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Understanding confirmation test failures for detecting pathogenic *E. coli*

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Abstract

FSIS currently declares seven serotypes of STEC as adulterants of raw non-intact beef products and product components in the USA. Collectively these STEC serotypes are referred to as pathogenic STEC (pSTEC) and Australian beef producers exporting to the USA must demonstrate their product has tested free of pSTEC prior to export. Testing for pSTEC typically relies on the molecular detection of three genetic markers; *stx*, *eae*, and a pSTEC O serotype. However, initial screening does not determine if a single organism (i.e pSTEC) is carrying all three genetic markers and attempts are made to isolate pSTEC during routine confirmation. Substantial differences between the pSTEC potential positive rate and the pSTEC confirmation rate have been observed and this study has attempted to identify reasons as to why this occurs. A total of 127 enrichment broths comprising 98 Big6 (*E. coli* O26, O45, O103, O111, O121, O145) and 29 *E. coli* O157 potential positives were analysed using immunomagnetic separation and colony hybridisation targeting *E. coli* that harbor *stx*, *eae*, or are of a pSTEC serotype. STEC, *eae*-containing *E. coli*, and *E. coli* of a pSTEC serotype that lack *stx* and *eae* were found in 40.8, 27.5 and 27.5% of Big6 samples and 20.7, 13.8 and 3.4% of *E.* coli O157 samples, respectively. It was common for samples to contain multiple isolates of *E. coli* harboring different genetic markers that would be detected during screening and which could account for the potential positive status of that sample. Whilst their presence alone doesn't indicate the absence of pSTEC, the data indicates that organisms other than pSTEC are likely to contribute significantly to the generation of potential positive samples during screening. These combinations may give rise to an elevated potential positive rate, increase the difference between potential positive and confirmed positive rates, and reduce the perceived effectiveness of currently established confirmation protocols. Whilst ongoing refinement of confirmation protocols is required to ensure maximum isolation rates are achieved from samples containing pSTEC, a focus should remain on identifying genetic or phenotypic differences of pSTEC that can be exploited during the screening process.

Executive Summary

The United States of America is Australia's second largest beef export market with shipments in 2011 reaching 167,820 tonnes at an estimated value of $774 million. Manufacturing beef or beef trim comprises a substantial proportion of beef exports and it is an export requirement that product is tested for *E. coli* O157 prior to being sent to the USA. As of June 2012 this requirement extended to an additional six serotypes of pathogenic STEC (pSTEC). One aspect of the pSTEC testing process that has come under additional scrutiny in recent times is the ratio of potential positives (i.e screening test positive) to confirmed positives. There is some concern that failure to convert potential positives into confirmed positives may result in contaminated product leaving Australia for export markets and this could result in a positive point of entry (POE) detection in the USA. Central to understanding the low conversion rate of potential positives to confirmed positives is an understanding of the power of the screening tests used for pSTEC. Current testing protocols analyse samples for up to three gene targets (*stx*, *eae*, and O serotype). The inherent flaw in current testing protocols is the assumption that if the gene targets of interest are present then they are likely to reside within the same organism. Previous studies have routinely demonstrated that beef samples can contain *E. coli* that harbor any combination of the genetic targets currently used to define pSTEC. It was demonstrated that a combinatorial approach to the detection of pSTEC did not result in the detection and isolation of pSTEC and isolates harbouring *stx* only or *eae* only were more likely to be isolated from beef sample enrichments that had tested positive for the gene targets consistent with pSTEC. Nevertheless, there is a lack of information on the combinations of genes and organisms present in potentially positive manufacturing beef samples. Such data would fill the knowledge gap that exists in relation to pSTEC testing and will more accurately quantify the percentage of potential positive samples that are sent for confirmation despite the enrichment having never contained pSTEC.

A total of 127 enrichment broths comprising 98 Big6 (*E. coli* O26, O45, O103, O111, O121, O145) and 29 *E. coli* O157 potential positives were analysed using PCR, immunomagnetic separation and colony hybridisation targeting *E. coli* that harbor *stx*, *eae*, or are of a pSTEC serotype. Potential positive samples were re-screened for the presence of *stx*, *eae* and the seven pSTEC serotypes. Detection of *stx* and *eae* occurred in 75 (77%) of 98 Big6 potential positives and in 12 (41%) of 29 *E. coli* O157 potential positives. Detection of at least one O serotype occurred in 55 (56%) of 98 Big6 potential positives and O157 was detected in just five (17%) of 29 *E. coli* O157 potential positives. When combined with the *stx* and *eae* results only 46 (47%) of 98 Big6 enrichment broths and two (7%) of 29 *E. coli* O157 enrichment broths met the criteria of a potential positive. Previous studies have noted a lack of agreement between different pSTEC test methods. In this study, the inability to detect pSTEC O serotypes was the most common factor affecting agreement between methods. Additional investigations are required to determine if the specific attributes of test systems, the relative competencies of laboratory staff, or the uniqueness of the microflora within manufacturing beef samples effect screening test results.

Attempts were made to isolate *E. coli* possessing any combination of *stx*, *eae*, and pSTEC O serotypes from all potential positive broths. At least one organism harboring any combination of *stx*, *eae*, or

pSTEC O serotype was isolated from 78 (80%) of 98 Big6 enrichment broths and from 12 (41%) of *E. coli* O157 enrichment broths. pSTEC were isolated from five (5.1%) of the 98 Big6 and one of the 29 *E. coli* O157 enrichments broths analysed. STEC, *eae*-containing *E. coli*, and *E. coli* of a pSTEC serotype that lack *stx* and *eae* were found in 40.8, 27.5 and 27.5% of Big6 samples and 20.7, 13.8 and 16.7% of *E.* coli O157 samples, respectively. A further breakdown of the combinations of genetic markers present in each of the target organisms reveals that the majority of markers reside alone in different *E. coli* isolates. The exception to this was the combination of *eae* with a pSTEC serotype which was isolated from 19.4% of Big6 and 13.8% of *E. coli* O157 enrichment broths. Multiple variations of a target organism type were identified within individual samples. One particular Big6 enrichment broth yielded a STEC, an *eae*-containing *E. coli*, an *E. coli* O145 isolate, an *E. coli* O103 isolate, and an *E. coli* O145 harboring *eae*. In general, it was common for samples to contain multiple isolates of *E. coli* harboring different genetic markers that would be detected during screening and which could account for the potential positive status of that sample.

The findings of this study adequately describe the difficulty facing companies and commercial laboratories that are using current methodologies for the detection and isolation of PSTEC from manufacturing beef samples. Whilst the presence of *E. coli* harboring combinations of pSTEC markers doesn't specifically indicate the absence of pSTEC, the data indicates that organisms other than pSTEC are likely to contribute significantly to the generation of potential positive samples during screening. These combinations may give rise to an elevated potential positive rate, increase the difference between potential positive and confirmed positive rates, and reduce the perceived effectiveness of currently established confirmation protocols. Whilst ongoing refinement of confirmation protocols is required to ensure maximum isolation rates are achieved from samples containing pSTEC, a focus should remain on identifying genetic differences of pSTEC that can be exploited during the screening process.

Contents

Project objectives

Investigate whether or not pathogenic *E. coli* are present in samples that test positive by screening methods and in turn will determine possible reasons for the failure to isolate pathogenic *E. coli* through routine testing. The direct outcomes of the project will include:

- An estimation of the number of confirmed pSTEC samples likely to be obtained from potential positive pSTEC samples
- An understanding of the range and combinations of key virulence markers in bacteria from potential positive pSTEC samples
- A comparison of the efficiency and appropriateness of testing strategies for pathogenic *E. coli*
- Generate data that can be used by industry (MLA, AMPC) and DAFF in discussions with trade partners relating to perceived failures in *E. coli* O157 or pSTEC confirmation

Milestone 3 is the final report for project G.FMS.0282. The data and conclusions of the report will assist in meeting the above mentioned objectives and will assist industry stakeholders by providing relevant information on which they can assess the effectiveness of their current testing strategies.

Success in achieving milestone

Collection and analysis of 98 Big6 and 29 *E. coli* O157 potential positive enrichment broths has been conducted and the results and conclusions are described below. The project aim of analysing 100 Big6 and 100 *E. coli* O157 potential positive enrichment broths was not achieved with the shortfall in *E. coli* O157 samples likely to be related to the enhanced conversion rate from potential positive to confirmed positive that these samples readily achieve in comparison to Big6 samples. Notwithstanding, the data and conclusions outlined in this report reinforce the challenge facing companies and commercial laboratories that are using current methodologies for the detection and isolation of pSTEC from manufacturing beef samples. It also confirms that the poor conversion rate from potential positive to confirmed positive is most likely to due not to the presence of a pSTEC isolate in the test broth but to the presence of multiple *E. coli* strains that each harbor the virulence markers on which the initial screening is based. This report is expected to satisfy the achievement criteria of Milestone 3 of G.MFS.0282 with an expectation that summary reports relating to the complimentary research being conducted as part of ongoing collaborations are to be provided to MLA by June 30, 2014.

Introduction

The United States of America is Australia's second largest beef export market with shipments in 2011 reaching 167,820 tonnes at an estimated value of $774 million. Manufacturing beef or beef trim comprises a substantial proportion of beef exports and it is an export requirement that product is tested for *E. coli* O157 prior to being sent to the USA. As of June 2012 this requirement extended to an additional six serotypes of pathogenic STEC (pSTEC). One aspect of the pSTEC testing process that has come under additional scrutiny in recent times is the ratio of potential positives (i.e screening test positive) to presumptive and/or confirmed positives. Samples that test positive during a screening test but subsequently fail to yield a confirmed isolate are called negative and able to be exported. There is some concern that failure to convert potential positives into confirmed positives may result in contaminated product leaving Australia for export markets and this could result in a positive point of entry (POE) detection in the USA. Several POE detections have already occurred and the current Australian red meat industry process of testing and confirming pSTEC has been questioned by the US Food Safety and Inspection Service who operate the POE testing program. A recent baseline survey on the prevalence of pSTEC in manufacturing beef concluded that the conversion rate of potential positives to confirmed positive is also low and similar concerns about Australian beef testing positive at POE for pSTEC are likely if data relating to why samples aren't confirmed as positive are not generated.

Central to understanding the low conversion rate of potential positives to confirmed positives is an understanding of the power (or lack thereof) of the screening tests used for pSTEC. Tests are conducted that identify one or more targets either through antigen/antibody reactions or via direct gene detection. In general terms, testing for pSTEC requires samples to be tested for up to three gene targets. The inherent flaw in current testing protocols is the assumption that if the gene targets of interest are present then they are likely to reside within the same organism. Indeed it would appear that the opposite situation is likely to exist in the majority of samples whereby the gene targets of interest, although all present in a single enrichment broth, reside in separate organisms. The impact of such a scenario on the conversion rate of potential positives to confirmed positives is unknown and should be immediately addressed in order to provide scientific evidence for confirmation test failures for pSTEC. Failure to adequately investigate this area could leave the Australian meat industry open to additional sampling measures from FSIS in order to address the perceived shortfall in the conversion rate for Australian manufacturing beef samples and any changes to the rate of POE test positives.

Previous MLA/CSIRO funded projects have routinely demonstrated that beef samples can contain *E. coli* that harbor any combination of the genetic targets currently used to define pSTEC. It was demonstrated that a combinatorial approach to the detection of pSTEC did not result in the detection and isolation of pSTEC and isolates harbouring *stx* only or *eae* only were more likely to be isolated from beef sample enrichments that had tested positive for the gene targets consistent with pSTEC (Barlow and Mellor 2010). A survey of retail beef and sheep meat (Barlow, Gobius et al. 2006) and a number of industry reports are consistent in their findings that key gene targets (in particular *stx* and *eae*) are often present in beef samples but are usually present in different strains of *E. coli*.

Despite this there has never been a concerted effort to detail the combinations of genes and organisms present in potentially positive beef samples. Such data would fill the knowledge gap that exists in relation to pSTEC testing and will more accurately quantify the percentage of potential positive samples that are sent for confirmation despite the enrichment having never contained pSTEC.

Materials and Methods

Sample collection

Samples that test potentially positive for pSTEC (*E. coli* O157 and Big6) must be sent to a commercial testing laboratory for confirmatory testing. Enrichments broths from which a pSTEC isolate is not recovered are reported as negative to the customer. Each enrichment is then de-identified and forwarded to CSIRO for further analysis. All enrichment broths are received by CSIRO within one week of the initial screening test and are accompanied by the following information

- Who conducted the initial screening test (i.e on site laboratory or commercial laboratory)
- Which test system was used
- What the enrichment broths were potentially positive for (i.e *E. coli* O157 and/or Big6), and
- What serotype(s) the enrichment broth is potentially positive for, if known.

Detection of pSTEC

Enrichment broths were tested for the presence of *stx*, *eae* and the pSTEC serotypes O26, O45, O103, O111, O121 and O145 using the primers and probes outlined in the FSIS laboratory guidebook MLG 5B.03 (http://www.fsis.usda.gov/PDF/MLG-5B.pdf) and for *E. coli* O157 using previously published primers and probes (Perelle, Dilasser et al. 2004).

Isolation of target organisms

IMS

Attempts were made using immunomagnetic separation (IMS) to isolate pSTEC from all potentially positive broths. IMS was performed using Assurance GDS Poly IMS – Top STEC (BioControl, USA) and/or Dynabeads (Invitrogen, Norway) specific for individual pSTEC serotypes. Dynabeads specific to pSTEC serotypes were used if serotype data was available following the pSTEC screening tests. Top STEC beads were used if the GDS system was used as the original test system or if no serotype information was available. All IMS was performed using an automated bead retriever (Invitrogen). The resulting bead-bacteria complexes were plated onto rainbow agar (Biolog, USA), cefixime-tellurite sorbitol MacConkey agar (CT-SMAC; Oxoid, UK), USMARC chromogenic agar medium (Kalchayanand, Arthur et al. 2013) and washed sheep blood agar supplemented with mitomycin C (WBAM; Sugiyama, Inoue et al. 2001). All agar plates were incubated at 37 ± 2°C for 24 h. Following incubation, representative colonies (up to 30 per sample) were picked from all plates and streaked onto sheep blood agar (SBA) and incubated overnight at 37°C ± 2°C for 24 h. The resulting colonies were tested for the presence of *stx* and *eae* using a published multiplex PCR (Paton and Paton 1998)

and for pSTEC serotypes using the detection PCR's listed above. All isolates that were shown to contain any combination of *stx*, *eae* or were of a pSTEC serotype were confirmed as *E. coli* and stored at -80°C.

Colony hybridisation

All enrichment broths were investigated further for the presence of *eae*-containing *E. coli*, STEC and *E. coli* of pSTEC serotypes using colony hybridisation as described previously (Barlow, Pemberton et al. 2004). DIG-labelled probes specific for *eae*, *stx*, and pSTEC O serotypes were prepared using a PCR DIG probe synthesis kit (Roche) as per the manufacturer's instructions and the primers listed in Table 1. Colony hybridisation of *eae* and *stx*-containing *E. coli* were performed independently of each other whereas colony hybridisation for the pSTEC O serotypes was conducted using a combined probe targeting all pSTEC O serotypes. Colonies that were identified as containing any of the genetic targets were streaked onto SBA and incubated overnight at 37°C ± 2°C for 24 h. Each isolate was then tested for the presence of *stx*, *eae* and pSTEC O serotypes using the PCR primers and probes listed above. All isolates that were shown to contain any combination of *stx*, *eae* or were of a pSTEC serotype were confirmed as *E. coli* and stored at -80°C.

Table 1. Primers used to generate DIG-labelled probes

Primer	Sequence (5' – 3')	Target	Reference
MK1 MK2	TTT ACG ATA GAC TTC TCG AC CAC ATA TAA ATT ATT TCG CTC	*stx*	(Karch and Meyer 1989)
Eae AF Eae AR	GAC CCG GCA CAA GCA TAA GC CCA CCT GCA GCA ACA AGA GG	*eae*	(Paton and Paton 1998)
RfbE (O157)-F RfbE (O157)-R	TTT CAC ACT TAT TGG ATG GTC TCA A CAG TGA GTT TAT CTG CAA GGT GAT	*rfbE* O157	(Perelle, Dilasser et al. 2004)
Wzx O26-F Wzx O26-R	GTA TCG CTG AAA TTA GAA GCG C AGT TGA AAC ACC CGT AAT GGC	*wzx* O26	http://www.fsis.usda.gov/PDF/MLG-5B.pdf
Wzx O45-F Wzx O45-R	CGT TGT GCA TGG TGG CAT TGG CCA AAC CAA CTA TGA ACT G	*wzx* O45	
Wzx O103-F Wzx O103-R	TTG GAG CGT TAA CTG GAC CT ATA TTC GCT ATA TCT TCT TGC GGC	*wzx* O103	
WbdI O111-F WbdI O111-R	TGT TCC AGG TGG TAG GAT TCG TCA CGA TGT TGA TCA TCT GGG	*wbdI* O111	
Wzx O121-F Wzx O121-R	AGG CGC TGT TTG GTC TCT TAG A GAA CCG AAA TGA TGG GTG CT	*wzx* O121	
Wzx O145-F Wzx O145-R	AAA CTG GGA TTG GAC GTG G CCC AAA ACT TCT AGG CCC G	*wzx* O145	

Results

A total of 98 Big6 and 29 *E. coli* O157 potential positive enrichment broths have been received and analysed. Enrichment broths were more likely to have been tested at an on-site laboratory at an abbatoir with 64 (65%) of 98 Big6 and 23 (79%) of 29 *E. coli* O157 potential positives (based on screening tests) tested by abattoir staff. Of the 64 Big6 potential positives to occur at on-site laboratories, 13 (20%) were generated using the BAX system and 42 (66%) using the GDS system. Information on the test system used was not supplied for nine (14%) of 64 samples. This compares with six (18%) of 34 from the BAX system and 28 (82%) of 34 from GDS for samples tested by a commercial laboratory. Similar trends were observed with *E. coli* O157 with on-site laboratories generating potential positives 22%, 43% and 35% using the Bax system, GDS system, or unknown test system respectively. Of the six potential positives identified by a commercial laboratory, two (33%) were generated using the BAX system with the remaining 4 (66%) identified by the GDS system. A summary breakdown of all samples analysed is shown in Appendix A.

Detection of pSTEC

Upon arrival at the CSIRO laboratory all enrichment broths were re-screened for the presence of *stx*, *eae* and the seven pSTEC serotypes (Figure 1). Detection of *stx* and *eae* occurred in 75 (77%) of 98 Big6 potential positives and in 12 (41%) of 29 *E. coli* O157 potential positives. Big6 potential positive samples that had been tested using the BAX system were less likely to test positive for *stx* and *eae* than samples initially tested using the GDS system. Six (32%) of 19 BAX tested samples compared with 11 (16%) of 70 GDS tested samples did not test positive for *stx* and *eae* during re-screening. Similarly, *E. coli* O157 potential positive samples that had been tested using the BAX system were less likely to test positive for *stx* and *eae* than samples initially tested using the GDS system. Four (57%) of seven BAX tested samples compared with four (29%) of 14 GDS tested samples did not test positive for *stx* and *eae* during re-screening. The differences between the BAX and GDS test systems, although not significant, were consistent regardless of whether the screening test was performed at an on-site laboratory or at a commercial laboratory. Detection of *stx* and *eae* in those samples for which the test system was unknown was poor with just three (33%) of nine Big6 and 2 (25%) of eight *E. coli* O157 samples testing positive for the presence of both markers.

Detection of at least one O serotype occurred in 55 (56%) of 98 Big6 potential positives and O157 was detected in just five (17%) of 29 *E. coli* O157 potential positives. When combined with the *stx* and *eae* results only 46 (47%) of 98 Big6 enrichment broths and two (7%) of 29 *E. coli* O157 enrichment broths met the criteria of a potential positive. Previous studies have noted a lack of agreement between different pSTEC test methods. In this study, the inability to detect pSTEC O serotypes was the most common factor affecting agreement between methods. Additional investigations are required to determine if the specific attributes of test systems, the relative competencies of laboratory staff, or the uniqueness of the microflora within beef sample effect screening test results.

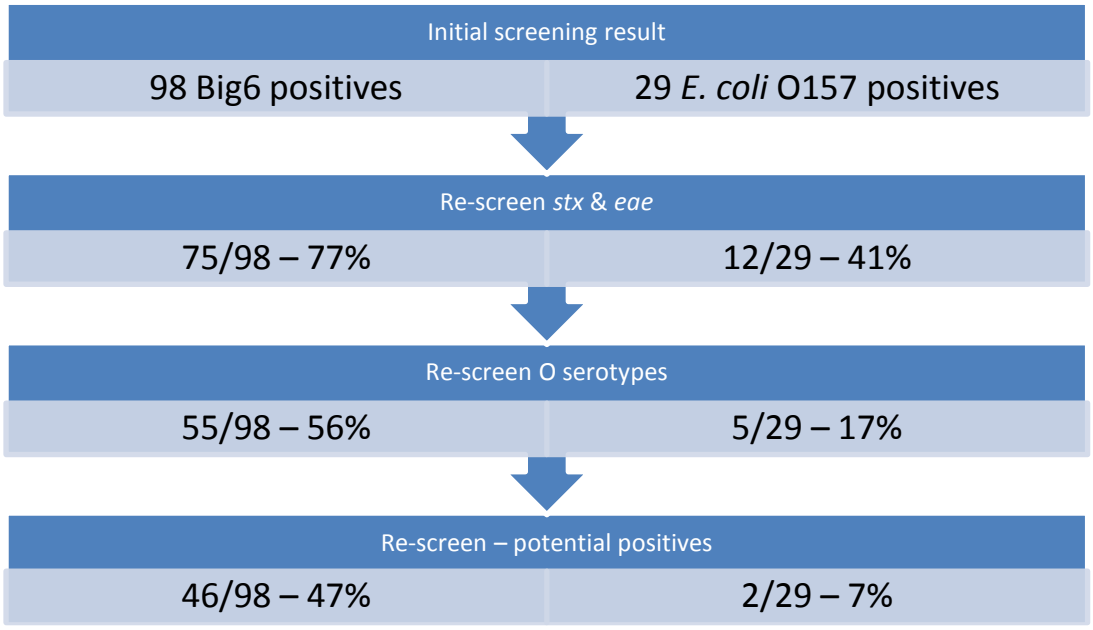

Figure 1. Results of re-screening potential positive broths

Isolation of target organisms

Attempts were made to isolate *E. coli* possessing any combination of *stx*, *eae*, and pSTEC O serotypes from 98 Big6 and 29 *E. coli* O157 potential positive broths. At least one organism harboring any combination of *stx*, *eae*, or pSTEC O serotype was isolated from 78 (80%) of 98 Big6 enrichment broths and from 12 (41%) of *E. coli* O157 enrichment broths. A summary of isolated organisms is shown below.

pSTEC

pSTEC were isolated from five (5.1%) of the 98 Big6 and one of the 29 *E. coli* O157 enrichments broths analysed. One of the pSTEC isolated from a Big6 sample was an *E. coli* O157 harboring *stx* and *eae*, however this broth was only sent for Big6 confirmation and therefore was not a target during the confirmation process. The remaining four pSTEC recovered from Big6 samples were all *E. coli* O26. One additional EHEC isolate that was not of a pSTEC serotype was isolated from an *E. coli* O157 potential positive sample. Five of the six pSTEC isolates recovered from either the Big6 or *E. coli* O157 samples were isolates using IMS. One pSTEC was unable to be isolated using IMS and was only recovered when colony hybridization targeting *eae* was used.

STEC

E. coli harboring *stx* were isolated from 46 (47%) of 98 Big6 and from eight (28%) of 29 *E. coli* O157 enrichment broths. The association of *stx* with a pSTEC serotype was low with only seven (7.1%) Big6 and one (3.4%) *E. coli* O157 sample yielding isolates that were of a pSTEC serotype and

contained *stx*. Multiple STEC were isolated from individual samples with up to four different STEC isolated from the same sample. These STEC were shown to differ based their virulence marker profiles. STEC were not recovered from any of the 18 Big6 and 14 *E. coli* O157 samples that tested negative for *stx* during re-screening.

Eae-containing *E. coli*

E. coli harboring *eae* were isolated from 47 (48%) of 98 Big6 and 8 (28%) of 29 *E. coli* O157 enrichment broths. Twenty-four (24%) of the 98 Big6 samples yielded *eae*-containing *E. coli* that were of a pSTEC serotype compared with 29 (30%) Big6 samples that yielded *eae*-containing *E. coli* of non-pSTEC serotypes. Six (6%) samples contained both an *eae*-containing *E. coli* of a pSTEC serotype and an *eae*-containing *E. coli* of a non-pSTEC serotype. *E. coli* of serotype O157 harboring *eae* but not *stx* were not recovered from any of the *E. coli* O157 samples. Isolates containing *eae* were not recovered from any of the 14 Big6 and 13 *E. coli* O157 samples that tested negative for *eae* during re-screening.

E. coli of pSTEC serotypes

Attempts were made to isolate *E. coli* belonging to a pSTEC serotype using IMS or colony hybridisation. Fifty-five *E. coli* belonging to pSTEC serotypes were recovered from 44 (45%) of 98 Big6 samples. Multiple serotypes were recovered from ten (10%) samples with one sample yielding *E. coli* O26, O45 and O103. *E. coli* O26 and O103 were most commonly isolated and were recovered from 21 and 19 samples, respectively. All pSTEC serotypes except *E. coli* O111 were isolated. Interestingly, *E. coli* O157 lacking *stx* and/or *eae* were isolated from just one (3.4%) of the 29 *E. coli* O157 samples tested.

Within sample combinations

With the exception of samples containing pSTEC, samples that contain an individual target organism such as an STEC, an *eae*-containing *E. coli*, or an *E. coli* of a pSTEC serotype will not result in that sample testing potentially positive for pSTEC. This study evaluated potential positive samples in an attempt to demonstrate that the key genetic markers used during PCR screening (i.e *stx*, *eae*, and O serotype) were present in different organisms within the same enrichment. Table 2 gives a breakdown of the number of different target organisms isolated per sample for both Big6 and *E. coli* O157 enrichment broths. For example, the presence of three targets would indicate that *E. coli* were isolated containing *stx*, *eae*, and of an O serotype, however, all three targets were not present in a single *E. coli* isolate. Enrichment broths that yielded pSTEC isolates were removed from this analysis although it should be noted that two of the five Big6 samples and the *E. coli* O157 sample that yielded pSTEC also contained additional target organisms. A comparison of the number of target organisms isolated from each sample did not identify any significant difference between the Bax and GDS systems.

Table 2. Presence of target organisms in potential positive enrichment broths

Enrichment Broth	N=	3 targets	2 targets	1 target	0 targets
Big6	93	9 (9.7)*	31 (31.3)	33 (35.5)	20 (21.5)
E. coli O157	28	2 (7.1)	3 (10.7)	6 (21.4)	17 (60.7)

* Figures in parentheses are percent

A further breakdown of the combinations of genetic markers present in each of the target organisms reveals that the majority of markers reside alone in different *E. coli* isolates (Table 3). The exception to this was the combination of *eae* with a pSTEC serotype which was isolated from 19.4% of Big6 and 13.8% of *E. coli* O157 enrichment broths. Multiple variations of a target organism type were identified within individual samples. One particular Big6 enrichment broth yielded a STEC, an *eae*-containing *E. coli*, an *E. coli* O145 isolate, an *E. coli* O103 isolate, and an *E. coli* O145 harboring *eae*.

Table 3. Combinations of genetic markers present in target organisms

Marker combination	Big 6 Enrichment broth (n=98)	*E. coli* O157 enrichment broth (n=29)
pSTEC	5 (5.1)*	1 (3.4)
Non-pSTEC EHEC	0 (0.0)	1 (3.4)
stx only	40 (40.8)	6 (20.7)
eae only	27 (27.5)	4 (13.8)
stx & pSTEC O serotype	2 (2.0)	0 (0.0)
eae & pSTEC O serotype	19 (19.4)	4 (13.8)
pSTEC O serotype only	27 (27.5)	1 (3.4)

* Figures in parentheses are percent

Summary and Conclusions

This study has analysed 98 Big6 and 29 *E. coli* O157 potential positive enrichment broths. Each of these broths did not yield pSTEC isolates during standard confirmation testing at a commercial lab. This study has determined that individual samples may often contain combinations of target organisms possessing the genetic markers commonly used in the determination of potential positive broths. Whilst their presence alone doesn't indicate the absence of pSTEC, the data indicates that organisms other than pSTEC are contributing significantly to the generation of potential positive samples during screening. These combinations may give rise to an elevated potential positive rate during routine screening, increase the difference between potential positive and presumptive positive rates, and reduce the perceived effectiveness of currently established confirmation protocols. A list of conclusions for the samples analysed thus far are below.

- Re-screening of potential positive broths determined that only 46 (47%) of 98 Big6 enrichment broths and two (7%) of 29 *E. coli* O157 enrichment broths met the criteria of a potential positive. Previous studies have noted a lack of agreement between different pSTEC test methods. In this study, the inability to detect pSTEC O serotypes was the most common factor affecting agreement between methods. Additional investigations are required to determine if the specific attributes of test systems, the relative competencies of laboratory staff, or the uniqueness of the microflora within beef sample effect screening test results.

- pSTEC isolates may be recovered from potential positive samples that originally do not yield pSTEC isolates during routine confirmation. However, the proportion of samples from which this occurs is small and additional materials and methods that involve the testing of thousands of colonies may be required.

- Potential positive samples that originally do not yield pSTEC isolates during routine confirmation often contain multiple isolates of *E. coli* harboring different genetic markers that would be detected during screening and which could account for the potential positive status of that sample. STEC, *eae*-containing *E. coli*, and *E. coli* of a pSTEC serotype that lacks *stx* and *eae* were found in 40.8, 27.5 and 27.5% of Big6 samples and 20.7, 13.8 and 3.4% of *E.* coli O157 samples, respectively.

- *E. coli* isolated from potential positive enrichment broths tend to contain only one of the three genetic markers (*stx*, *eae*, a pSTEC serotype) typically used to identify potential positive enrichment broths. The exception to this is the regular finding of *eae* in *E. coli* of pSTEC serotypes with *E. coli* O26 harboring *eae* regularly isolated.

- Whilst ongoing refinement of confirmation protocols is required to ensure maximum isolation rates are achieved from samples containing pSTEC, a focus should remain on identifying genetic differences of pSTEC that can be exploited during the screening process.

Overall progress of the project

This project was expected to analyse up to 100 Big6 and 100 *E. coli* O157 potential positive broths that have not yielded a pSTEC isolate during routine confirmation. A total of 98 Big6 and 29 *E. coli* O157 broths have been investigated and described in this report. The shortfall in *E. coli* O157 undoubtedly represents the higher conversion rate of potential positives to confirmed positives at the commercial laboratory than was observed with the Big6 samples. During the course of this project a number of international research collaborations were established and additional research is being conducted to further explore the variability of target organisms in potential positive broths that do not yield pSTEC isolates at confirmation. Collaborative agreements are currently in place with Roka Bioscience, Neogen and ANSES and summary reports of these research efforts will be provided to MLA in lieu of the outstanding samples proposed for this project.

Recommendations

The findings of this study adequately describe the difficulty facing companies and commercial laboratories that are using current methodologies for the detection and isolation of pSTEC from manufacturing beef samples. This report is expected to satisfy the achievement criteria of Milestone 3 of G.MFS.0282 with an expectation that summary reports relating to the complimentary research being conducted as part of ongoing collaborations are to be provided to MLA by June 30, 2014.

Future research

Methods for the detection and isolation of pSTEC from beef cattle samples continue to evolve. Substantial interest exists in identifying additional genetic and phenotypic markers of pSTEC that can be exploited to enhance detection and isolation. Notwithstanding, there appears to be a lack of data relating to the composition of the samples routinely subjected to testing and their associated microflora on which existing and novel test strategies are to be implemented. Conducting a metagenomic analysis of pSTEC enrichment broths would enable a greater understanding of the variability of the key genetic markers (O-types, *eae* and *stx*) present and could identify if refinements to methodologies are required to address any unique aspects of Australian microflora. Furthermore, there is increasing evidence to suggest that some of *eae*-containing *E. coli* that lack *stx* should be termed potential EHEC and be classified as distinct from their current grouping as atypical EPEC. Many of these organisms are of Big6 serotypes and consequently the implications around EHEC evolution as well as the impact on pSTEC testing of Australian beef samples is requiring of research effort. An in-depth analysis of the microflora and their associated genetic composition should be viewed as priority research areas if the goal of developing and validating existing and novel approaches to pSTEC testing is to be realised.

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Appendix A: Sample breakdown

Table A1. A summary of initial and re-screening results and the isolate gene combinations recovered from Big6 potential positive broths

		Initial Screening			Re-screening			Isolate gene combinations*		
Sample	**Category**	**Test by**	**Test System**	**Potential Positive for**	***stx***	***eae***	**Serotype**	***stx*†**	***eae***	**Serotype**
1	Big 6	Commercial	Bax	O121	+	+	O121	$+_2$	-	-
								-	+	-
2	Big 6	Commercial	Bax	O26 & O145	-	+	O26	-	+	O26
3	Big 6	Commercial	Bax	O26 & O45	-	+	O26	-	+	O26
								-	-	O26
4	Big 6	Commercial	Bax	O45	-	-	O45	-	-	-
5	Big 6	Commercial	Bax	O26	+	+	O26	**$+_1$**	**+**	**O26**
6	Big 6	Commercial	Bax	O103	+	+	O103	$+_2$	-	-
								-	+	O103
7	Big 6	Customer	Bax	O103 & O45	+	+	O103, O45	$+_2$	-	-
								-	+	-
								-	-	O103
8	Big 6	Customer	Bax	O45	+	+	None Detected	-	-	-
9	Big 6	Customer	Bax	O103 & O45	-	-	None Detected	-	-	O103
								-	-	O45
10	Big 6	Customer	Bax	O103 & O121	+	+	O121 & O103	-	+	-
								-	-	O103
								-	-	O121
11	Big 6	Customer	Bax	O103	+	+	None Detected	$+_{1\&2}$	-	-
12	Big 6	Customer	Bax	O121 & O103 & O45	-	+	O45	-	+	-
								-	-	O103
13	Big 6	Customer	Bax	O121 & O45	+	+	O121	-	-	-

Sample	Category	Initial Screening			Re-screening			Isolate gene combinations*		
		Test by	Test System	Potential Positive for	*stx*	*eae*	Serotype	*stx*†	*eae*	Serotype
14	Big 6	Customer	Bax	O121 & O103	+	+	O121 & O103	-	+	-
15	Big 6	Customer	Bax	O121 & O26	-	+	None Detected	-	+	-
16	Big 6	Customer	Bax	O26 & O45 & O103	+	+	O26 & O45 & O103	-	+	O26
								-	+	$O26_{(ehx)}$‡
								-	+	-
								-	-	O103
								-	-	O45
17	Big 6	Customer	Bax	O121 & O26	+	+	O121	-	-	O121
18	Big 6	Customer	Bax	O121 & O103	+	+	O121 & O103	$+_{1\&2}$	-	-
19	Big 6	Customer	Bax	O26 & O103	+	+	O26 & O103	-	+	O26
20	Big 6	Commercial	GDS	Big6	+	+	O103	-	+	O103
21	Big 6	Commercial	GDS	Big6	+	+	None Detected	$+_1$	-	-
								-	+	-
22	Big 6	Commercial	GDS	Big6	+	+	None Detected	**$+_2$**	**+**	**O157**
23	Big 6	Commercial	GDS	Big6	-	-	None Detected	-	-	-
24	Big 6	Commercial	GDS	Big6	+	+	O103	$+_2$	-	-
								-	-	O103
25	Big 6	Commercial	GDS	Big6	+	-	None Detected	$+_2$	-	-
								-	-	O103
								-	-	O121
26	Big 6	Commercial	GDS	Big6	+	+	O26 & O45	$+_2$	-	-
								-	+	O26
								-	+	-
								-	-	O103
27	Big 6	Commercial	GDS	Big6	+	+	O26	$+_{1\&2}$	-	-

Sample	Category	Initial Screening Test by	Test System	Potential Positive for	Re-screening *stx*	Re-screening *eae*	Re-screening Serotype	Isolate gene combinations* *stx*†	Isolate gene combinations* *eae*	Isolate gene combinations* Serotype
								-	+	O26
28	Big 6	Commercial	GDS	Big6	+	+	O26	$+_{1\&2}$	-	-
29	Big 6	Commercial	GDS	Big6	+	+	O103	-	-	O103
30	Big 6	Commercial	GDS	Big6	+	+	O145	-	+	O145
31	Big 6	Commercial	GDS	Big6	+	+	O26 & O45 & O103	$+_{1\&2}$	-	-
								-	+	-
32	Big 6	Commercial	GDS	Big6	+	+	None Detected	-	-	-
33	Big 6	Commercial	GDS	Big6	+	+	O45	$+_{2}$	-	-
								-	-	O45
34	Big 6	Commercial	GDS	Big6	+	-	O26	$+_{1\&2}$	-	-
								$+_{2}$	-	O26
								$+_{2}$	-	-
								$+_{1}$	-	-
35	Big 6	Commercial	GDS	Big6	+	+	O45	$+_{1}$	-	-
								$+_{2}$	-	-
36	Big 6	Commercial	GDS	Big6	+	+	O121	-	-	O121
37	Big 6	Commercial	GDS	Big6	+	+	O121 & O45	-	+	-
								-	-	O45
38	Big 6	Commercial	GDS	Big6	+	+	O103	$+_{2}$	-	-
								-	+	-
39	Big 6	Commercial	GDS	Big6	-	-	None Detected	-	-	-
40	Big 6	Commercial	GDS	Big6	+	+	None Detected	-	-	-
41	Big 6	Commercial	GDS	Big6	+	+	None Detected	-	-	-
42	Big 6	Commercial	GDS	Big6	+	+	None Detected	-	-	-
43	Big 6	Commercial	GDS	Big6	+	+	None Detected	$+_{2}$	-	-
44	Big 6	Commercial	GDS	Big6	+	+	None Detected	$+_{1}$	-	-
45	Big 6	Commercial	GDS	Big6	+	+	None Detected	$+_{2}$	-	-

Sample	Category	Initial Screening			Re-screening			Isolate gene combinations*		
		Test by	Test System	Potential Positive for	*stx*	*eae*	Serotype	*stx*†	*eae*	Serotype
46	Big 6	Commercial	GDS	Big6	-	+	None Detected	-	-	-
47	Big 6	Commercial	GDS	Big6	-	+	O26	-	+	O26
48	Big 6	Customer	GDS	Big6	+	+	None Detected	$+_1$	-	-
49	Big 6	Customer	GDS	Big6	-	-	None Detected	-	-	-
50	Big 6	Customer	GDS	Big6	+	+	O26	-	+	O26
								-	-	O26
51	Big 6	Customer	GDS	Big6	-	-	None Detected	-	-	-
52	Big 6	Customer	GDS	Big6	+	+	None Detected	-	+	-
								-	-	O103
53	Big 6	Customer	GDS	Big6	+	+	None Detected	$+_2$	-	-
								-	+	-
54	Big 6	Customer	GDS	Big6	+	+	None Detected	**$+_1$**	**+**	**O26**
55	Big 6	Customer	GDS	Big6	+	+	None Detected	$+_{1\&2}$	-	-
								-	+	-
56	Big 6	Customer	GDS	Big6	+	+	O103	$+_{1\&2}$	-	-
57	Big 6	Customer	GDS	Big6	+	+	O103	$+_2$	-	O103
								-	-	O103
58	Big 6	Customer	GDS	Big6	+	+	None Detected	-	+	-
59	Big 6	Customer	GDS	Big6	-	-	None Detected	-	-	-
60	Big 6	Customer	GDS	Big6	+	+	None Detected	$+_{1\&2}$	-	-
61	Big 6	Customer	GDS	Big6	+	+	O26 & O103	$+_2$	-	-
62	Big 6	Customer	GDS	Big6	+	+	O45	$+_2$	-	-
63	Big 6	Customer	GDS	Big6	+	+	O145 & O157	-	+	O145
64	Big 6	Customer	GDS	Big6	+	+	O45	-	+	-
65	Big 6	Customer	GDS	Big6	+	+	O45 & O103	$+_2$	-	-
								-	+	O26

Sample	Category	Initial Screening			Re-screening			Isolate gene combinations*		
		Test by	Test System	Potential Positive for	*stx*	*eae*	Serotype	*stx*†	*eae*	Serotype
66	Big 6	Customer	GDS	Big6	+	+	None Detected	$+_{1\&2}$	-	-
67	Big 6	Customer	GDS	Big6	+	+	O121	-	+	O26
								-	+	-
								-	-	O121
68	Big 6	Customer	GDS	Big6	+	-	None Detected	$+_2$	-	-
69	Big 6	Customer	GDS	Big6	+	+	None Detected	$+_{1\&2}$	-	-
								$+_2$	-	-
								-	+	-
70	Big 6	Customer	GDS	Big6	+	+	O103 & O145	$+_2$	-	-
								-	eae	O145
								-	eae	-
								-	-	O145
								-	-	O103
71	Big 6	Customer	GDS	Big6	+	+	O121 & O103 & O157	$+_2$	-	-
72	Big 6	Customer	GDS	Big6	+	-	O103 & O157	$+_{1\&2}$	-	-
								$+_2$	-	-
73	Big 6	Customer	GDS	Big6	+	+	O26 & O103	-	+	O26
								-	-	O103
74	Big 6	Customer	GDS	Big6	+	+	O26 & O103	$+_2$	-	-
								-	+	O26
75	Big 6	Customer	GDS	Big6	+	+	None Detected	-	+	-
76	Big 6	Customer	GDS	Big6	+	+	O103	-	-	O103
77	Big 6	Customer	GDS	Big6	+	+	O26 & O103	**$+_1$**	**+**	**O26**
								-	-	O103
78	Big 6	Customer	GDS	Big6	+	+	O26 & O45	-	-	O26
79	Big 6	Customer	GDS	Big6	+	+	O45	$+_2$	-	-

Sample	Category	Initial Screening			Re-screening			Isolate gene combinations*		
		Test by	Test System	Potential Positive for	*stx*	*eae*	Serotype	*stx*†	*eae*	Serotype
								-	+	-
								-	-	O26
								-	-	O45
80	Big 6	Customer	GDS	Big6	+	+	O45	$+_2$	-	-
								-	+	-
81	Big 6	Customer	GDS	Big6	+	+	None Detected	$+_2$	-	-
82	Big 6	Customer	GDS	Big6	+	+	None Detected	-	-	-
83	Big 6	Customer	GDS	Big6	+	+	None Detected	-	+	-
84	Big 6	Customer	GDS	Big6	+	+	None Detected	$+_{1\&2}$	-	-
								$+_2$	-	-
								-	+	-
85	Big 6	Customer	GDS	Big6	+	+	None Detected	-	-	-
86	Big 6	Customer	GDS	Big6	+	+	None Detected	-	-	-
87	Big 6	Customer	GDS	Big6	+	+	O103	-	-	O103
88	Big 6	Customer	GDS	Big6	+	+	None Detected	**$+_1$**	**+**	**O26**
								-	eae	-
89	Big 6	Customer	GDS	Big6	+	+	O26	-	+	O26
90	Big 6	Customer	Unknown	Big6	+	+	O26 & O45	$+_2$	-	-
								-	+	O26
91	Big 6	Customer	Unknown	Big6	-	+	None Detected	-	+	-
92	Big 6	Customer	Unknown	Big6	-	+	O121	-	-	O121
93	Big 6	Customer	Unknown	Big6	-	+	O121	-	+	-
94	Big 6	Customer	Unknown	Big6	-	-	None Detected	-	-	-
95	Big 6	Customer	Unknown	Big6	+	+	None Detected	-	-	-
96	Big 6	Customer	Unknown	Big6	+	+	O103	$+_2$	-	-
								-	-	O103

Sample	Initial Screening Category	Test by	Test System	Potential Positive for	Re-screening *stx*	*eae*	Serotype	Isolate gene combinations* *stx*†	*eae*	Serotype
97	Big 6	Customer	Unknown	Big6	-	-	None Detected	-	-	-
98	Big 6	Customer	Unknown	Big6	+	-	None Detected	-	-	-

* pSTEC isolates are shown in bold and each isolate is represented by a different row; † subscript number refers to the *stx* type within each isolate; ‡ two different isolates of O26 carrying eae were defined based on the presence/absence of the additional virulence marker *ehx*

Table A2. A summary of initial and re-screening results and the isolate gene combinations recovered from *E. coli* O157 potential positive broths

Sample	Initial Screening Category	Test by	Test System	Re-screening *stx*	*eae*	Serotype	Isolate gene combinations* *stx*†	*eae*	Serotype
1	O157	Customer	Bax	+	-	O157	-	-	-
2	O157	Customer	Bax	+	+	None Detected	$+_2$	+	-
3	O157	Customer	Bax	-	-	None Detected	-	-	-
4	O157	Customer	Bax	+	+	O103	-	-	-
5	O157	Customer	Bax	-	-	None Detected	-	-	-
6	O157	Commercial	Bax	-	-	None Detected	-	-	-
7	O157	Commercial	Bax	-	-	O157	-	-	O157
8	O157	Customer	GDS	-	-	None Detected	-	-	-
9	O157	Customer	GDS	+	+	O145 & O157	**$+_2$**	**+**	**O157**
							-	+	O145
10	O157	Customer	GDS	+	+	O45	-	+	-
11	O157	Customer	GDS	+	+	O103 & O145	$+_2$	-	-
							-	+	O145
							-	+	-
							-	-	O145
							-	-	O103

Initial Screening				Re-screening			Isolate gene combinations*		
Sample	Category	Test by	Test System	*stx*	*eae*	Serotype	*stx*†	*eae*	Serotype
12	O157	Customer	GDS	+	+	O121 & O103 & O157	$+_{2}$	-	-
13	O157	Customer	GDS	+	-	O103 & O157	$+_{1\&2}$	-	-
							$+_{2}$	-	-
14	O157	Customer	GDS	+	+	None Detected	$+_{2}$	-	-
15	O157	Customer	GDS	+	+	None Detected	-	-	-
16	O157	Customer	GDS	+	+	None Detected	-	+	-
17	O157	Customer	GDS	+	+	None Detected	$+_{1\&2}$	-	-
							$+_{2}$	-	-
							-	+	-
18	O157	Commercial	GDS	-	-	None Detected	-	-	-
19	O157	Commercial	GDS	+	+	None Detected	-	-	-
20	O157	Commercial	GDS	-	+	None Detected	-	-	-
21	O157	Commercial	GDS	-	+	O26	-	+	O26
22	O157	Customer	Unknown	+	+	O26 & O45	$+_{2}$	-	-
							$+_{2(ehx)}$‡	-	-
							-	+	O26
23	O157	Customer	Unknown	-	-	None Detected	-	-	-
24	O157	Customer	Unknown	-	+	None Detected	-	-	-
25	O157	Customer	Unknown	-	-	None Detected	-	-	-
26	O157	Customer	Unknown	-	-	None Detected	-	-	-
27	O157	Customer	Unknown	-	-	None Detected	-	-	-
28	O157	Customer	Unknown	+	-	None Detected	-	-	-
29	O157	Customer	Unknown	-	+	None Detected	-	-	-

* pSTEC isolates are shown in bold; † subscript number refers to the *stx* type within each isolate; ‡ two different STEC were defined based on the presence/absence of the additional virulence marker *ehx*