Seminar report

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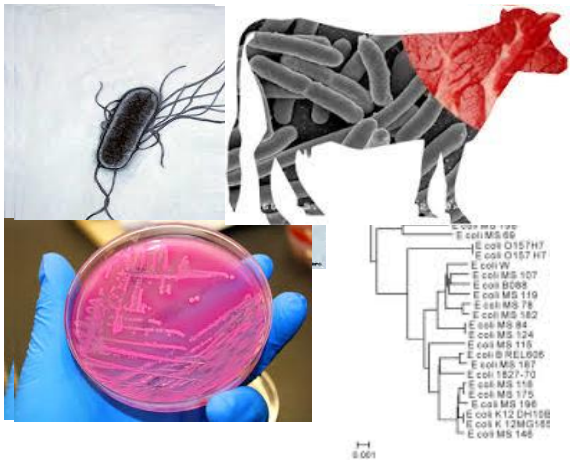

Shiga toxin-producing *Escherichia coli* in manufacturing beef: Where have we been? Where should we be going?

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Abstract

The US Food Safety Inspection Service(FSIS) began testing for the 6 O-types of Shiga toxin-producing *Escherichia coli* (STEC) in June 2012. As a result, the Australian beef export industry began testing product that was subject to FSIS testing at point-of-entry into the USA. These 'Big 6' strains, were additional to *E. coli* O157 that had been subjected to testing since late 2007. The test methods for the Big 6 strains are not as simple, easily performed, or reliable as E. coli O157, and test methods, both rapid methods, and confirmation methods, have developed over the past five years.

Meat & Livestock Australia convened a seminar to review the current position with testing in June 2017, and the proceedings are presented here. The prevalence of STEC are reviewed, the nature of the organisms and detection methods are explained and a recent comparison of test methods is presented. The seminar looks at the testing system that has been implemented in New Zealand, and the direction that STEC testing may take internationally, as the significance of these microbes are reviewed, and further new molecular methods are implemented.

The seminar should provide industry practitioners with information that will help them to make decisions about approaches to testing for their business, and provide the industry an opportunity to consider how to respond to new approaches being implemented based on molecular biology and an understanding of public health implications of STEC.

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1 National STEC testing – what does it tell us?

1.1 Background

Why do we test for Shiga toxin-producing *Escherichia coli* (STEC) in exported beef for grinding?

- The United States Food Safety and Inspection Service (FSIS) declared *E. coli* O157:H7 to be an adulterant in 1994 in raw ground beef and beef components
- FSIS declared six additional serotypes (O26, O45, O103, O111, O121, O145) to be adulterants in 2012
- Canada introduced controls for O157:H7/NM in raw beef products in 2014
- Australia meets these requirements through equivalence arrangements
- The US and Canada require pre-export testing and undertake port-of-entry testing
- Other markets conduct STEC port-of-entry testing (e.g. EU, Japan, Singapore)

What does our STEC testing program involve?

- STEC testing of beef intended for grinding prior to export to Canada or the US
- Each lot tested for O157:H7 and if applicable, non-O157 (subject to HACCP assessment)
- Tested in department approved laboratories using approved methods
- Lots cannot exceed 700 cartons (approx. 19,000 kg)
- N60 sampling (five samples from minimum 12 cartons)
- Tested lot loaded into a single shipping container
- Monthly government verification samples also taken from each establishment exporting to Canada or the US

What happens if STEC is detected in a lot prior to export?

Company testing

- Product in the lot is retained and condemned or subjected to a validated process to achieve five log reduction in *E. coli* (≥69.4˚C for 10 s)
- HACCP reassessment

Government verification testing

- Product in the lot is retained and condemned or subjected to a validated process to achieve five log reduction in *E. coli* (≥69.4˚C for 10 s)
- Investigation and identification of corrective actions
- Follow-up testing
- Actions summarised in the department's Critical Incident Response Guideline

1.2 National STEC data

STEC data in beef for grinding in Australia show that prevalence is low and has decreased in the five years since 2012 (Fig. 1). Observed increases in prevalence over this period were more likely to occur over the summer months, although overall prevalence is low and fluctuations over time may not be

significant. The predominant serotypes detected were *E. coli* O157 and O26, with very few detections of *E. coli* O45, O103, O111, O121 and O145. There are similarities in the prevalence fluctuations of *E. coli* O157 and non-O157 over time (Fig. 1 and 2).

The highest observed prevalence peaks occurred in late 2012 and mid 2013 (Fig. 3). These peaks were mainly attributed to detections in beef from establishments in Victoria (25 detections), Queensland (8) and New South Wales (7). The reasons for peaks in the data are not easy to identify and may be multi-factorial. An analysis of rainfall in Victoria may partially explain the increase in detections in that state in 2012/13. However, Victorian detections decreased from mid-2014 and do not show obvious trends against rainfall data from that time.

STEC prevalence in Australian beef compares favourably with data from the US and Europe (Table 1). *E. coli* O157 prevalence in Australia was 0.18% over the past five years, compared to 0.32% in the US and 0.24% in Europe over similar periods. A similar trend is apparent for prevalence of non-O157 serotypes.

Fig. 1. Confirmed STEC in company tested beef for grinding (2012-16)

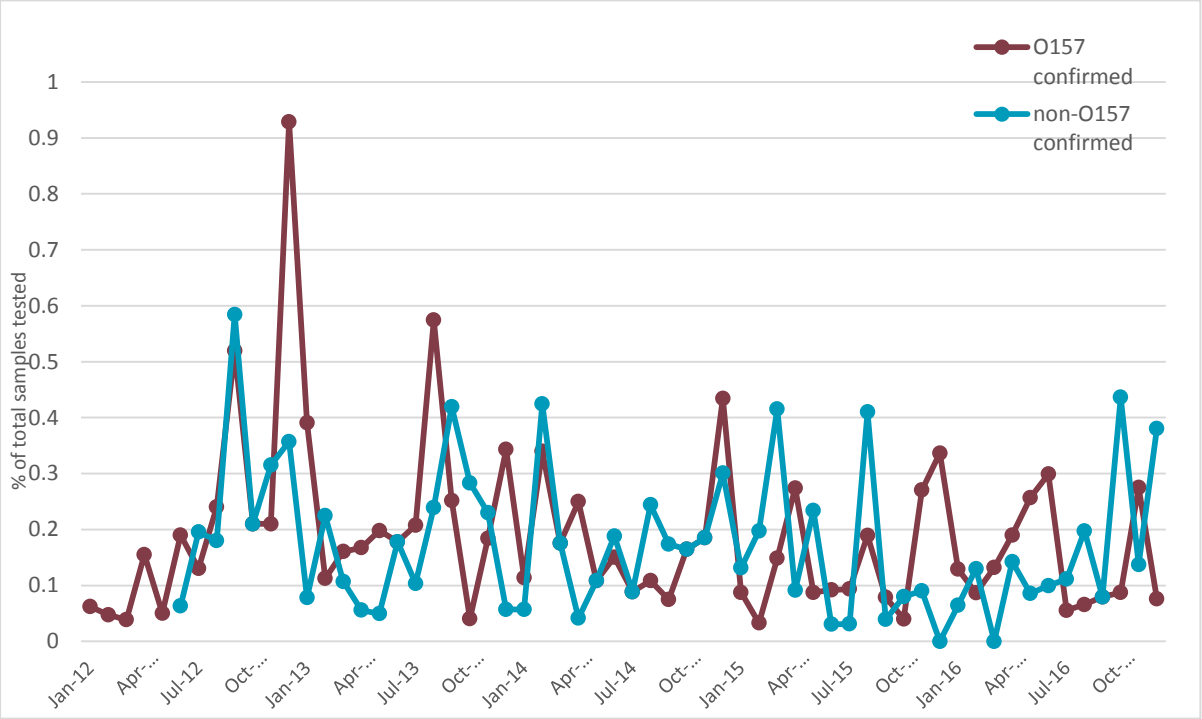


Fig. 2. Confirmed non-O157 serotypes in company tested beef for grinding (2012-16)

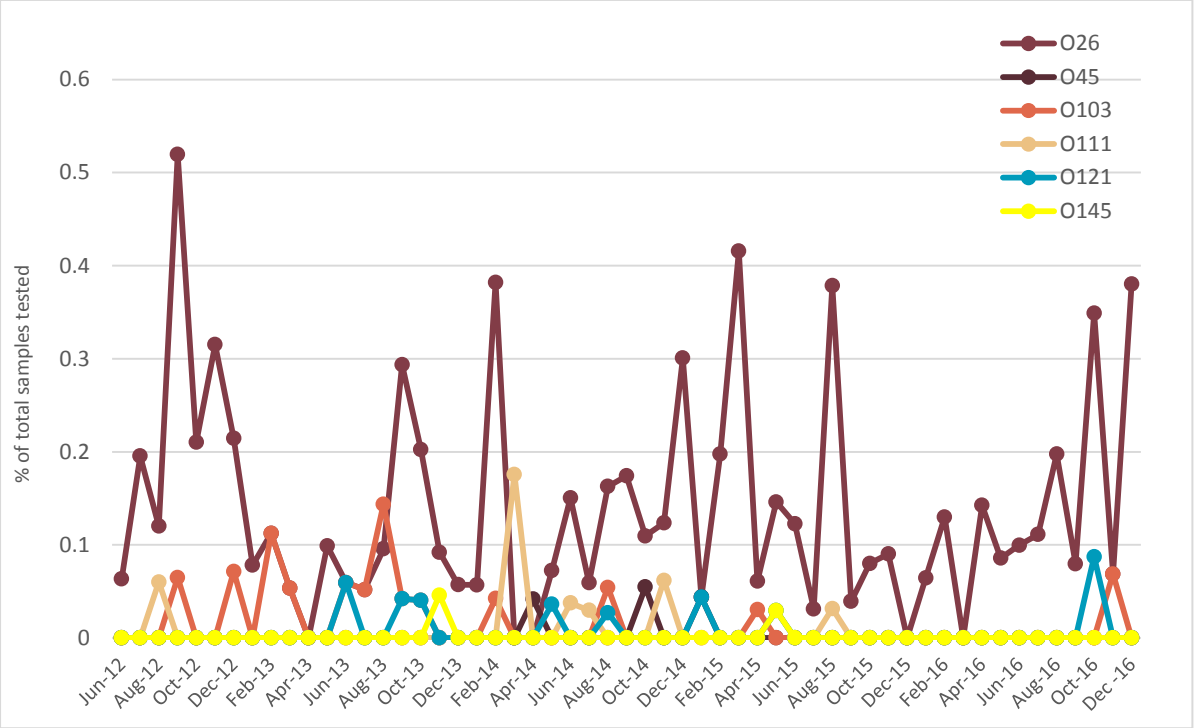


Fig. 3. Confirmed STEC in company tested beef for grinding (2012-16)

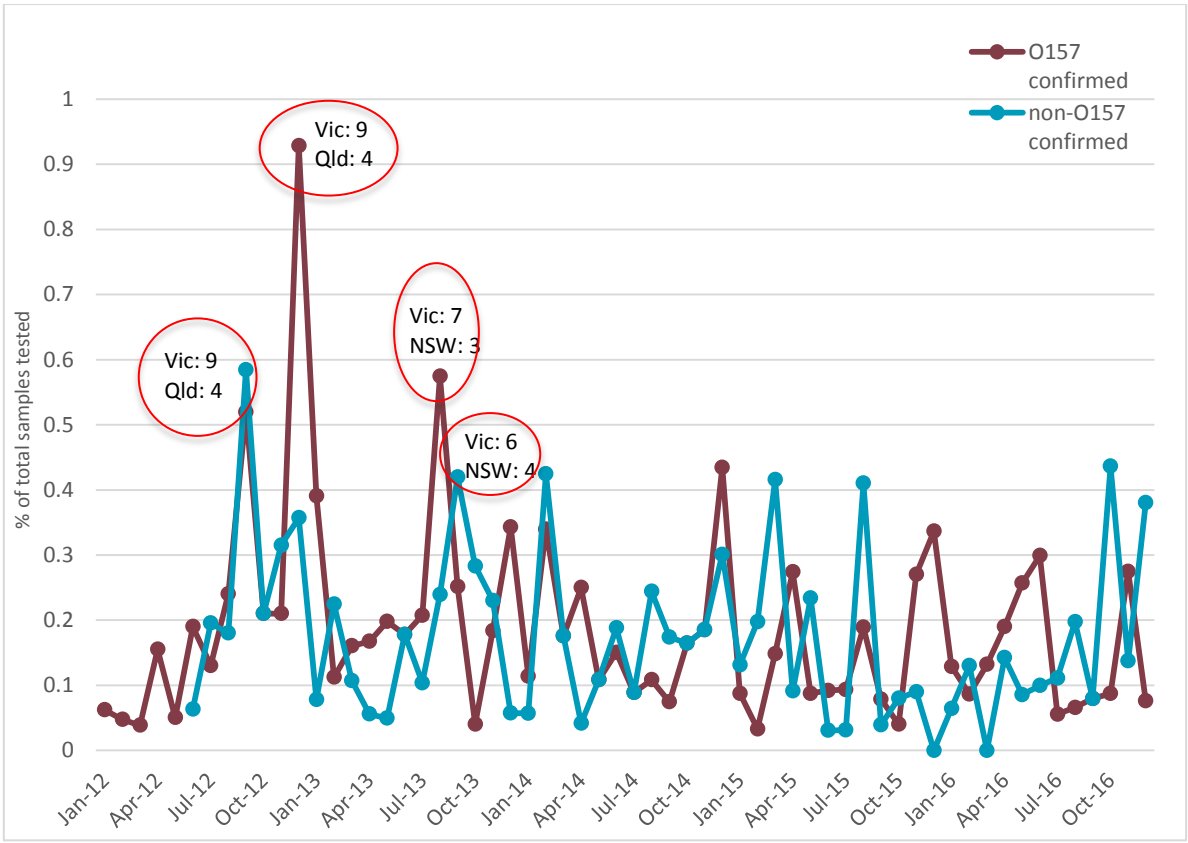


Fig. 4. Confirmed STEC in company tested beef for grinding and monthly rainfall – Victoria (2012-16)

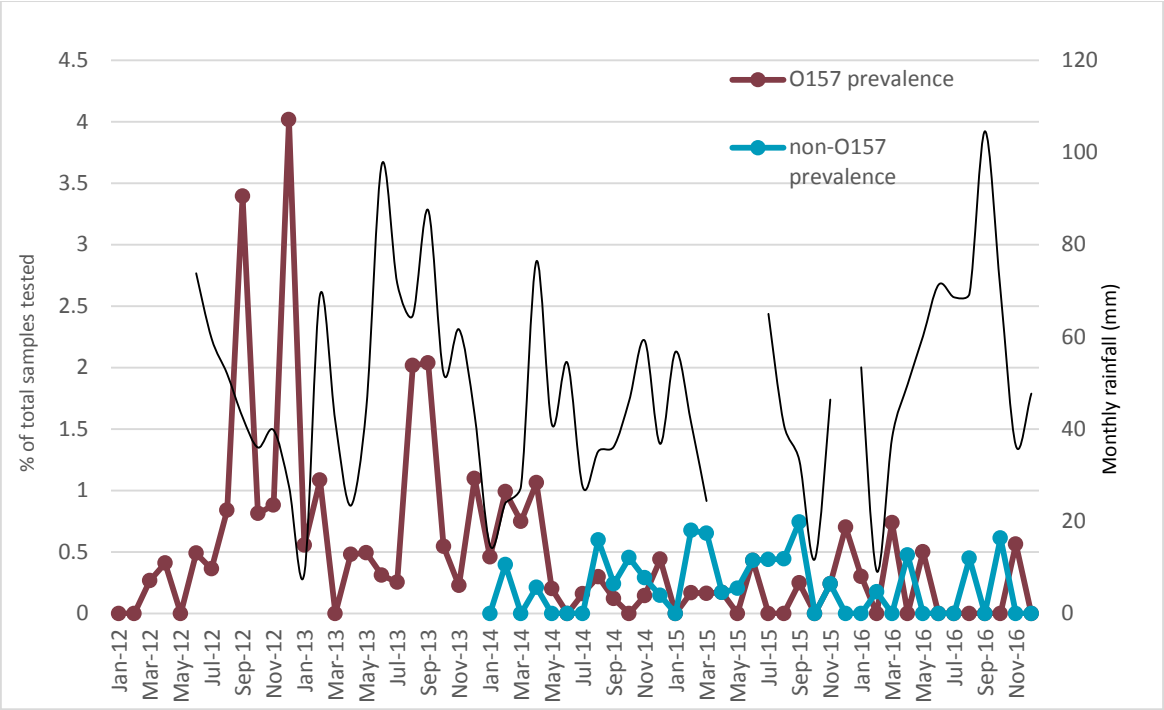


Table 1. STEC prevalence in beef – Australia, USA and Europe

Country	Year	Product	STEC	n	Prevalence (%)	Reference
Australia	2012-16	Trim	O157	136,144	0.18	DAWR
			Non-O157		0.17*	
USA	2012-15	Trim	O157	10,025	0.32	FSIS[1]
			Non-O157		0.74	
Europe	2007-09	Fresh beef	O157	37,998	0.24	EFSA/ECDC[2]
			Non-O157**		0.78	

*Estimate
**O26, O103, O111, O145

[1] Mamber, S.W., Alexander, N., Chen, W.S., McGinn, J., Taylor, T., Manis, L., Jarosh, J., Wong, B., Campbell, T. and Whitaker, R. (2016) *Escherichia coli* O157:H7 and Non-O157 Shiga Toxin-producing *E. coli* (STEC) in Beef Manufacturing Trimmings Samples (MT60 Sampling Project) Analyzed by the Food Safety and Inspection Service from Fiscal Years 2012 to 2015. Poster Presentation at International Association for Food Protection Annual Meeting.

[2] http://ecdc.europa.eu/en/publications/Publications/1106_TER_EColi_joint_EFSA.pdf

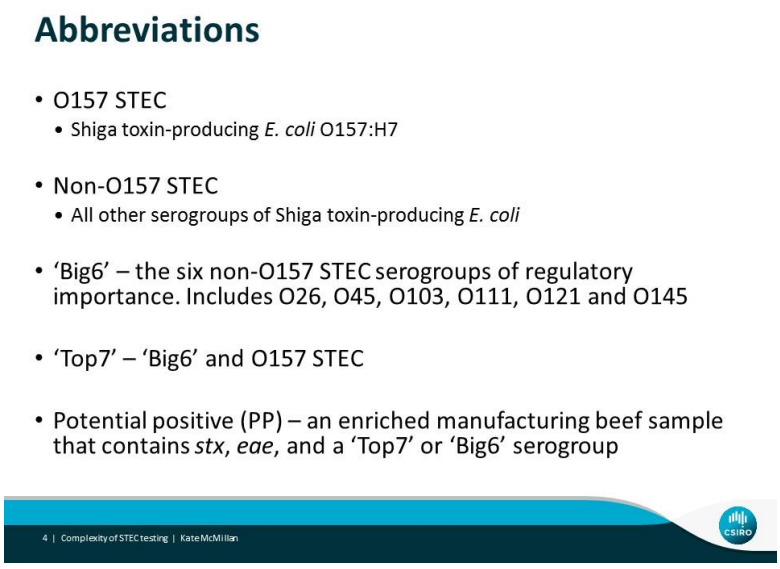
1.3 Conclusions

- STEC prevalence in Australian beef is low and decreasing
- There is some observed association between prevalence of *E. coli* O157 and non-O157 serotypes in Australian beef over the past five years
- It is difficult to find associations between spikes in prevalence and environmental factors given the low prevalence and the fact that the cause may be multi-factorial
- STEC prevalence in Australian beef compares favourably with prevalence in beef from major overseas markets

2 The complexity of STEC testing

2.1 Background

The majority of *E. coli* that humans or animals carry are harmless, however some carry genes that enable them to cause disease. *E. coli* that produce Shiga toxins (stx) are termed Shiga toxigenic *E. coli* (STEC). Some strains of STEC appear to have greater potential to cause human disease than others. This subset includes STEC belonging to certain serogroups (e.g. O157, O26, O111) and have additional virulence mechanisms (e.g. *E. coli* attaching and effacing gene; eae). In 2012, FSIS expanded its regulations from just testing for O157 to include an additional six serogroups, O26, O45, O103, O111, O121 and O145 which are colloquially known as the 'Big6' or non-O157 STEC. Companies exporting beef for grinding to countries with STEC regulations maybe required to conduct pre-export testing for STEC.



2.2 Australian perspective

There are many STEC test systems commercially available. The Australian beef industry typically uses two systems:

- BAX system real-time PCR STEC Suite (Hygiena)
- Assurance GDS MPX STEC assays (BioControl)

Samples that test positive using these systems are classified as 'potential positives' (PP) and subsequently proceed for culture confirmation at a Department of Agriculture and Water Resources approved laboratory.

In Australia, samples that are PP for O157 are more often confirmed than samples that are PP for non-O157 STEC.

2.3 STEC testing is now more complex

Prior to 2012 – testing for O157 only was fairly straight forward

- O157 does not ferment sorbitol so easy to identify on plates
- Most O157 strains are likely to have *stx* and *eae*
- Only looking for one serogroup – easier to detect, isolate and confirm

Post 2012 – testing for O157 and non-O157 STEC

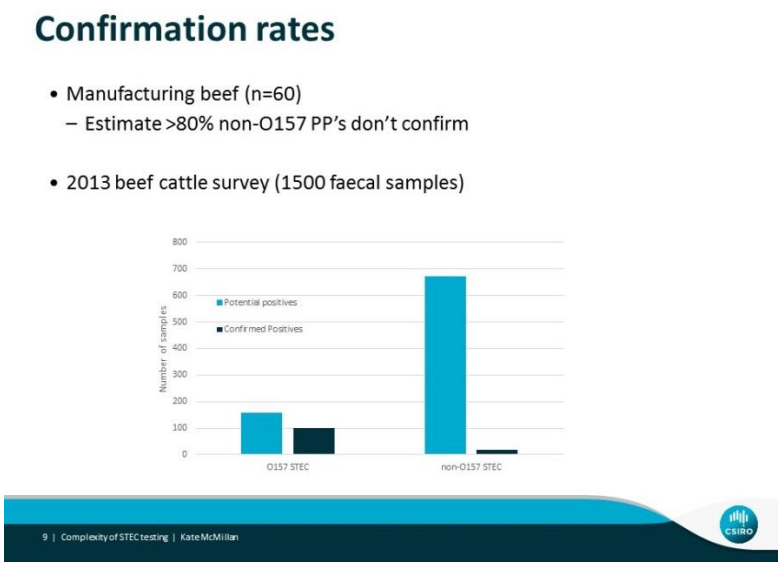
- Non-O157 have no distinguishing features to exploit e.g sorbitol
- Now looking for multiple serogroups not just O157
- Not all strains have *stx* and *eae*
- Very hard to distinguish from harmless *E. coli* during culture confirmation

2.4 Low confirmation rates

Most STEC testing protocols look for *stx*, *eae* and O serogroups. A positive screening test therefore only indicates that these genetic targets are present in the sample, it can't tell us if they are in the same cell or if that cell is an *E. coli*.

A survey of STEC in Australian cattle faeces conducted in 2013 had a low conversion rate of PP to confirmed positives[3]. Of the 1,500 samples tested, 44.5% were PP for non-O157 STEC but only 1.3% were culture confirmed as non-O157 STEC.

[3] https://www.mla.com.au/research-and-development/search-rd-reports/final-report-details/Product-Integrity/Understanding-confirmation-test-failures-for-detecting-pathogenic-E-coli/1167



2.5 Samples that don't culture confirm

Low conversion rates of PP's to confirmed positives can give rise to the following questions:

- Was there an error with the screening test (i.e. a false positive) or
- Did the confirming lab miss the STEC?

The answer to at least the first question is most likely to be no.

PP samples likely contain a variety of *E. coli* that in combination carry *stx*, *eae* and belong to one of the targeted serogroups. Therefore, a PP that does not confirm positive is not necessarily a 'false' positive as the test correctly identified the presence of the right combination of targets. Table 1 shows the variety of *E. coli* possessing STEC markers associated with samples that were PP but did not confirm.

Table 1. STEC virulence marker combinations in *E. coli* recovered from potential positive manufacturing beef enrichment broths (of the broths tested none confirmed positive for the targeted STEC).

***E. coli* isolates with STEC markers**	**Prevalence (n=93)**
STEC (*eae* and *stx*)	0 (0.0%)
stx only	40 (43.0%)
eae only	26 (28.0%)
stx & non-O157 serogroup	2 (2.2%)
eae & non-O157 serogroup	19 (20.4%)
Non-O157 serogroup only	26 (28.0%)

2.6 Conclusions

- STEC screening systems detect genetic markers to identify potential positive samples. They do not tell us if the genetic markers are in the same *E. coli.*
- Conversion rates of PP's to confirmed positives are low for non-O157 PP's as most often the genetic targets identified by the STEC screening systems are present in different isolates of *E. coli.*
- Culture confirmation of non-O157 STEC is a laborious lengthy procedure as it attempts to identify a small group of *E. coli* that appear similar to harmless *E. coli*.

3 Comparison of STEC detection systems

3.1 Background

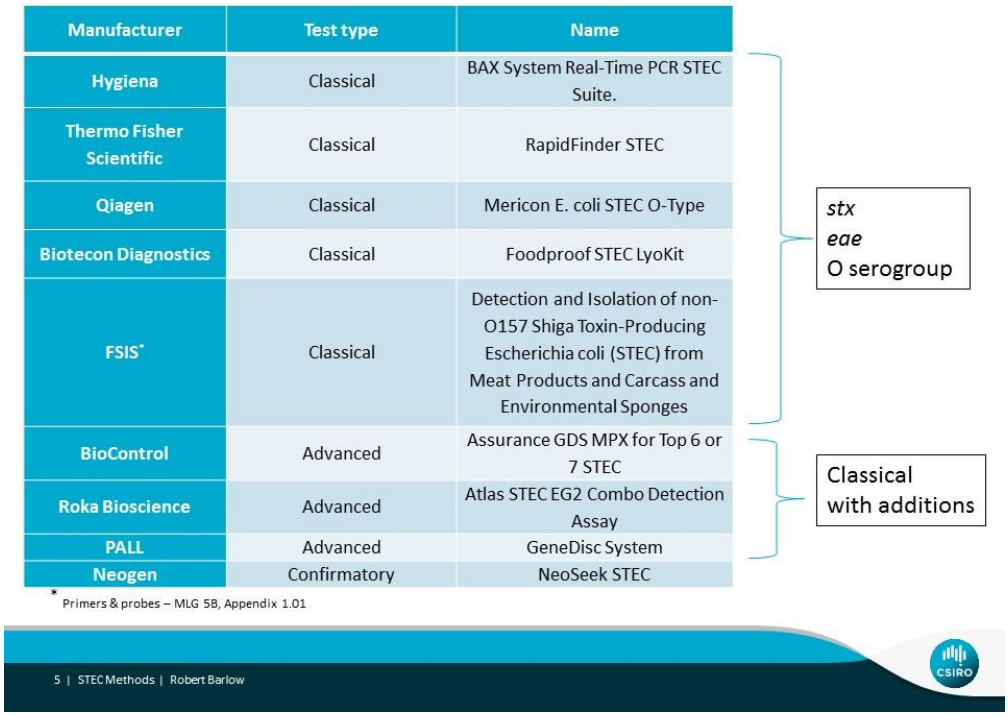
Australian beef exporters have been conducting pre-export testing of manufacturing beef lots destined to the US since the expansion of the STEC regulations in June, 2012. In general, Australian exporters use one of two test systems (BAX or GDS) to initially screen lots for the presence of STEC, with screen positives being subsequently culture confirmed at a Department of Agriculture and Water Resources laboratory. This approach has served the Australian beef industry well and assists in maintaining access into markets, such as the USA, that have regulations relating to the presence of STEC in beef destined for grinding. Our understanding of STEC is increasing due to advances in analytical technologies (genomics). From a STEC testing perspective this has supported the development of more sensitive and specific testing systems. Some of these systems employ detection strategies identifying the three markers commonly used to define STEC (i.e. *stx*, *eae* and O serogroup) whereas other systems are using additional or alternative markers to enhance the specificity of the test system in an attempt to reduce the numbers of PP's that are sent for culture confirmation. Additionally, there are STEC test systems that remove the need for culture confirmation completely by assaying a sample for large numbers of genetic targets that are then aligned with known STEC profiles. Assessing the performance of these systems in an Australian context will enable the effectiveness of currently used systems to be determined and may identify those systems that can reduce the number of PP's without compromising the ability to identify positive lots.

3.2 What does a good test look like?

STEC test systems can be broadly categorised as classical (few targets), advanced (more targets) or confirmatory (lots of targets). Test systems with most value to the industry are those that are able to reduce the number of PP samples while still able to identify samples that actually contain STEC.

3.3 Study design

- 100 manufacturing beef enrichment samples that were PP for non-O157 STEC
- Tested using the following STEC screening systems:
  - BAX system real-time PCR STEC suite (Hygiena)
  - RapidFinder STEC (ThermoFisher Scientific)
  - Mericon *E. coli* STEC O-type (Qiagen)
  - Foodproof STEC Lyokit (Biotecon Diagnostics)
  - Non-O157 STEC from meat products (FSIS)
  - Assurance GDS MPX (BioControl)
  - Atlas STEC EG2 combo detection assay (Roka Bioscience)
  - GeneDisc system (PALL)
- Tested using the following STEC confirmation system
  - NeoSeek STEC (Neogen)
- Performance measured by:
  - Ability to detect samples that were culture confirmed, and
  - Total number of PP's



Manufacturer	Test type	Name
Hygiena	Classical	BAX System Real-Time PCR STEC Suite.
Thermo Fisher Scientific	Classical	RapidFinder STEC
Qiagen	Classical	Mericon E. coli STEC O-Type
Biotecon Diagnostics	Classical	Foodproof STEC LyoKit
FSIS*	Classical	Detection and Isolation of non-O157 Shiga Toxin-Producing Escherichia coli (STEC) from Meat Products and Carcass and Environmental Sponges
BioControl	Advanced	Assurance GDS MPX for Top 6 or 7 STEC
Roka Bioscience	Advanced	Atlas STEC EG2 Combo Detection Assay
PALL	Advanced	GeneDisc System
Neogen	Confirmatory	NeoSeek STEC

3.4 Results

- 100 non-O157 PP samples collected between July 2016 and January 2017 – 61 generated by BAX and 39 by GDS
- 12 samples culture confirmed as O26

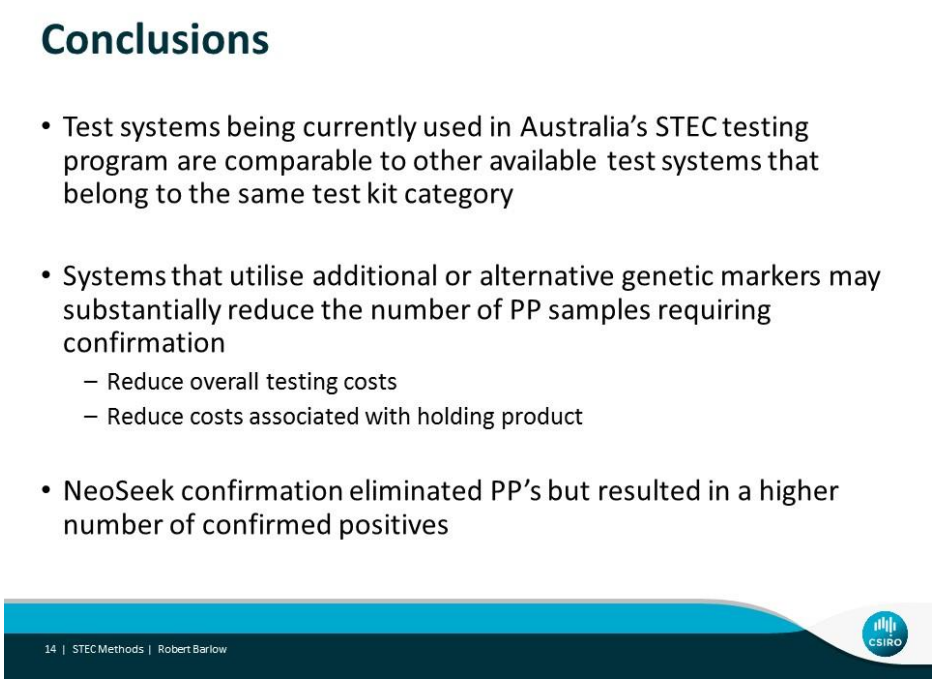
- The majority of STEC screening systems detected 11 of the 12 culture confirmed samples, the exception being GDS which detected 10 of the 12 confirmed samples. The Qiagen and PALL systems detected all confirmed samples for which they generated a test result (Table 1). All systems detected 10 of the 12 culture confirmed samples with variable results obtained for the remaining two culture confirmed samples.
- The advanced test systems of Roka, GDS, and PALL target additional or alternative genetic markers during screening. The use of these systems reduced the number of PP's without affecting the ability to detect culture confirmed samples (Table 1).
- This study used enrichments broths recommended by the GDS or BAX test systems. When comparing performance of test systems in this study it is necessary to consider:
    - Recommended enrichment media were not used for all tests
    - Recommended enrichment protocols were not used for all tests
    - Enrichment broths may change over time affecting what can be detected
- NeoSeek STEC was the only non-culture confirmation method evaluated. Using NeoSeek 16 samples were identified as positive for non-O157 STEC, this included 11 of the 12 culture confirmed samples.

Table 1. Detection of culture confirmed positives and overall positives by STEC test systems.

Test system	Test category	Non-O157 confirmed positives detected	Positives
FSIS	Classical	11/12	85
QIAGEN	Classical	12/12	82
BAX	Classical	11/12	67
RAPIDFINDER	Classical	11/12	64
BIOTECON	Classical	11/12	64
GDS	Advanced	10/12	56
PALL	Advanced	10/10*	42/94*
ROKA	Advanced	11/12	39
NEOSEEK	Confirmatory	11/12	16

* A software malfunction resulted in no result being generated for six samples, two of which culture confirmed.

3.5 Conclusions



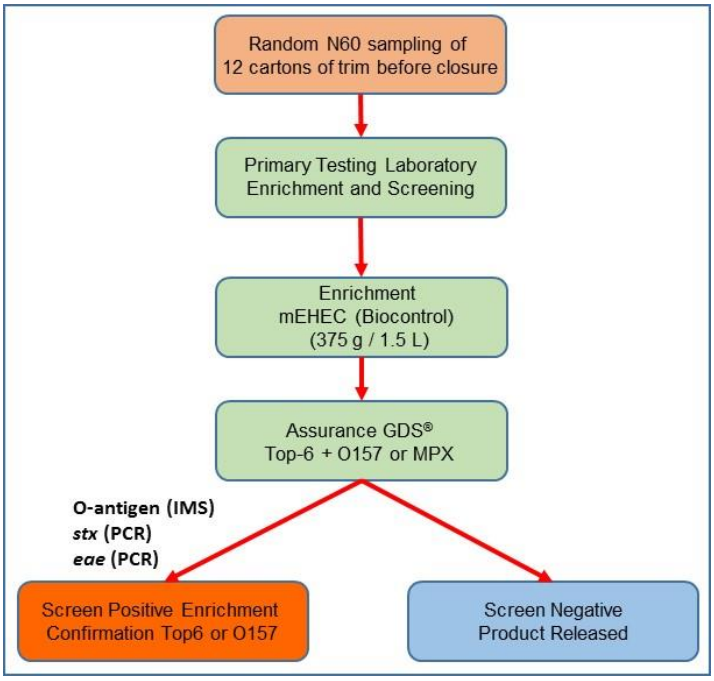
4 New Zealand STEC Monitoring Programme

4.1 Summary

- New Zealand has a "test and hold" STEC monitoring programme for all manufacturing grade beef (both adult and veal) exported to the US for use in non-intact products.
- Product export certification for the US requires valid STEC test results from laboratories approved under the MPI Recognized Laboratory Programme (RLP).
- All stages of the testing programme are regulated and monitored by MPI
- A random N60 sample (5 pieces of surface meat from 12 cartons) is taken per lot.
    - This is generally equivalent to a day's production
    - Carried out by certified samplers
    - Laboratories responsible for, and audit, sampling procedures
- Screen testing is carried out by six IANZ accredited (ISO17025) laboratories located throughout New Zealand.
- Currently the only screen method approved for use is Assurance GDS® (Biocontrol)
    - Validated for O157:H7 initially in 2009; validated for Top6 (O26, O103, O111, O121, O45, O145) in 2012
- Confirmation up until 2015 was by culture (FSIS- MLG5B.05)
    - From 2016
        - adult beef is by culture (FSIS- MLG5B.05)
        - young veal is by molecular (NeoSEEK)
- US product disposition is based on the presence or absence of Top7 STEC

4.2 Screening procedure

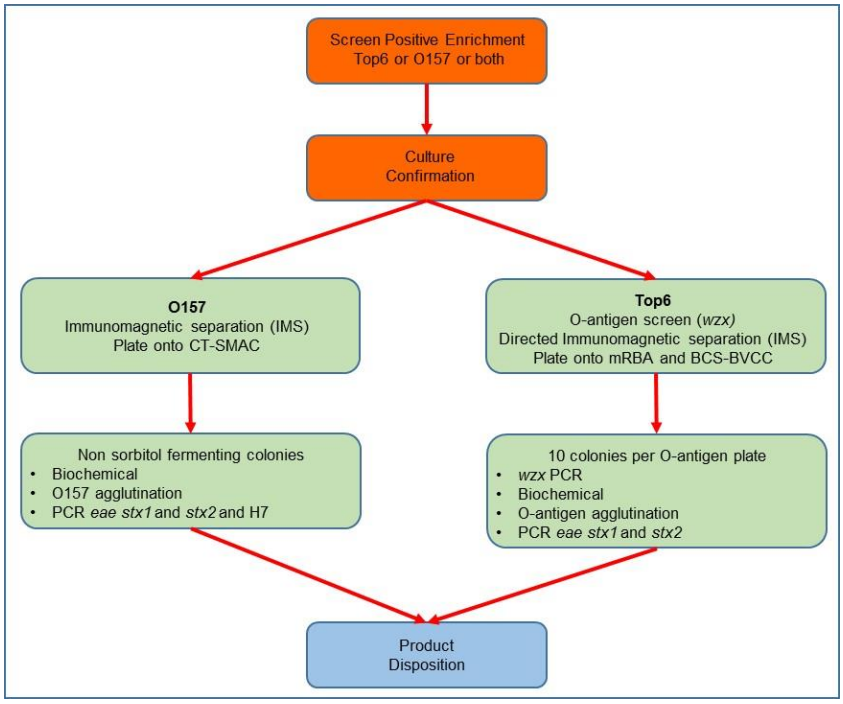
- All samples are screened for the presence of STEC O157 or Top6



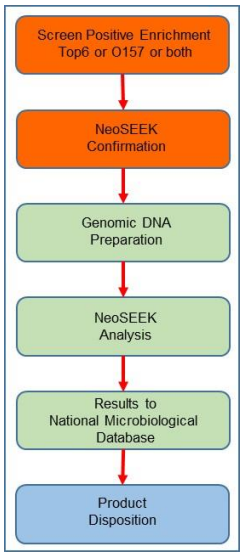
4.3 Confirmation procedure

- Samples that are positive for both Top6 and STEC O157 must go through confirmation for both
- For Top6 – only one positive isolate is required from any of the 6 serotypes

<u>Adult beef</u>

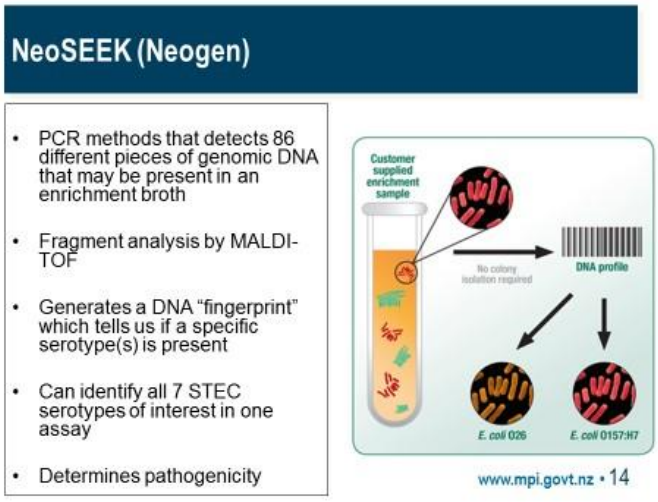


Veal



4.4 NeoSEEK Analysis

- NeoSEEK is a molecular STEC confirmation method, (GeneSEEK, Neogen Corporation)
- Has FSIS NOL and has A2LA (ISO17025:2005) accreditation
- Has been validated for use on enrichment broths containing meat
- Further extensively validated (and updated) for use in New Zealand



- Output is in the form of a table that indicates (Figure 5)
  - STEC – toxigenic bacteria with specific O-antigen present
  - NON - O-antigen bacteria present but not toxigenic/pathogenic
  - Blank – no bacteria of that serotype present

NeoSeek STEC						non		Eae+	Stx+
NeoSeek STEC			non	STEC		non	non	Eae+	Stx+
NeoSeek STEC	non								

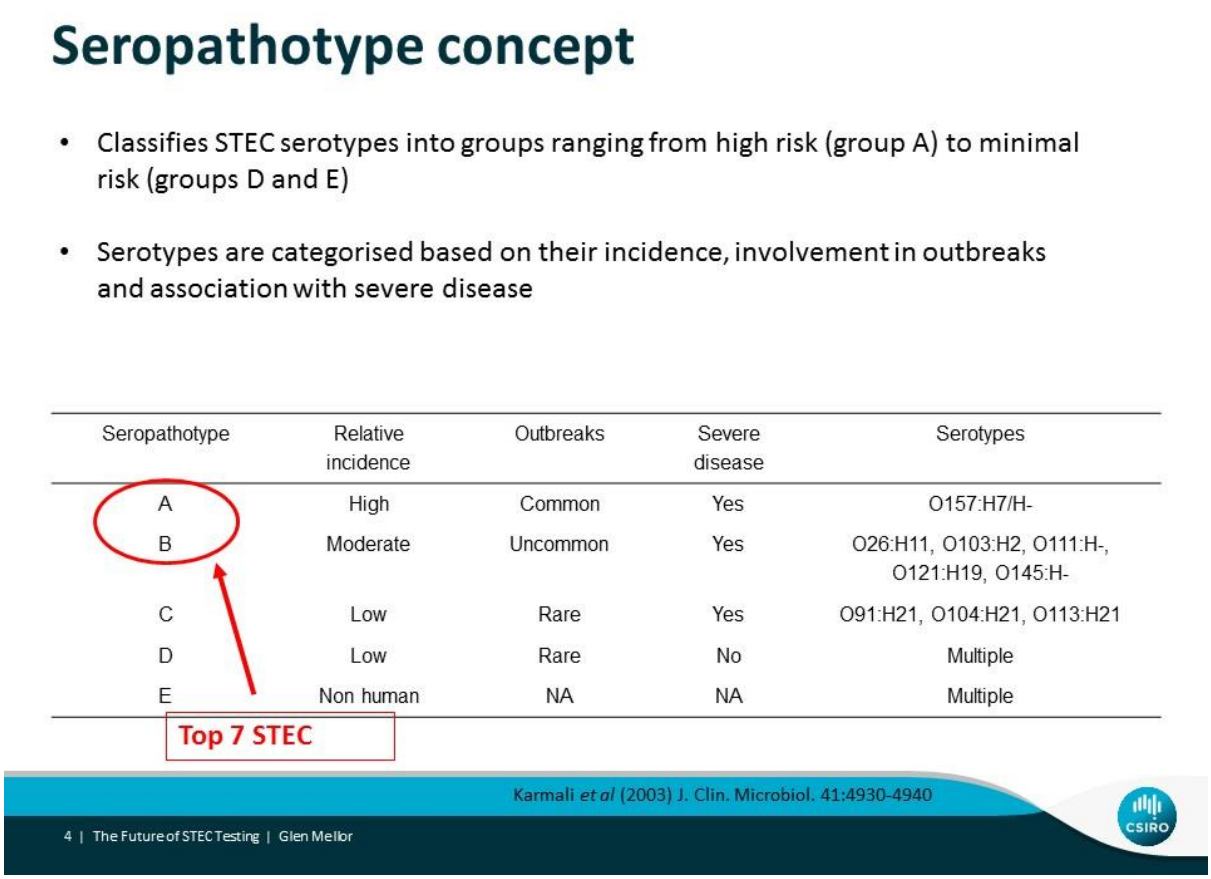
- Significant advantages for meat industry
  - Time from sampling to product disposition
  - Cost of Compliance decreased
  - Significant cost savings in production
- Significant advantages for MPI
  - Increased product assurance
  - Alignment of screen and confirmation
  - Improved specificity and sensitivity
  - Future proof technologies

5 The future of STEC testing

5.1 Current concept

The addition of the non-O157 serogroups to the STEC testing program in 2012 was a response to human illness data that demonstrated that these serogroups were responsible for the majority of non-O157 STEC related disease. Human illness data from the USA in 2013 supported the regulatory response with 48.5% of STEC-associated illness attributable to O157 and 44.6% attributable to the non-O157 serogroups. Identification of the specific serogroups for inclusion in the STEC testing

program followed on from an earlier classification concept known as the seropathotype concept, where serogroups are categorised based on their incidence, involvement in outbreaks and association with disease. The current STEC regulations assume that all STEC belonging to a particular serogroup have the same disease causing potential. However, there is evidence to suggest that within serogroups STEC may have differing ability to cause severe human disease.



5.2 Not all STEC are equal

The advent of genomic sequencing is enabling relationships between STEC to be further understood. For example, by analysing small variations in the genetic composition of O157 isolates they can be grouped into very specific groups or Clades. Some of these groups correlate highly with human disease and outbreaks (hypervirulent) and others do not. Indeed, some groups of isolates appear unlikely to cause disease in humans[4]. The genetic differences between isolates that are highly associated with human disease and those that aren't can be defined and tested for.

[4] https://www.mla.com.au/research-and-development/search-rd-reports/final-report-details/Product-Integrity/E-coli-subtyping-data-collection/106

5.3 Molecular risk assessment?

The disease potential that a STEC has is governed by the virulence genes it carries and not by its serogroup. Molecular risk assessment has evolved as our understanding of exactly what is required to cause severe human disease has increased. That is, defining risk based on the presence of genetic markers and not on a STEC's affiliation with a particular serogroup as was previously the case.

5.4 Future testing systems

- NeoSeek – highly adaptable i.e. capable of rapidly integrating new genetic targets. Measures PCR amplicon size based on mass therefore avoiding the issues of using probes as in real-time PCR applications.
- Droplet digital PCR – partitions the samples into 1000's of droplets (single cells) and tests each droplet for genetic targets. Would allow genetic targets to be linked i.e. have confidence that *stx* and *eae* are in the same *E. coli.*
- Desktop sequencers – USB connected device that is rapid and requires minimal hands-on effort. Suitable for analysing 100's to 1000's of genes.

5.5 Conclusions

- Comparisons of STEC that cause human disease with those that generally do not allows us to identify the genetic factors that contribute most to human disease.
- Categorising STEC based on molecular risk will likely see a shift away from serogroup focused testing.
- Future testing platforms will increase the speed of testing primarily by removing the need for culture confirmation. Reductions in the costs of sequencing systems and the simplification of conducting these tests will aid the integration of future test systems into food production businesses.

6 Future typing methods – here now

6.1 Background

Technological and computational advances in the sequencing of DNA has transformed most of the biological sciences, particularly microbiology. Since the first commercial next generation sequencing (NGS) equipment became available (~2007), whole genome sequencing (WGS) has become a standard application in most microbial research laboratories. These advances have not been limited to the realm of research, NGS is rapidly becoming the "gold standard" technology for public health and food regulatory agencies around the world. The recent proliferation in the use of WGS for typing bacterial pathogens involved in food borne disease outbreaks in the USA, Canada, Europe and the UK indicates that it will become the standard technology for disease investigation globally.

6.2 Applications of NGS technology

- Tracking and identification of bacterial isolates using techniques like:
    - Single nucleotide polymorphism (SNP) analysis – in which every difference between the isolate strain and a reference strain are determined
    - Whole genome multi-locus sequence typing (wgMLST) – in which a reference database of gene types across 1000's of genes is established and all isolates are scored against these references. NOTE – June 8, 2017 PulseNet published a review paper suggesting that wgMLST is their preferred method to replace PFGE (Eurosurveillance Vol. 22, Issue 23, 2017)
- Predict functions e.g., antimicrobial resistance
- Analyse large microbial community (determine who is there without culturing)
- Numerous other applications + research tools

6.3 Industry adoption

This technology will replace commonly used methods such as Pulsed Field Gel Electrophoresis (PFGE), Multi Locus Sequence Typing (MLST) and Multi Locus Variable number tandem repeat Analysis (MLVA). The adoption of NGS based methods to the typing and testing of foodborne microbes is certain, only the extent of the disruption to current testing regimes and regulations remains to be determined. The Australian Red Meat Industry will need to be aware of the potential issues and benefits that the adoption of a new technology will bring.

6.3.1 Issues caused by NGS/WGS

- The end of serotyping
    - Classification systems will need to be revamped
    - New regulations will need to be discussed
- The end of PFGE – PulseNet
    - Now transitioning to WGS
    - Higher level of discrimination with WGS
        - New definitions of "relatedness"
        - Better understanding of the biogeographic variability
- New standards
    - New regulation, accreditation and standards needed
        - Laboratory data generation
        - Computational analysis (statistical, bioinformatic, phylogenetic)

6.3.2 Local Issues

- Australia lags behind the US, Canada, Europe on NGS implementation
- Limited baseline data for Australian food pathogens – may impact assumptions on isolate origin

- Date handling and availability
    - Who will access the data
    - How/where will it be stored (off-shore cloud?)
- WGS methods are moving forward for health applications – will the food sector have a voice in what is developed?
-

6.3.3 Benefits of WGS / NGS

- More certainty on accuracy of source tracking / typing data
    - False positive PFGE should end
    - Regional differences likely to be detectable
- Faster identifications and analysis
    - Sequence data can be transported electronically
    - Analysis can be automated
- Early detection of emerging food-borne pathogens

6.3.4 Cost to industry

- The cost to industry in 2016 on confirmed STEC positives lots sent for heat treatment is estimated to be more than $1.3 million for the raw material only. It does not include other cost factors such as cost of production and additional labour for diverting of positive lots.

6.4 Next steps

NGS based methods represent the next logical step in the development of typing methodologies. Initially, typing was dominated by culture based methods that examined biochemical or physiological characteristics. This was followed by methods such as serotyping that examined the nature of important surface molecules on the cells. Then methods that used the genetic composition of the cells for typing were deployed such as PFGE, MLVA, and MLST. Technological changes have simply permitted a greater quantity of genetic information to be examined; so the current NGS based methods can be equated to an extremely high resolution version of PFGE. Although the research community has a myriad of applications for NGS, the public health community appears to be adopting a slow and steady approach toward applying NGS to the development of extremely accurate typing systems. Coincident with this, several older technologies such as PFGE will no longer be used. Methods such as serotyping will cease to be used in the very near future and necessitate some significant changes in the way microbes are typed. This will in turn lead to the requirement for some sweeping changes to regulations and standards.