          |      |              |         |
| Waikato                                    | 0.09 | 0.03, 0.29   | <0.001* |
| Taranaki                                   | 0.11 | 0.03, 0.39   | <0.001* |
| Manawatu-Wellington                        | 0.23 | 0.06, 0.87   | 0.030*  |
| Canterbury                                 | 0.19 | 0.05, 0.72   | 0.014†  |
| Southland                                  | 0.30 | 0.08, 1.13   | 0.076   |
| **Number of calves** in calf pen: Increase of one calf | 1.04 | 1.01, 1.07   | 0.003*  |
| **Temperature**: Difference between inside pen vs. outside the calf housing area (increase of 1°C) |      |              |         |
| Temperature                                | 1.20 | 0.96, 1.49   | 0.114#  |
| **Age**: Young calves (2 to 9 days of age) vs. older calves (10 to 21 days of age) |      |              |         |
| Age                                        | 0.43 | 0.27, 0.68   | <0.001* |

*Significant variable (p<0.05)

#Confounding factor for calf pen humidity, left in model

†Likelihood-ratio test of factor

Random effects variance: Calf pen within Farm (Variance = 1.09), Farm (Variance = 1.34)
Table 4: Bacterial isolation of STEC and non-STEC isolates of serogroup O157 and O26 *E. coli* from fecal calf enrichment broths

| Serogroup | Number of samples detected as STEC by NeoSEEK | Number of isolates | STEC Isolate Recovered* | STEC Isolate Recovered* | Overall STEC Recovery |
|-----------|-----------------------------------------------|--------------------|-------------------------|-------------------------|-----------------------|
| O157      | 29                                            | 14/29 (48%)        | 14/14 (100%)            | 14/29 (48%)            |                       |
| O26       | 109                                           | 70/109 (64%)       | 49/70 (70%)             | 49/109 (45%)           |                       |

*At least one isolate was recovered from the enrichment broth

Table 5: PERMANOVA analysis of core genome (SNP distance matrix), accessory genome (presence or absence of accessory genes), and virulence genes by region (n=5) and farm (n=18)

| Factor evaluated | Genomic dataset | df  | Pseudo-F | p-value | Component of variation |
|------------------|-----------------|-----|----------|---------|------------------------|
| Region           | Core            | 4   | 1.9      | 0.0975  | NS                     |
|                  | Accessory       | 4   | 2.69     | 0.0016  | 11.6%                  |
|                  | Virulence       | 4   | 1.36     | 0.245   | NS                     |
| Farm             | Core            | 17  | 28.6     | 0.0001  | 88.5%                  |
|                  | Accessory       | 17  | 9.3      | 0.0001  | 69.7%                  |
|                  | Virulence       | 17  | 24.7     | 0.0001  | 86.8%                  |

Residual variation: core (11.5%), accessory (30.2%), virulence (13.2%)
