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Haumaru Kai Aotearoa

PREVALENCE OF TOP 7 STEC IN BEEF IN NEW ZEALAND AND GENETIC ANALYSIS OF *E. coli* O157(2021)

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Scientific Interpretative Summary

This Scientific Interpretative Summary is prepared by New Zealand Food Safety (NZFS) risk assessors to provide context to the following report for MPI risk managers and external readers. This is an annual report which summarise the N60 Top 7 STEC Regulatory programme for 2021 and ensures compliance to the requirements as set out in the US OMAR.

Shiga toxin-producing *Escherichia coli* (STEC) are a group of foodborne pathogens that is composed of at least 200 serotypes. Of these *E. coli* O157:H7 and the Top 6 STEC (O26, O45, O103, O111, O121, and O145) serotypes have been determined to be adulterants in raw bulk manufacturing beef meat, including veal, in the United States of America and thus, the USA specifies procedures for detection of Top 7 STEC in beef and veal intended for import into that country. *E. coli* O157:H7 is still recognised as the most significant of the STEC group, both for the severity of the illness it causes, and for the number of outbreaks and individual cases occurring on an annual basis, both here and overseas.

The US Overseas Market Access Requirements (OMAR) details the Top 7 testing requirements that need to be met for export to the USA, including the typing of any isolates of *E. coli* O157:H7 that have been detected in either beef or veal during routine monitoring in New Zealand (NZ) by whole genome sequencing (WGS), with the provision of providing typing profiles to USA regulators upon request.

ESR continued to complete STEC culture confirmation on any screen-positive beef enrichment and collate the data into this annual report. In addition, MPI requested *E. coli* O157:H7 isolation was to be attempted for all veal enrichments that were confirmed as *E. coli* O157:H7 by NeoSEEK® and that the resulting isolates along with those from adult beef enrichments would be typed by WGS and bionformatic analysis performed and reported.

In 2021, 239 beef enrichments underwent Top 7 STEC culture confirmation procedures. Of these 182 were serogroup positive for at least one of the Top 7, and of these only 39 were confirmed as culture positive. As with previous years, the most common STEC serotype identified was O26, followed by O157 and O103.

In addition, MPI requested that culture isolation for the serotype O157 for 17 veal samples was performed. In total 31 *E. coli* O157:H7 isolates were sequenced; 25 of which were confirmed as STEC isolates (17 from bovine and 6 from veal) and 6 were O157 serotype positive but classed as non-pathogenic.

Sequencing Type (ST) 11 was the dominant sequence type identified (24 of 25) with one being ST10084. None of the beef isolates were genetically related but it was noted that two beef isolates were related (within five single nucleotide polymorphisms (SNP) difference) to three clinical isolates from 2021. No strong epidemiological link was identified to explain this finding, however ESR did note that:

> "between 25 and 38 new 5-SNP clusters of human cases are being detected annually in NZ, mostly comprising less than five cases suggesting that within the broader cluster groups NZ STEC O157 are a heterogeneous group. Generally, NZ cattle and human isolates group together and

occasionally cluster very closely. This is not unexpected as internationally bovine species are regarded as a significant source for this pathogen".

Comparison of the non-STEC with STEC serogroup O157 as defined by the presence or absence of H7 revealed that there were significant differences in the core genome with H7-positive O157 clustering as a distinct group. Further STEC O157:H7 separated into three separate clades depending on the type of *stx* gene present (*stx 1a, 2a* and *2c*). All bovine STEC O157 isolates from 2021 were found to have the *mdf*(A), an acquired macrolide–lincosamide–streptogramin B resistance gene, which is also common in NZ clinical STEC isolates. No other resistance genes were identified in this group.

There were no requests in 2021 for NZ WGS data in the investigation of overseas outbreaks associated with meat. However, the library of molecular profiles held by *PulseNet Aotearoa New Zealand* will continue to inform both investigations of USA outbreaks as required and source attribution studies of human cases in New Zealand.

Science for Communities

PREVALENCE OF TOP 7 STEC IN BEEF IN NEW ZEALAND AND GENETIC ANALYSIS OF *E. coli* O157 (2021)

Prepared for the Ministry for Primary Industries

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EXECUTIVE SUMMARY

For the 2021 calendar year, the Enteric Reference Laboratory (ERL) tested a total of 238 meat screen-positive enrichment broths and one pure presumptive *Escherichia coli* (*E. coli*) O157 culture referred from adult bovine samples from six laboratories. These samples were tested as a part of N60 Top 7 STEC Regulatory Programme where testing of bovine meat trim for Top 7 STEC is required prior to export to the United States of America. The samples comprised 17 broths referred for *E. coli* O157 immunomagnetic separation (IMS) and culturing only; 199 broths referred for Top 6 (serogroups O26, O45, O103, O111, O121, O145) Shiga toxin-producing *E. coli* (STEC) testing; and 22 broths referred for Top 7 STEC (Top 6 serogroups plus *E. coli* O157) testing.

Thirty-nine samples (38 enrichment broths and the one pure *E. coli* O157 culture) were confirmed culture positive for a Top 7 STEC serotype with three different STEC serogroups observed: O103 (n=1); O26 (n=21); and O157 (n=17).

Of the 221 sample broths screened for Top 6 STEC serogroups (submitted for Top 6 or Top 7 testing), 182 (82%) yielded a positive multiplex real-time polymerase chain reaction (RT-PCR) result for one or more of the serogroups tested. One hundred and sixty of these 182 samples failed however, to yield colonies following isolation that fulfilled the criteria for Top 6 STEC confirmation (biochemically reacting as an *E. coli;* and serogroup confirmed by titration; and positive for *eae* and *stx1* and/or, *stx2* by PCR). Twenty three of the 39 broths (59%) received for either O157 IMS or Top 7 testing failed to yield results that fulfilled the the United States, Department of Agriculture (USDA) criteria for STEC O157.

One hundred and ten primary laboratory screen-positive bobby veal broths were received and then stored frozen (-80°C) at ERL. At the end of the 2021 bobby calf season, New Zealand Food Safety (NZFS) requested 17 samples be defrosted and cultured for *E. coli* O157. NeoSeek™ confirmation results provided by NZFS indicated that nine of these 17 broths were confirmed positive and eight were not positive for STEC O157. Eight of the nine NeoSeek™ confirmed positive broths yielded viable STEC O157 colonies. Of the eight NeoSeek™ negative broth samples, six yielded viable *E. coli* O157 colonies following culture isolation that did not meet the USDA STEC definition.

Thirty-one *E. coli* O157 isolates (eight bobby calf and 17 bovine STEC isolates; and six bobby calf non-STEC isolates) underwent whole genome sequencing (WGS). The sequenced genomes were analysed using ESR's GNReporter pipeline, core genome multi-locus

sequencing typing (cgMLST) and single nucleotide polymorphism (SNP) cluster analysis pipelines. Twenty-four STEC O157 isolates were identified as sequence type (ST) 11, the remaining STEC O157 isolate was identified as ST10084. None of the STEC O157 bovine isolates obtained for 2021 were genetically closely related (less than 5 SNP differences). However, two separate STEC O157 bovine isolates were observed to be genetically closely related (less than 5 SNP differences) with a total of three human clinical isolates collected within 10 weeks of the bovine samples.

A review of NZ clinical STEC strains isolated in 2021 demonstrated that NZ clinical strains do not fit the "Top 7" STEC model with many other serotypes, some of which are *eae* negative, being isolated from clinical cases presenting with serious illness. As the NZ STEC genomic database grows, so too will our understanding of this organism's transmission pathways, but additional source data are necessary to improve our knowledge.

1. Introduction

On June 4th, 2012, six Shiga toxin-producing *Escherichia coli* (STEC) serogroup (O26, O45, O103, O111, O121, and O145), in addition to *E. coli* O157:H7, were declared adulterants in certain types of raw beef meat in the United States of America (USA). Consequently, New Zealand (NZ) beef exported to the USA has to be tested for the presence of these serogroups collectively referred to as the "Top 7 STEC". The Enteric Reference Laboratory (ERL) at ESR carries out confirmation of presumptive positive meat enrichment broths from adult bovine samples as part of the N60 Top 7 STEC Regulatory Programme. ERL staff enter result data into the NZ National Microbiological Database (NMD) via E-STAR, thus fulfilling requirements under the Ministry for Primary Industries (MPI) Overseas Market Access Requirements (OMAR): United States of America, amendment 21, May 2021, Part 2 Schedule 1.

Prior to May 2021, the United States, Department of Agriculture (USDA) definition of a positive Top 7 STEC sample was as follows:

- The sample is positive for non-O157 STEC if the isolate is agglutination positive for one or more of the Top 6 non-O157 STEC serogroups, positive for *stx1* and/or *stx2* and *eae*, positive for one or more of the six non-O157 serogroup genes and biochemically identified as *E. coli*. If the isolate and all additional colony picks from plating media are ultimately determined to be negative for either *stx* or *eae*, and the Top 6 serogroup genes, the sample is negative for Top 6 non-O157 STEC.
- The sample is considered positive for *E. coli* O157, if the isolate is biochemically identified as an *E. coli* that is serologically and genetically determined to be "O157" and meets at least one of the following criteria:

  1) Positive for Shiga toxin production

  2) Positive for Shiga toxin gene(s) (*stx*)

  3) Genetically determined to be "H7"

This was amended when the Microbiology Laboratory Guidebook (MLG) 5C (2021) was updated. The definition for a positive Top 7 STEC isolate from May 2021 is as follows:

- CONFIRMED POSITIVE STEC: A sample is considered positive for STEC when the *E. coli* isolate belongs to one of the seven targeted serogroups and contains a *stx* gene and an *eae* gene.

In response to initiatives by the United States Department of Agriculture – Food Safety and Inspection Service (USDA-FSIS) to further control *E. coli* O157:H7 in the USA beef supply, New Zealand Food Safety, Ministry for Primary Industries (NZFS), and industry agreed in January 2008 to type all *E. coli* O157:H7 isolates detected under the NZ *E. coli* O157:H7 regulatory monitoring programme by pulsed field gel electrophoresis (PFGE), and to provide a summary of the PFGE profiles to the USDA-FSIS as required. From 2016, this molecular typing was expanded to include typing derived from whole genome sequencing (WGS) methods. Following a transition period of two years (2016-2017), STEC O157 isolates have been typed solely by WGS methods from 2018 onwards.

In 2018, the sequence of a USDA-FSIS isolate which was suspected of having originated from NZ was included in genomic analysis (Wright *et al.*, 2019). At the request of NZFS, the sequence has also been included in subsequent years' genomic analysis.

This report summarises:

1. The results from confirmatory Top 7 STEC testing undertaken at ESR on adult bovine trim samples collected as a part of the NMD programme during 2021. These data provide an indication of potential prevalence of Top 7 STEC in NZ adult beef samples.
2. The WGS analyses of *E. coli* O157 isolates cultured from 2021 adult bovine and bobby veal enrichments, compared to NZ bovine and clinical isolate O157 data available to date.
3. NZ bovine Top 6 STEC data with available information from NZ clinical non-O157 STEC cases.

Please note throughout this report a number of terms for *E. coli* O157 are used as follows:

1. STEC O157 – meets the May 2021 USDA adulterant definition – (*stx* and *eae* positive)
2. *E coli* O157:H7 – meets the pre-May 2021 USDA adulterant definition (*eae* and O157 and H7 positive with or without *stx* genes)
3. Non-STEC O157 – all *E coli* O157 which do not meet the current USDA adulterant definition
4. *E coli* O157 - all of the above

2. Methods

2.1 TESTING METHODS FOR MEAT ENRICHMENT BROTHS

An aliquot of each N60 enrichment carcass trim meat broth for confirmatory testing was referred to ESR's Enteric Reference Laboratory (ERL) for one of the following specified tests:

1. Top 6 STEC confirmation: testing for serogroups O26, O45, O103, O111, O121, O145
2. Top 7 STEC confirmation: testing for serogroups O26, O45, O103, O111, O121, O145 and O157
3. *E. coli* O157 immunomagnetic separation (IMS): ESR's testing process begins by performing IMS solely for *E. coli* O157 on the sample.

Prior to the USDA change in definition described above, submitted enrichment broths received for Top 6 or Top 7 STEC were tested using methods in accordance with the United States OMAR (US OMAR) Amendment 20 (August 2020) with reference to the Microbiology Laboratory Guidebook (MLG) 5C (2019).

Once the Top 7 STEC definition changed, testing and reporting was based on the updated Microbiology Laboratory Guidebook 5C.01 (May 2021) and the updated US OMAR – amendment 21 (May 2021).

On receipt at ESR, all consignments were checked to ensure the samples were within the acceptable temperature range of 0 – 10˚C. All samples that did not meet this criterion were rejected and the referring laboratory notified via telephone to resend the sample.

A boiled extract of a well-mixed aliquot from each enrichment broth for Top 6 or Top 7 testing was screened using a multiplex real-time polymerase chain reaction (RT-PCR) assay. The RT-PCR detects the *wzx* gene that encodes the O-antigen flippase within the O-antigen gene cluster for *E. coli* serogroups O26, O45, O103, O121, O145; the *wbdI* gene that encodes for glycosyl transferase in the O111 antigenic cluster and the *rfbE* gene for O157. The assay was performed using an Applied Biosystems ABI7500 platform. A full ERL procedures document (based on MLG 5 C, 2019) is available on request.

For isolation by culture of Top 6 or *E. coli* O157 STEC strains, RT-PCR screen-positive enrichments are subjected to an IMS step using commercially available beads coated with antibodies specific to each serogroup detected. Testing for the Top 6 STEC was performed using RapidChek® CONFIRM™ STEC IMS Kit (Romer Labs Inc., Union, MO, USA) and *E. coli* O157:H7 IMS testing was performed using Dynabeads™ anti-*E. coli* O157 from Applied

Biosystems (Thermofisher Scientific, Carlsbad, California, USA). These two products have been evaluated and validated for NZ use (Wright, 2019).

To isolate Top 6 STEC, pathogen-bead complexes concentrated by IMS were plated onto both modified Rainbow Agar (mRBA; prepared in-house using Rainbow® Agar O157 (Biolog Inc., Hayward, CA, USA) supplemented with 5.0 mg/L novobiocin and 0.05 mg/L cefixime trihydrate) and blood agar with vancomycin, cefixime, and cefsulodin (BVCC; Fort Richard, Auckland, NZ). Cefixime Tellurite-Sorbitol MacConkey agar (CT-SMAC; Fort Richard), was used for the isolation of *E. coli* O157:H7/STEC O157. All plating media were incubated at 37˚C for 20-24 hours.

2.1.1 Confirmation of Top 6 STEC

Following incubation, 10 colonies representing all colonial phenotypes present were sub-cultured from each mRBA and BVCC plate onto Tryptic Soy Agar (TSA; Fort Richard). TSA plates were incubated at 37˚C, overnight.

Following overnight incubation, a pool of each of the 10 sub-cultures from TSA was screened for the presence of *stx1*, *stx2, eae,* and *hlyA* genes using a conventional multiplex PCR assay (Paton and Paton 1998).

If a pool was negative for *stx1*, *stx2,* and *eae*, five of the 20 isolates from within that pool were tested using O-group specific slide agglutination (*E. coli* OK antisera, SSI Diagnostica, Hillerød, Denmark) to confirm whether the targeted serogroup had been recovered. If the five isolates were all negative, then a further five, up to a total of 20 isolates were tested until a positive was identified.

If a pool was positive for *stx1* and/or *stx2* and *eae,* then all isolates from within that pool were tested with O-group specific OK antisera for the serogroup under test. Isolates that were observed to be agglutination positive were individually screened for *stx1*, *stx2*, *eae,* and EHEC-*hly*A genes. Presumptive Top 6 STEC (positive for *eae* and *stx1* and/or *stx2* genes) were confirmed as *E. coli* using biochemical testing and titration of the O antigen.

All isolates obtained through IMS were tested for the presence of *stx1*, *stx2, eae* and EHEC-*hlyA* genes, either in a pool or individually. However, if more than one serogroup was *stx1* and/or *stx2* and *eae* positive, final confirmation was performed on only one serogroup per meat enrichment broth to limit costs to industry. Therefore, it is possible that some meat enrichment broths were positive for more than one Top 6 STEC serogroup.

2.1.2 Confirmation of *E. coli* O157

As above, this method was revised in May 2021 as per the revised US OMAR (amendment 21) and as per the revised MLG (5C.01):

January – May 2021:

Non-sorbitol fermenting colonies from CT-SMAC were tested using O157-group specific OK antisera and slide agglutination, with a target of six O157 agglutination positive colonies per sample. O157 agglutination positive colonies were sub-cultured onto TSA and retested following overnight incubation at 37˚C to avoid false positive agglutination that may occur when testing isolates from selective agar (SSI Diagnostica product information). O157 agglutination positive colonies were tested biochemically to confirm that these were *E. coli*. Serological titration was performed to confirm O157 serogroup positivity and PCR to confirm H7. The presence of the *stx1*, *stx2, eae,* and EHEC-*hlyA* genes in individual isolates was determined using a multiplex PCR (Paton and Paton 1998).

May 2021 Onwards:

Ten non-sorbitol and sorbitol fermenting colonies from CT-SMAC were sub-cultured onto TSA and incubated overnight at 37°C. All colonies on TSA were slide agglutinated using O157-group specific OK antisera. The presence of the *stx1*, *stx2, eae,* and EHEC-*hlyA* genes in individual O157 agglutination positive isolates was determined using a multiplex PCR as above (Paton and Paton 1998). Isolates that were *stx 1* and/or *2, eae*, and O157 agglutination positive were tested biochemically to confirm they were *E. coli* and serological titration was performed to confirm O157 serogroup positivity.

All tests were performed according to ERL standard operating procedures. The biochemical testing data are not part of this project and hence are not summarised in the attached spreadsheet.

Pure presumptive *E. coli* O157 isolates referred to ERL are confirmed using the same steps.

2.2 BOBBY VEAL TRIM ENRICHMENT BROTHS

Bobby veal carcass trim enrichment broth samples which were Top 7 STEC screen positive were received by ESR during the 2021 calendar year. Following receipt, an 8 ml aliquot of each sample was frozen at -80°C until required.

At the conclusion of the 2021 bobby calf season, NZFS requested that 17 specific enrichment broths be tested to isolate *E. coli* O157. The 17 enrichment broths were thawed to room temperature and *E. coli* O157 IMS testing, and confirmation testing was performed as per the methods outlined in Section 2.1.2. The IMS product from the eight unconfirmed samples was also plated on to the following additional culture media: mRBA, BVCC and MacConkey agar (Fort Richard, Auckland, NZ).

2.3 METHODS FOR GENOTYPING *E. coli* O157 ISOLATES

Genomic DNA was extracted from *E. coli* O157 isolates, using the Chemagic™ 360 extraction platform (PerkinElmer, Waltham, MA, USA). The genomes were sequenced on the Illumina NextSeq platform using the Nextera XT library kit (Illumina, San Diego, CA, USA). Sequencing quality assessment and initial analysis, which included species identification, *de novo* assembly, and sequence type assignment, were performed using Nullarbor v 2.0[1].

The ESR in-house pipeline GNReporter (Wright *et al*., 2020) was used to determine serotype, virulence genes, and antimicrobial resistance genes. The 7-gene multi-locus sequence type (ST) was inferred using WGS data according to the Achtman scheme (Wirth *et al*., 2006).

To determine the genetic relationship between *E. coli* O157 isolates, single nucleotide polymorphism (SNP) analysis was performed using two approaches:

1. SnapperDB v 1.0.5[2]: *E. coli* O157 sequence reads are mapped against the *E. coli* O157:H7 Sakai reference genome (NC_002695.1). A SNP address is generated for each isolate (Figure 1). In general, the SNP address represents a genetic distance metric-based on shared SNPs an isolate of interest has compared to other *E. coli* O157 genomes within the ESR database. ESR uses the SNP address method routinely for recognising clusters of clinical *E. coli* O157 and O26 isolates using a 5 SNP difference cut-off as per the recommendations of Larkin *et al.* (2017).

2. Snippy 4: an additional core-SNP analysis method (Seeman *et al.*) which assists in phylogeny. The SNP data are uploaded into the Microreact platform (Argimón *et al*., 2016) to visualise clustering information.

Both of these SNP analyses are done by mapping to the Sakai reference strain (STEC O157:H7 strain, Sakai GenBank accession BA000007).

[1] https://github.com/tseemann/nullarbor.
[2] https://academic.oup.com/bioinformatics/article/34/17/3028/4961427

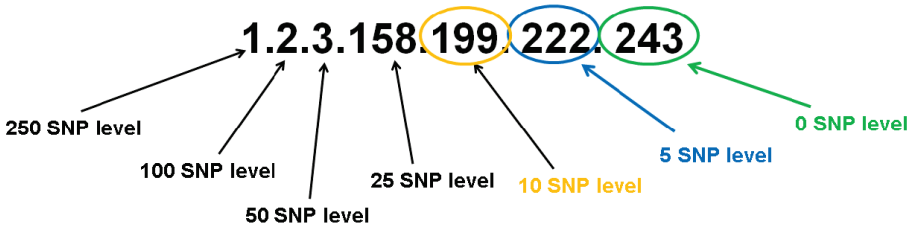

Figure 1: How to "read" a SNP address (Copied from Larkin *et al.*, 2017: – Figure 2).

The SNP address is a series of numbers, displayed sequentially representing the number of SNP differences in decreasing order, which represent a "cluster" of isolates that are within a threshold number of SNPs from each other (Figure 1). Thresholds are 250, 100, 50, 25, 10, 5 and 0 SNPs. Strains that share only the first number in the SNP address are within 250 SNPs of each other and strains that share the first, second and third set of numbers are within 50 SNPs of each other and so on. If two strains have the same seven-character SNP address, it means that, in the context of the reference genome, there are no identifiable SNP differences between these strains (i.e., they are genetically indistinguishable). Isolates that fall into the same 5-SNP cluster will have no more than 5 SNPs that are different.

A less discriminatory clustering tool was also applied to all the bovine *E. coli* O157 isolates from 2016 – 2021. The cgMLST scheme was downloaded from pubMLST and cgMLST allele calling performed using chewBBACA (version 2.8.5). The scheme recognises 2513 loci within core genes and as such gives a much higher resolution than the 7-gene multi-locus sequencing typing (MLST) scheme, but a lower resolution than SNP analysis. Core-genome MLST differences were visualised using minimum spanning trees within GrapeTree[3].

[3] https://github.com/achtman-lab/GrapeTree

3. Results

3.1 LABORATORY CONFIRMATION TESTING RESULTS

In the spreadsheet that accompanies this report, the following data are presented for each of 238 meat enrichment broths and single presumptive *E. coli* O157 isolate submitted to ERL for the 2021 calendar year:

1) ERL sample number
2) Sample status
3) Temperature on receipt
4) Date collected
5) Date received by ERL
6) Transit time
7) Sample source
8) Sample type
9) Submitting client
10) Test requested
11) Final result for requests for Top 6, O157 IMS; and the O157 result for a Top 7 request
12) Final result additional (only used for Top 7 requests to show the Top 6 final result)
13) STEC toxin PCR results for colonies isolated from mRBA and BVCC Agar (Top 6), or CT-SMAC (O157) (only shown on the worksheets for the positive serogroup)
14) Real-time PCR (RT-PCR) results for Top 6/7 testing.

No information on the primary screening method used by the submitting laboratory was provided on the submission form accompanying the referred sample. However, all samples submitted in 2021 for Top 7 STEC screening under the MPI N60 Top 7 STEC Regulatory Programme were screened using Biocontrol Assurance® GDS kits (MPI, personal communication).

3.1.1 Summary of samples received

Six laboratories submitted 238 STEC screen-positive beef enrichment broth samples collected during the 2021 calendar year (Table 1) – a similar number to 2020 (Table 1). The 2021 samples comprised 17, 199, and 22 broths that were tested for *E. coli* O157 IMS, Top 6 STEC, and Top 7 STEC, respectively. Collectively, for 2021, a total of 221 broth samples were tested for Top 6; and 39 broth samples were tested for *E. coli* O157.

A further 24 broth samples were received but excluded from testing as their temperature on receipt exceeded 10˚C (range 10.4˚C – 19.4˚C). One additional sample was excluded as it was not accompanied by a vial for temperature measurement. Repeat aliquots of all 25 broths were subsequently received at an acceptable temperature for testing to be completed.

Table 1: Number of beef enrichment broths tested for confirmation during 2016-2021 by submitting laboratory

Primary Laboratory (location[2])	Number of Enrichment Broths Received[1]					
	2016	**2017**	**2018**	**2019**	**2020**	**2021**
ANZCO FOODS Kokiri	1	0	0	0	0	0
AsureQuality (AK)	66[3]	78	119	127	122	68
AsureQuality (HM)	2	0	3	1	0	0
AsureQuality (CH)	9	22	31	50	37	96
Eurofins NZ Laboratory Services (AK)	10	0	0	0	3	4
Eurofins ELS (LH)	15	22	53	45	33	31
Eurofins NZ Laboratory Services (CH)	7	11	29	14	16	14
Hill Laboratories (BN)	1	0	0	0	0	0
Hill Laboratories (CH)	24	35	41	35	26	25
Total	135	168	276	272	**237**	**238**

[1]Includes broths submitted for Top 6 STEC, Top 7 STEC, or *E. coli* O157 confirmation

[2] AK: Auckland; HM: Hamilton; CH: Christchurch; LH: Lower Hutt; BN: Blenheim

[3] Includes bobby veal carcass enrichment samples

In addition, one laboratory referred multiple isolates from a single enrichment sample that was presumptive *E. coli* O157 for confirmation.

3.1.2 Top 6 Results

Of the 221 sample broths screened for Top 6 STEC serogroups (submitted for Top 6 or Top 7 testing), 182 (82%) yielded a positive RT-PCR result for one or more of the serogroups tested.

Of the 182 RT-PCR positive samples, Top 6 STEC were culture confirmed for 22 samples, with the majority (21 samples) being O26 (Table 2). STEC O26 was the serogroup most commonly identified across 2015 – 2021 (Table 4), with 13.2% samples confirmed as STEC O26 across the seven-year period.

For some samples multiple STEC serogroups were detected, therefore the total number of positive detections (n=337 – Table 2) is greater than the total number of positive samples (n=182).

Table 2: Summary of Top 6 confirmed samples and Top 6 RT-PCR serogroup positive samples.

Serogroup	Number of STEC culture confirmed samples/number of samples with RT-PCR serogroup positive.	
	2020#	**2021**
O26	17/55 (31%)	21/65 (34%)
O103	7/81 (9%)	1/82 (1.2%)
O45	0/86	0/116
O111	0/0	0/0
O121	0/58	0/61
O145	1/3 (33%)	0/13
Total broths Top 6 positive/total broths tested	**25/219 (11%)**	**22/221 (10%)**

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Table 3 shows the Top 6 results for the 221 enrichment samples submitted for Top 6 or Top 7 STEC testing by the referring laboratory.

Table 3: Summary of screen results for beef enrichment broths received for Top 6/7 STEC confirmation.

Submitting Laboratory	Total Top 6/7 samples submitted	Number of samples confirmed positive Top 6 STEC
AsureQuality (AK)	63	7 confirmed as O26
AsureQuality (CH)	89	5 confirmed as O26
Eurofins (AK)	4	1 confirmed as O26
Eurofins ELS	29	2 confirmed as O26 1 confirmed as O103
Eurofins (CH)	12	3 confirmed as O26
Hill (CH)	24	3 confirmed as O26
Total	**221**	**22**

AK: Auckland; CH: Christchurch

The target serogroups were isolated by IMS from all of the Top 6 RT-PCR-positive samples. It was observed that many isolates that were positive by serogroup specific slide agglutination failed to yield positive results for the presence of *eae* and *stx1* and/or *stx2* by PCR, so were neither further identified nor serogroup-confirmed by titre. These samples were reported as "No Top 6 detected".

On occasion it was noted that at least one of the pools of 10 isolates selected for testing for each sample, yielded positive results for *eae* and *stx1* and/or *stx2* by PCR but none of the 10 isolates were positive for the target serogroup, and *eae,* and *stx*1 and/or 2 when tested individually. These samples were also reported as "No Top 6 detected".

Table 4 provides a summary of the number of sample enrichment broths that were confirmed as Top 6 STEC-containing received within years 2016 – 2021. Once a Top 6 serogroup is confirmed, other potential positive serogroups identified during the RT-PCR screen are not further investigated. This approach has not changed in the seven years shown.

Table 4: Summary of the number of samples that were confirmed STEC Top 6 by ERL for 2015 – 2021.

STEC Serogroup	2015[a*]	2016[b*]	2017[c]	2018[d]	2019[e]	2020[f]	2021	Total
O26	92 (25.6%)	22 (23.7%)	11 (8.7%)	26 (10.2%)	12 (4.9%)	17 (7.8%)	21 (9.5%)	201 (13.2%)
O103	8 (2.2%)	0	2 (1.6%)	4 (1.6%)	4 (1.6%)	7 (3.2%)	1(0.5%)	26 (1.7%)
O145	1 (0.3%)	0	0	0	0	1 (0.5%)	0	2 (0.1%)
O45	0	0	0	1 (0.4%)	0	0	0	1 (0.07%)
No Top 6 STEC	259 (71.9%)	71 (76.3%)	114 (89.7%)	223 (87.8%)	227 (93.4%)	194 (88.5%)	199 (90.0%)	1287 (84.8%)
Total	**360**	**93**	**127**	**254**	**243**	**219**	**221**	**1517**

[a]Dufour 2016; [b]Wright and Gilpin 2017; [c]Wright *et al.,* 2018; [d]Wright, *et al.,* 2019; [e]Wright *et al.,* 2020; [f]Wright *et al.,* 2021

[*] Includes bobby veal carcass enrichment samples

Fourteen of the 21 STEC O26 isolates, and the single O103 isolate had the toxin profile of *stx1*+ *stx2*- *eae*+. The remaining seven STEC O26 isolates had the toxin profile of *stx1*-, *stx2*+, *eae*+.

3.1.3 STEC O157 results

Table 5 shows the results from the 39 enrichment broths referred for STEC O157 confirmation (Top 7 and O157 IMS) and one presumptive *E. coli* O157 culture.

None of the 17 broths submitted for only O157 IMS yielded a confirmed STEC O157.

Sixteen of the 22 samples submitted for Top 7 STEC confirmation were culture confirmed as positive for STEC O157 by ERL. The single presumptive *E. coli* O157 culture was also confirmed as STEC O157:H7 (Table 5).

Table 5: Summary of results for total enrichment broths and presumptive STEC O157 cultures received for *E. coli* O157 confirmation (Top 7 or O157 IMS)

Submitting Laboratory	Number of broth samples received	Number of pure isolates received	Number of samples confirmed for STEC O157
AsureQuality (AK)	15	0	8
AsureQuality (CH)	12	0	3
Eurofins AK	0	0	0
Eurofins NZ ELS	6	0	4
Eurofins NZ Laboratory Services (CH)	3	0	0
Hill (CH)	3	1	2
Total	**39**	**1**	**17 (includes the pure culture isolate)**

3.1.4 Sample age

Sample age on broth receipt (number of days between primary sample collection and broth receipt at ESR) and positive yield is shown in Table 6. The majority (n=220) of enrichment broths were received within seven days of primary sample collection, with a median of five days. Four of the seven enrichment broths that were received 11+ days after primary sample collection were from the Christmas/New Year period. The oldest sample from which a Top 7 STEC was confirmed (O157) was seven days old.

Table 6: Summary of confirmed Top 7 STEC positive yield based on broth sample age on receipt for confirmatory testing

Broth age on receipt at ESR	Total samples received	O26 +	O103 +	O157 +	Total positive
1 – 3 days	72	7	1	10	18
4 – 6 days	126	12	0	5	17
7 – 9 days	31	2	0	1	3
10 – 12 days	3	0	0	0	0
13 - 21 days	6	0	0	0	0
Total	**238**	**21**	**1**	**16**	**38**

Sample age is not critical for referred purified cultures; therefore, these have not been included in the above analysis.

3.2 BOBBY VEAL TRIM ENRICHMENT BROTHS

One hundred and ten bobby veal enrichment broths were received and stored during the 2021 season. At the end of the bobby calf season, 17 stored bobby veal broths were subjected to IMS testing for O157, at the request of NZFS. These broths had all tested screen positive for O157 at the primary testing laboratory, and nine had been subsequently confirmed as STEC O157 by NeoSeek™. STEC O157 was isolated from eight of these nine enrichment broths. Six of the eight unconfirmed broths yielded isolates of *stx* negative *E. coli* O157, and no O157 positive organisms were retrieved from the remaining three samples.

3.3 WHOLE GENOME SEQUENCING RESULTS FROM THE *E. COL*I O157 ISOLATES

Quality parameters including sequencing quality, coverage depth, assembled genome size and number of contigs, indicated that all WGS outputs were of sufficient quality for analysis.

3.3.1 STEC O157 isolates toxin types

The results obtained from WGS analysis for all STEC O157 isolates were consistent with the laboratory results for the phenotypic serotype identification, as well as the virulence genes detected by conventional multiplex PCR (*stx1*, *stx2, eae,* EHEC-*hlyA*) (Table 7). Among the 25 STEC O157 isolates (17 bovine and eight bobby), three were positive for *stx1* only, 16 were positive for *stx2* only, and six were positive for both *stx1* and *stx2*. WGS analysis showed that all nine STEC O157 isolates that were *stx1* positive were subtype 1a. Of the 22 STEC O157 isolates that were *stx2* positive, 15 were subtype 2a and seven were subtype 2c.

All 17 bovine STEC O157 isolates were identified as ST11, as were seven of the eight bobby veal isolates; the remainder being ST10084.

Table 7: Serotype, core virulence genes and *stx* subtypes of STEC O157 isolates as inferred using whole genome sequence data.

Isolate	Source*	Serotype	*stx1*	*stx1* subtype	*stx2*	*stx2* subtype	*eae*	EHEC-*hly*A	MLST type
21ER0652	Bovine	O157:H7	-	-	+	a	+	+	11
21ER0741	Bovine	O157:H7	+	a	+	a	+	+	11
21ER0957	Bovine	O157:H7	-	-	+	c	+	+	11
21ER0995	Bovine	O157:H7	-	-	+	c	+	+	11
21ER1087	Bovine	O157:H7	-	-	+	c	+	+	11
21ER1224	Bovine	O157:H7	+	a	+	a	+	+	11
21ER1954	Bovine	O157:H7	-	-	+	a	+	+	11
21ER2330	Bovine	O157:H7	-	-	+	c	+	+	11
21ER2416	Bovine	O157:H7	+	a	-	-	+	+	11
21ER2649	Bovine	O157:H7	-	-	+	a	+	+	11
21ER2656	Bovine	O157:H7	-	-	+	a	+	+	11
21ER2957	Bovine	O157:H7	+	a	+	a	+	+	11
21ER2966	Bovine	O157:H7	-	-	+	c	+	+	11
21ER3593	Bovine	O157:H7	-	-	+	c	+	+	11
21ER3944	Bovine	O157:H7	-	-	+	a	+	+	11
21ER4136	Bovine	O157:H7	-	-	+	c	+	+	11
21ER4237	Bovine	O157:H7	+	a	-	-	+	+	11
21ER2054	Bobby	O157:H7	-	-	+	a	+	+	11
21ER2244	Bobby	O157:H7	+	a	+	a	+	+	11
21ER2499	Bobby	O157:H7	-	-	+	a	+	+	11
21ER2582	Bobby	O157:H7	-	-	+	a	+	+	11
21ER2690	Bobby	O157:H7	+	a	+	a	+	+	11
21ER3031	Bobby	O157:H7	-	-	+	a	+	+	11
21ER3138	Bobby	O157:H7	+	a	-	-	+	+	11
21ER3345	Bobby	O157:H7	+	a	+	a	+	+	10084

*bovine = adult bovine; bobby = bobby veal/calf

3.3.2 Non-STEC O157

The six non-STEC O157 comprised O157:H12 (n=4); O157:H42 (n=1); and one presumptive *Escherichia fergusonii* which was O157 slide agglutination positive, and the presence of the O157 gene was confirmed by WGS analysis. All six isolates were negative for *stx*1, *stx*2, *eae* and *hly*A.

3.3.3 STEC O157 cluster analysis

Genomic sequences of the *E. coli* O157 isolates underwent cluster analysis using core genome SNP analysis (SnapperDB and Snippy 4). Table 8 shows the SNP address findings for the 2021 isolates. None of the bovine/bobby isolates from 2021 were observed to cluster within 5-SNP each other.

Table 8: Seven-digit SNP addresses of 2021 *E. coli* O157 bovine isolates.

Isolate number	250 SNP	100 SNP	50 SNP	25 SNP	10 SNP	5 SNP	0 SNP
21ER2690[b]	1	3	5	5	486	649	1045
21ER0741[B]	1	3	5	5	853	878	897
21ER2957[B]	1	3	5	5	929	964	994
21ER4237[B]	1	3	5	32	965	1005	1037
21ER3138[b]	1	3	5	32	979	1022	1055
21ER2416[B]	1	3	5	32	1009	1057	1095
21ER1224[B]	1	3	5	38	872	898	920
21ER2244[b]	1	3	5	38	978	1021	1054
21ER3345[b]	1	3	5	139	980	1023	1056
21ER3944[B]	2	2	4	772	960	998	1030
21ER2499[b]	2	2	8	779	974	1017	1050
21ER2656[B]	2	2	21	756	927	962	992
21ER2582[b]	2	2	44	778	972	1015	1048
21ER1954[B]	2	2	441	749	919	952	1098
21ER0652[B]	2	6	6	282	1010	1058	1096
21ER2054[b]	2	6	6	742	971	1013	1046
21ER3031[b]	2	26	61	173	976	1019	1052
21ER2649[B]	2	26	72	144	152	959	989
21ER2330[B]	5	7	7	352	1006	1054	1092
21ER2966[B]	5	7	7	757	930	965	995
21ER1087[B]	5	10	193	313	871	897	919
21ER0995[B]	5	10	431	731	862	888	909
21ER3593[B]	5	10	451	767	951	988	1020
21ER4136[B]	5	10	456	776	964	1003	1035
21ER0957[B]	5	160	430	730	861	887	908
21ER2781[bn]	11	111	287	796	1003	1049	1085
21ER3172[bn]	11	169	463	793	1000	1046	1082
21ER2583[bn]	43	167	459	787	993	1038	1073
21ER2827[bn]	43	170	464	794	1001	1047	1083
21ER2691[bn]	44	168	462	792	999	1045	1081
21ER3761[bn]	45	171	466	797	1007	1055	1093
SRR6760761	46	173	474	815	1049	1099	1151

[B] Bovine [b] bobby veal sample [n] non-STEC

The international *E. coli* O157:H7 USDA-FSIS sequence that was first included in the 2018 data set (SRR6760761, Table 8) was observed to possess >250 SNP differences to any of the 2021 NZ bovine *E. coli* O157 isolates.

Table 9 shows the bovine STEC O157 isolates from 2016 onwards that form 5-SNP clusters with other bovine and/or human clinical isolates. During SNP-analysis, isolates that share less than 5 SNP differences with clinical isolates within a database are assigned a 'ClusterID'. The year within the ClusterID denotes the year of isolation of the first isolate in the cluster, while the second number denotes the number of that specific cluster within that year. Clusters that comprise bovine isolates only have been designated as Bovine n (n being the number of bovine clusters seen to date). The SNP address for an isolate can change as more isolates are added to a database, but the ClusterID does not change, allowing for ongoing tracking of clusters. There are now >1000 NZ human clinical isolates in ESR's *E. coli* O157 WGS database for all years from 2016 onwards.

Table 9: STEC O157 5-SNP clusters which include bovine isolates.

			SNP address							
ID	**Source**	**ClusterID**	**250**	**100**	**50**	**25**	**10**	**5**	**0**	**ST Achtman**
18ER0975	Bovine	STEC_2018_C_30	2	2	102	147	155	257	260	11
18ER1141	Bovine	STEC_2018_C_06	2	6	33	137	145	145	247	11
18ER2368	Bovine	STEC_2018_C_26	1	3	5	139	147	147	357	10084
18ER3198	Bovine	STEC_2018_C_26	1	3	5	139	147	147	784	10084
18ER3276	Bovine	STEC_2018_C_26	1	3	5	139	147	147	654	10084
19ER1832	Bovine	STEC_2017_C_04	5	10	106	597	673	681	726	11
19ER2971	Bovine	Bovine 1	1	3	30	33	34	34	34	11
19ER3923	MPI project (405953)	Bovine 1	1	3	30	33	34	34	37	11
20ER0003	Bovine	STEC_2020_C_03	2	6	6	31	32	32	32	11
20ER0567	Bovine	Bovine 2	1	3	5	5	486	649	658	11
21ER2690	Bobby	Bovine 2	1	3	5	5	486	649	1045	11
21ER3593	Bovine	STEC_2021_C_27	5	10	451	767	951	988	1020	11
21ER1954	Bovine	STEC_2021_C_19	2	2	441	749	919	952	1098	11

Some of these clusters are new (highlighted yellow in the table) and some have been discussed in previous reports, but all are summarised below for completeness.

- STEC_2018_C_30 comprises two isolates: bovine 18ER0975 and a clinical isolate from January 2021.
- STEC_2018_C_06 comprises bovine 18ER1141 and two clinical isolates from 2018.

- STEC_2018_C_26 comprises bovine isolates 18ER2368, 18ER3198, 18ER3276 and two clinical isolates from 2019.
- STEC_2017_C_04 comprises bovine 19ER1832 and a clinical isolate from 2017.
- STEC_2021_C_19 (new cluster) comprises bovine 21ER1954 and two clinical isolates collected within six weeks of the bovine sampling.
- STEC_2021_C_27 (new cluster) comprises bovine 21ER3593 and a clinical isolate collected within 10 weeks of the bovine sampling

Figure 2 represents a cluster diagram using cgMLST of 128 *E. coli* O157 New Zealand beef isolates whole genome sequenced by ESR to date (2016-2021). Significant differences at core-genes (>2300) were observed between isolates identified as H7 and non-H7.

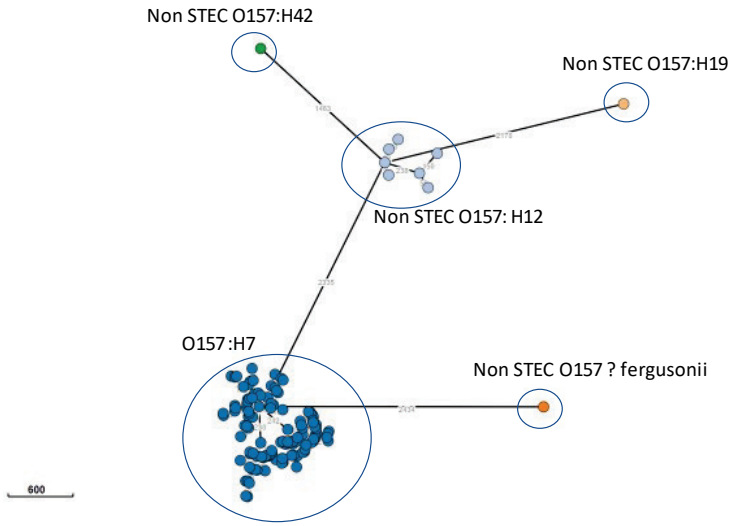

Figure 2. Minimum spanning tree generated from cgMLST of 128 *E. coli* O157 New Zealand isolates from 2016-21, grouped by H antigen type and visualised in GrapeTree.

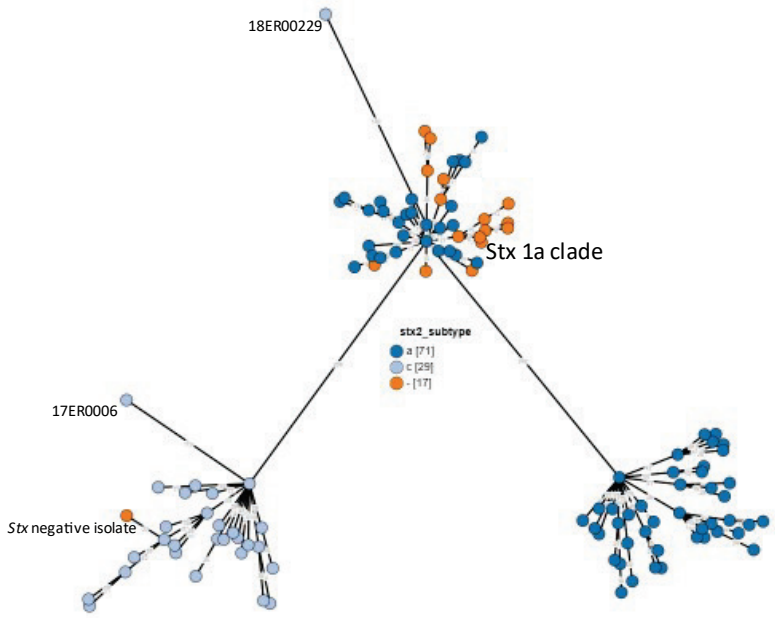

Figure 3. Minimum spanning tree generated from cgMLST of 118 O157:H7 New Zealand isolates from 2016-21, grouped by *stx* subtype, and visualised in GrapeTree.

In Figure 3, cgMLST of 118 *E. coli* O157:H7 isolates resulted in three distinct broad cluster groups with >230 core-gene differences observed between each group. One group comprised isolates that were all *stx1a* positive, some of which are also *stx*2a positive. One isolate (18ER00229) that was identifed as *stx1a*-, stx*2c*+ was observed as an outlier and had 183 core-gene differences from the other isolates within this group.

A second broad cluster group comprised isolates identified as a *stx*1a-, stx2c+ group. One isolate (17ER0006) that was identified as *stx*1a+, stx2c+ was observed as an outlier and had 113 core-gene differences to the other isolates within this group. An *stx* negative *E. coli* O157:H7 isolate (19ER3218) was observed to cluster (within 25 core-gene differences of others in this group) suggesting it may have derived from an *stx*2c+ strain.

Figure 4 is a cluster diagram using cgMLST of 45 *E. coli* O157:H7 that were identifed as *stx1a* positive and displays the relatedness of different isolates over the years tested. The centre cluster is that designated STEC_2018_C_26.

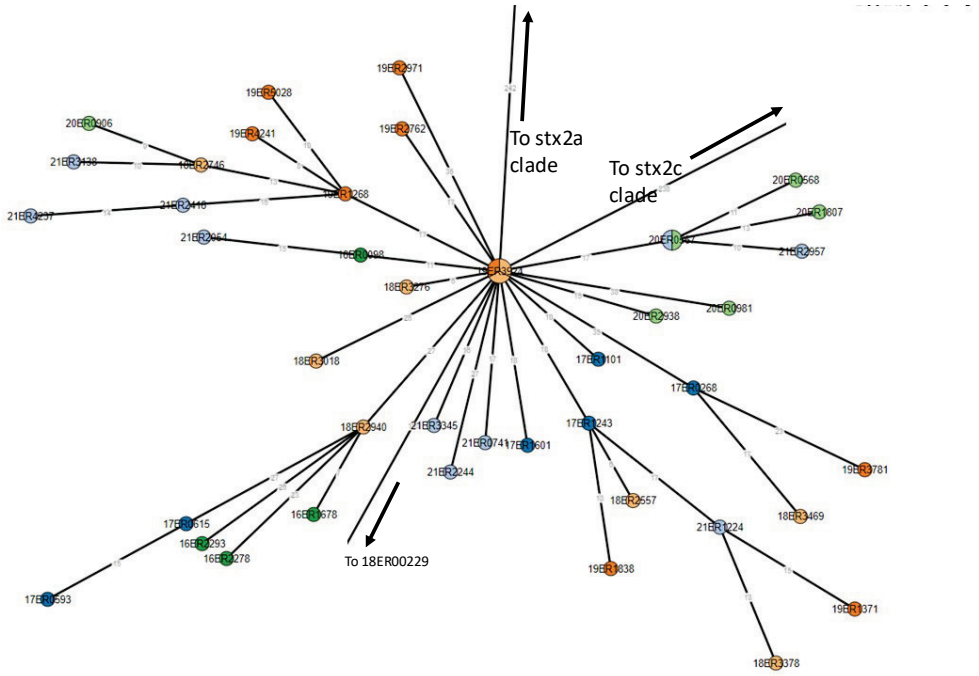

Figure 4. Minimum spanning tree generated from cgMLST of 45 *E. coli* O157:H7 *stx*1a positive New Zealand isolates from 2016-21, with isolates with less than 10 core-gene differences grouped together, and branches shown in logarithmic scale visualised in GrapeTree.

3.3.4 STEC O157 Antimicrobial resistance genes

The WGS reads from all 31 *E. coli* O157 isolates were screened for the presence of antimicrobial resistance genes. All bovine isolates from 2021 were found to have the *mdf*(A) gene, an acquired macrolide–lincosamide–streptogramin B resistance gene (Van Hoek *et al.*, 2011). This gene is common in NZ clinical STEC isolates (ESR, unpublished data). No other resistance genes were identified in this dataset.

4. Discussion

4.1 Top 7 STEC Confirmation

The number of meat enrichment broths received by ESR for confirmation testing for Top 6, Top 7 STEC or *E. coli* O157 varies each year (Table 1). Samples tested at ESR pre-2017 were sourced from both bobby calf and adult beef carcasses. All samples received for confirmatory testing from 2017 - 2021 were sourced only from adult beef carcasses. Bobby veal have previously been shown to carry more Top 7 STEC serotypes per sample than adult beef (MPI, personal comms). This change in sample population has therefore affected the frequency of Top 7 STEC confirmed at ESR as seen in Table 4.

Top 6 STEC serogroups were detected by RT-PCR in 82% of the samples received at ESR for Top 6 and Top 7 testing. However, isolates screened from 160 (88%) of these samples failed to yield positive results for *eae* and *stx1* and/or *stx2* by PCR and were therefore reported as "No Top 6 or Top 7 detected". This confirmation rate of 12% of samples screened by IMS and 10% of all samples received for confirmatory testing is comparable to previous years and not dissimilar to the 10% recently reported by Dr Mick Bosilevac, Nebraska USDA, in a 2022 Webinar presentation: STEC: Challenges in Controlling, Detecting and Resolving Contamination hosted by Biomerieux.com, regrettably the webinar had not been made publicly available at the time of finalising this report .

The potential reasons for this apparent poor recovery of Top 7 STEC during confirmation testing include:

a. The submitted broths contained STEC belonging to serotypes other than the Top 6/7 in conjunction with non-toxigenic *E. coli* strains belonging to the Top 6/7 serogroups. In 2006, Cookson *et al.*, (2006) reported a variety of serotypes of STEC found in NZ cattle and sheep. Whilst their study sample was small and not all isolates were sero-typable by the methods used, the only Top 6 serogroup the authors reported was O26. In 2001, a study of point-of-sale raw meats purchased from Dunedin supermarkets found STECs in 12% of beef samples tested (Brooks *et al.*, 2001) but none of these belonged to the Top 6 serogroups. Browne (2018) reported *stx*-negative O26 isolates were found in a cross-sectional study of dairy cattle across NZ.

b. The screening test used in the primary testing laboratories detects genomic DNA from all bacteria in the enrichment population. It is possible therefore that the target genes are located in different bacterial cells within the enrichment. That is, one organism is

positive for *stx* genes whilst another is positive for the *eae* gene, therefore the sample gives a positive screen result.

c. Even though there is an IMS step in the screening process, there remains the possibility that the submitted broths contained *stx* positive organisms belonging to other genera. The review of Mauro and Koudelka (2011) noted that *stx* carriage was not limited to *E. coli* and can be broadly distributed amongst other bacterial species.

d. The RT-PCR detected organisms other than *E. coli* with the same target O-antigen. It is known that other Enterobacterales can share somatic antigens with *E. coli* (Stenutz *et al*., 2006). This was evidenced in the presumptive *E. fergusonii* reported here.

e. The isolates had "lost" their Shiga-toxin genes. These are carried on mobile elements which can be lost during culture passaging (Bielaszewska *et al*.,2007).

f. The numbers of live STEC cells in the sample were too low to be isolated by culture. This could be due to the screening laboratories' IMS concentration step increasing the sensitivity of the screening PCR beyond the capability of the confirmatory culture. Alternatively, all PCRs amplify DNA from both living and dead cells; thus, it is possible that the primary laboratory detected *stx* toxin genes from bacteria which were dead or sub-lethally injured at the time of confirmatory testing at ESR.

g. Organisms grown and left in enrichment broth lose viability over time. The median age for all broths was five days upon ESR receipt, and the median age for positive broths was three days (Table 6). The oldest broth from which a Top 7 STEC serogroup (STEC O157) was isolated was seven days. Nine broths were more than nine days old on receipt and of these two were >/=20 days old.

h. In the case of serogroup O157, primary testing laboratories refer O157 screen positive enrichments for confirmation that may have negative primary *stx* and *eae* results. The confirmation of six non-STEC O157 from 2021 NZ bobby samples demonstrates the range and frequency of other non-STEC O157 serotypes and explains why O157 screen positive enrichments may fail to yield confirmed STEC O157 (17/17 samples received for O157 IMS in 2021).

Delays in sample dispatch and/or extended sample transport times can have two very different negative effects:

1. False negative results being reported due to reduced organism viability.
2. Increased storage time for beef products, waiting for US eligibility certification and release for export.

It would be ideal if broths could reach ESR within seven days of collection; however there are courier issues around public holidays and weekends.

The number of confirmations for individual Top 7 serogroups is variable over time but it is noted that STEC O26 increased from 17 confirmations in 2020 to 21 in 2021 and that STEC O157 also increased - from nine confirmations in 2020 to 17 in 2021. Contrary to this trend, confirmations of STEC O103 decreased from seven in 2020 to one in 2021.

4.2 Whole genome sequencing analysis of *E. coli* O157 isolates

There were three *E. coli* O157:H7 toxin profiles observed from the 25 bovine and bobby STEC O157 isolates in 2021: *stx1*+ *stx2*-; *stx1*– *stx2*+; and *stx1*+ *stx2*+. All *stx1*+ isolates were subtype 1a. Subtype 2a accounted for 15 *stx2*+ isolates, the remaining seven *stx2*+ isolates (all bovine) being subtype 2c.

Six years (2016 – 2021) of whole genome sequencing results (128 isolates) show that NZ bovine/bobby *E. coli* O157 generally fall into three distinct genomic groups:

a. Those that are *stx*1a positive regardless of the *stx*2 result share the same first two digits of the SNP address – 1.3.x.x.x.x.x indicating they are within 100 SNP of each other.

b. Isolates that are *stx*1 negative and *stx*2a positive form a separate group that fall within 250 SNP of each other (SNP address: 2.x.x.x.x.x.x)

c. Isolates that are *stx*1 negative *stx*2c positive form a third group that all fall within 250 SNP of each other (SNP address 5.x.x.x.x.x.x).

Using SNP-analysis, it is observed that these genomic groups are greater than 250 SNP from each other. Core-genome MLST analysis also resulted in similar genomic groupings with >230 core-gene differences observed between the groups (Figure 3).

There were two STEC O157 isolates (17ER0006 (*stx*1a+2c+) and 18ER0229 – stx2c+) that were observed as outliers, with >250 SNP and >100 core-genome differences from any other isolate in the dataset. These isolates dated from before the introduction of the current ESTAR data-capture platform; but should be traceable through its predecessor. It would be interesting to know more about the origin of these isolates as outliers are also rare in the clinical isolate group, usually in cases with a history of overseas travel (ESR, unpublished data).

Non-STEC O157 were isolated from six of the eight unconfirmed Neoseek™-tested enrichments from 2021. These were all serotypes other than O157:H7 and were all negative

for *eae*, *stx*1 and *stx*2. Sequence based SNP cluster analysis showed these six to be genomically distinct from NZ STEC O157 strains with >250 SNP from any of the above genomic groupings (Table 7) and >2300 core-gene differences by cgMLST analysis (Figure 2). These results suggest that these *E. coli* isolates belong to completely separate lineages and are not recent derivatives of NZ STEC O157 strains. This level of SNP and core-gene difference was also seen with non-STEC O157 from previous years, with the exception being 19ER3218 which met the former adulterant description of an *E. coli* O157:H7, being *eae* positive and H7 positive but negative for both *stx*1 and *stx*2 (Wright *et al*. 2020). This isolate clusters with isolates that are *stx*1 negative *stx*2c positive (group C above) suggesting it may be a derivative of a *stx*2c strain.

This project demonstrates the value of multilayer genomic typing: SnapperDB SNP analysis provides superior fine clustering to cgMLST analysis but does not give a ready way of showing just how different beyond ">250 SNP". The use of cgMLST is useful in higher level differentiation.

The vast majority of STEC O157 in NZ from clinical and bovine sources sequenced to date (>1100) have been ST11. However, in 2018, three bovine isolates were observed to possess a single SNP difference in the *adk* allele, but otherwise had an MLST profile consistent with ST11 (Wright *et al*. 2019). One NZ bovine isolate from 2019 was also found to have this same profile which has been subsequently designated ST10084. (Wright *et al.* 2020). Bovine and clinical isolates belonging to ST10084 have so far invariably been positive for both *stx*1a and *stx*2a; and cluster within 10 SNPs of each other. Among the >200,000 *E. coli* uploaded from international sources to Enterobase (enterobase.warwick.ac.uk – Zhou *et al.* 2020) only one belongs to ST10084, the clinical case from NZ in 2019 which was used to confer the ST to this profile. This observation may suggest that this ST is unique to NZ.

The international *E. coli* O157:H7 USDA-FSIS sequence that was first included in the 2018 data set (SRR6760761, Table 8) was observed to possess >250 SNP differences to any of the 2021 NZ bovine *E. coli* O157 isolates. This isolate continues to have a unique SNP address in comparison to all NZ isolates (bovine and human), confirming that the international isolate is not genetically related to any NZ *E. coli* O157 sequenced to date.

4.3 COMPARISON OF BOVINE STEC O157 ISOLATES WITH CLINICAL ISOLATES

While NZ's borders remained closed in 2021 because of the COVID-19 pandemic, lockdowns had far less impact on clinical enteric testing in 2021 compared with 2020; and the NZ clinical

STEC notifications rebounded to a similar level to 2018 with 913 cases confirmed (ESR, internal communication). Further, as of December 2021, >80% of the clinical human faecal samples in NZ were being tested for STEC via a number of different multiplex PCR methods replacing culture-based methods.

Many different serotypes are associated with clinical STEC infection in NZ, some of which are localised in their prevalence. In 2021, the 10 most frequently confirmed serogroups by ESR from NZ human samples were (in order of prevalence): O157 (n=193); O26 (n=131); O128 (n=82); O146 (n=30); O91 (n=28); O38 (n=27); O103 (n=25); O174 (n=15); O176 (n=14); O5 (n=13)[4].

Other than the *E. coli* O157, O26 and O103 described above there were only six other clinical cases in 2021 where a Top 7 serogroup was isolated in NZ: O145 (n=4), O121 (n=1) and O45 (n=1).

A review of illness severity data for NZ STEC clinical cases notified to Episurv (the national notifiable disease surveillance database) between January 2016 and December 2021 (Lake *et al*. 2021) identified that only two of the USDA Top 7 STEC serogroups (O157 and O26) were amongst the serogroups associated with haemolytic uraemic syndrome (HUS). Seven non-Top 7 STEC serotypes were isolated from HUS cases in NZ during that period.

The NZ clinical case definition of STEC infection[5] does not require the laboratory confirmation of the *eae* gene and NZ case data show there are many STEC serotypes other than the USDA Top 7 STEC isolated from clinical cases presenting with serious illness and a significant number of these are *eae*-negative (Lake *et al*. 2021).

The pathoserotype STEC O26 *stx2a*+ decreased in bovine STEC samples, from 9/17 in 2020 (53%) to 7/21 (33%) in 2021. This pathoserotype was isolated from 63 clinical cases in 2021 and was seen in 12 cases of HUS between 2016 and 2021 (Lake *et al*. 2021). To-date all NZ clinical isolates have been ST21, subtype *stx2a*. ESR is routinely sequencing all clinical STEC and running weekly clustering analysis of STEC O26. Sequence information on O26 bovine isolates would be a useful addition to this dataset.

The bovine *E. coli* O157:H7 isolates from 2016 – 2021 were compared with genomes from >1000 NZ human STEC O157 isolated between 2016 and December 2021. Amongst the 2021

[4] ESR as yet unpublished data
[5] NZ Communicable Disease Control Manual https://www.health.govt.nz/our-work/diseases-and-conditions/communicable-disease-control-manual/verocytotoxin-or-shiga-toxin-producing-escherichia-coli-vtec-stec accessed 28 March 2022.

isolates, there were two 5-SNP clusters comprising both bovine and clinical isolates collected from within the same three-month period. STEC_2021_C_19 included bovine 21ER1954 and two clinical isolates collected within six weeks of the bovine sampling; and STEC_2021_C_27 included 21ER3593 and a clinical isolate collected within 10 weeks of the bovine sampling.

With regard to STEC_2021_C_19, the two clinical cases presented 10 days apart in the Auckland region, and one was hospitalised with haemorrhagic colitis. Both reported recent beef consumption. For NZ-notified cases where a food history has been recorded between 60% and 70% of STEC cases from the last five years reported having consumed beef (EpiSurv data, 13 May 2022).

A detailed history has not been recorded for the clinical case associated with STEC_2021_C_27 who is from the Canterbury region. This is not unexpected as Public Health Units' resources have been directed towards COVID-related investigations, and also some cases are lost to follow up.

ESR's growing experience with SNP based clustering shows that where two apparently unrelated isolates fall within 5-SNPs they are likely to have a recent shared history.

Routine public health surveillance performed by ESR on behalf of the Ministry of Health has shown that between 25 and 38 new 5-SNP clusters of human cases are being detected annually in NZ, mostly comprising less than five cases suggesting that within the broader cluster groups NZ STEC O157 is a heterogeneous group. Generally, NZ cattle and human isolates group together and occasionally cluster very closely. This is not unexpected as internationally bovine species are regarded as a significant source for this pathogen (Lake *et al*. 2021).

The SNP address is utilised by PHE (now the UK Health Security Agency) epidemiologists and microbiologists as the primary method for identifying microbiological clusters of gastrointestinal infections in England to detect potential outbreak events (Chattaway *et al*. 2019). Case isolates that fall within a 5-SNP single linkage cluster are considered likely to have been exposed to a common source of contamination (Dallman *et al*., 2021) – Dallman used this classification:

- Household: a case who shared the same household as another case.
- Outbreak: a case belonging to a 5-SNP cluster of cases where the source of the outbreak was determined.

- Community Cluster: a case with an isolate belonging to the same 5-SNP single linkage cluster as an isolate from another case where a common source was not determined
- Sporadic: a case with an isolate that did not belong to a 5-SNP single linkage cluster as an isolate from another case.

NZ follows this analysis by prioritising more intensive investigation of cases that are within a 5-SNP cluster as opposed to singletons – those with no other isolate within 5 SNPs.

The different WGS analysis tools used in this project have allowed a high level of discriminatory assessment of NZ isolates and the ability to readily compare genomes internationally. Ongoing WGS based analysis will allow investigation of these observations further.

5. References

Argimón, S.; Abudahab, K.; Goater, R.J.E.; Fedosejev, A.; Bhai, J.; Glasner, C.; Feil, E.J.; Holden, M.T.G.; Yeats, C.A.; Grundmann, H.; *et al*. 2016 Microreact: Visualizing and sharing data for genomic epidemiology and phylogeography. Microb. Genom., 2,e000093

Bielaszewska M, Prager R, Köck R, Mellmann A, Zhang W, Tschäpe H, Tarr PI, Karch H. Shiga toxin gene loss and transfer in vitro and in vivo during enterohemorrhagic Escherichia coli O26 infection in humans. Appl Environ Microbiol. 2007 May;73(10):3144-50. doi: 10.1128/AEM.02937-06. Epub 2007 Mar 30. PMID: 17400784; PMCID: PMC1907125.

Bosilovac, M. STEC: Challenges in Controlling, Detecting and Resolving Contamination. A Webinar hosted by Biomerieux.com, March 2022.

Brooks HJL, Mollison BD, Bettelheim KA, Matejka K, Paterson KA, Ward VK. 2001 Occurrence and virulence factors of non-O157 Shiga toxin-producing *Escherichia coli* in retail meat in Dunedin, New Zealand. Letters in Applied Microbiology, 32, 118-22.

Browne AS. 2018. A food chain approach to control of Shiga toxin-producing *Escherichia coli* in New Zealand: 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.

Chattaway MA, Dallman TJ, Larkin L, Nair S, McCormick J, Mikhail A, Hartman H, Godbole G, Powell D, Day M, Smith R, Grant K. 2019. The transformation of reference microbiology methods and surveillance for *Salmonella* with the use of whole genome sequencing in England and Wales. Frontiers in Public Health Vol 7DOI=10.3389/fpubh.2019.00317

Cookson AL, Taylor SCS, Bennett J, Thomson-Carter F, Attwood GT. 2006 Serotypes and analysis of distribution of Shiga-toxin producing *Escherichia coli* from cattle and sheep in the lower North Island, New Zealand. NZVJ,. 54:2, 78-84.

Dallman TJ, Greig DR, Gharbia SE, Jenkins C. 2021 Phylogenetic structure of Shiga toxin-producing *Escherichia coli* O157:H7 from sub-lineage to SNPs. *Microb Genom*.;7(3):mgen000544. doi:10.1099/mgen.0.000544

Dufour M. 2016. Summary of the data from the 2015 STEC Top-6 and E. coli O157 very young calf season. MPI Report FW 15056

Lake R, Cressey P, Rivas L, Wright J, Anglemyer A, Horn B, Paine S, 2021 Better Undertanding of Foodborne STEC infections. MPI Report FW21011.

Larkin L, Mikhail A. McCormick J. 2017**.** SNP cluster detection and monitoring with Gastro Data Warehouse Guidance Manual. National Gastrointestinal Infections Department National Infection Service Public Health England**.**

Mauro SA, Koudelka GB. 2011. Shiga Toxin: Expression, Distribution, and Its Role in the Environment. *Toxins* 3.6 (2011): 608–625.

Microbiology Laboratory Guidebook 5C. 2019 Detection, Isolation and Identification of Top Seven Shiga Toxin-Producing *Escherichia coli* (STEC) from Meat Products and Carcass and Environmental Sponges. 5C United States Department of Agriculture Food Safety and Inspection Service, Office of Public Health Science,

Microbiology Laboratory Guidebook 5C.01 2021 Detection, Isolation and Identification of Top Seven Shiga Toxin-Producing *Escherichia coli* (STEC) from Meat Products and Carcass and Environmental Sponges. 5C United States Department of Agriculture Food Safety and Inspection Service, Office of Public Health Science,.

Paton AW, and Paton JC. 1998. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx*$_1$, *stx*$_2$, *eaeA*, enterohemorrhagic *E. coli hlyA*, *rfb*$_{O111}$, and *rfb*$_{O157}$. J. Clin. Microbiol. 36**:**598-602.

Seemann, T., Goncalves da Silva, A., Bulach, DM., Schultz, MB., Kwong, JC., Howden, BP. Snippy, rapid bacterial SNP calling and core genome alignments. Availabe online: https://github.com/tseemann/snippy (accessed on 13th December 2020).

Stenutz R, Weintraub A, Widmalm G. 2006. The structures of *Escherichia coli* O-polysaccharide antigens. FEMS Microbiol Rev 30 382–403.

van Hoek AH, Mevius D, Guerra B, Mullany P, Roberts AP, Aarts HJ. 2011 Acquired antibiotic resistance genes: an overview. Front Microbiol.; 2:203. Published 2011 Sep 28. doi:10.3389/fmicb.2011.00203

Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, Karch H, Reeves PR, Maiden M, Ochman, Achtman, M. 2006. Sex and virulence in Escherichia coli: an evolutionary perspective. *Molecular microbiology*, *60*(5), 1136–1151. https://doi.org/10.1111/j.1365-2958.2006.05172.x

Wright J. (2019) Validation of immuno-magnetic beads for Top 7 Shiga toxin-producing *Escherichia coli* confirmation testing MPI Report FW 19007.

Wright J, Gilpin B. 2017 Prevalence of Top 6 and *E. coli* O157 in beef in New Zealand and genetic analysis of *E. coli* O157 2016 MPI Report FW 17024

Wright J, Gilpin B, Draper J, Wang J, Ren X. 2018. Prevalence of Top 6 STEC and *E. coli* O157 in beef in New Zealand and Genetic analysis of *E. coli* O157 2017 MPI Report FW18051.

Wright J, Gilpin B, Ren X, Dupont P 2019 Prevalence of Top 6 and *E. coli* O157 in Beef in New Zealand and Genetic Analysis of *E. coli* O157 2018 MPI Report FW 19025

Wright J, Strydom H, Wang J 2020 Prevalence of Top 6 and *E. coli* O157 in Beef in New Zealand and Genetic Analysis of *E. coli* O157 2019 MPI Report FW20015

Wright J, Strydom H, Wang J, X Ren 2021 Prevalence of Top 6 and *E. coli* O157 in Beef in New Zealand and Genetic Analysis of *E. coli* O157 2020 MPI Report FW21003.

Zhou Z, Alikhan NF, Mohamed K, the Agama Study Group, Achtman M 2020 The EnteroBase user's guide, with case studies on *Salmonella* transmissions, *Yersinia pestis* phylogeny and *Escherichia* core genomic diversity. Genome Res. 30:138-152.