final report

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Pathogenic Shiga toxin producing *E. coli* (pSTEC) other than O157 (non-O157 STEC) in manufacturing beef - Baseline survey and method comparison

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Abstract

A survey of Australian manufacturing beef was conducted to determine the prevalence of pathogenic Shiga toxin-producing E. coli (pSTEC) belonging to serotypes referred to as the Big 6 (O26, O45, O103, O111, O121, and O145), declared by the US Food Safety and Inspection Service (FSIS) as adulterants in meat destined for grinding. The survey was conducted using four different screening methods, which were compared for their suitability in screening Australian manufacturing beef. FSIS Microbiology Laboratory Guidebook confirmation methods were followed as far as possible. There were 1197 samples, which were sub-sampled to enable testing on one of four test systems, resulting in a total of 4320 individual tests being conducted. One positive screening test was confirmed to contain a Big 6 serotype (E. coli O26). Two samples with a negative screening test were also found to contain a Big 6 serotype (E. coli O26). The prevalence of Big 6 strains in Australian beef is estimated to be approximately 0.02%. Screening tests yielded an average positive rate of 2.2%, with considerable differences between methods. Confirmation could take several days and incur significant expense.

Executive summary

Background

The Food Safety Inspection Service (FSIS) of USDA has declared six types of *E. coli*, in addition to *E. coli* O157 (nonO157 STEC), to be adulterants in beef that is intended for grinding or needle tenderisation. FSIS will implement a point-of-entry and domestic testing program, which will cause customers of Australian beef to require certificates of analysis, and AQIS (DAFF Biosecurity) to implement a testing program similar to the one that has been implemented for *E. coli* O157.

Objectives

The objectives of this project were to:

1. Provide an initial estimate of the prevalence of the Big 6 STEC strains in Australian manufacturing beef, including an understanding of the prevalence of positive results at the screening test stage and subsequent likelihood of confirmation.
2. Gain an understanding of the performance of commercially available screening tests for the Big 6 STEC, and subsequent confirmatory testing.

Methods

The methods chosen as screening tests for this project were from diagnostics companies that are active in the market and claimed to have already had validated methods (at least internal studies for sensitivity) for detecting the Big 6 STEC. Confirmation of screening test positive results were performed using methods based on the FSIS method.

Prevalence

There were 1197 samples, which were sub-sampled to enable testing on one of four test systems, resulting in a total of 4320 individual tests being conducted, after accounting for results which were excluded. One positive screening test was confirmed to contain a Big 6 strain (*E. coli* O26). Two samples with a negative screening test were also confirmed to contain a Big 6 strain (*E. coli* O26). The prevalence of Big 6 strains in Australian beef is estimated to be approximately 0.02%.

Comparison of methods

Screening tests systems yielded an average potential positive rate (screening test positive) of 2.2%, with considerable differences between methods (0.6-5.9%).

Significance to beef processors

Manufacturing beef will be potentially positive for a Big 6 STEC regularly and product would need to be held pending confirmation test results. Confirmation could take several days and incur significant expense. The most likely outcome of the confirmatory testing would be that Big 6 pSTEC would not be detected.

Contents

1 Background

1.1 Introduction

The Food Safety Inspection Service (FSIS) of USDA has declared six types of *E. coli* (the so-called Big 6), in addition to *E. coli* O157, to be adulterants in beef that is intended for grinding or needle tenderisation (FSIS, 2011). FSIS will implement a point-of-entry and domestic testing program for meat from animals slaughtered on or after 4 June 2012, which will cause customers of Australian beef to require certificates of analysis, and AQIS (DAFF Biosecurity) to implement a testing program similar to the one that has been implemented for *E. coli* O157. Essentially, the FSIS decision will require Australia to respond in the same way as for *E. coli* O157; i.e. most likely the same products involved, with the same testing regimes, and same product disposition rules.

The FSIS has a published method, MLG 5B.01, for its own laboratories that describes the full process for detection and isolation i.e. from screen testing to confirmation of pSTECs in meat products (refer Appendix 4). Outside of the FSIS, there has been no routine widespread test method for the Big 6. There have not been any previous systematic baselines studies across the industry on prevalence of these strains on product either in the US or in major beef producing countries, including Australia. In Australia, MLA commissioned CSIRO to examine the prevalence of *E. coli* O157 and non-O157 serotypes in cattle faeces but not on product (Barlow and Mellor, 2010).

There are a number of commercially available screening tests developed by diagnostics companies, however, these have recently come to market, have not been used in laboratories in Australia, and they have not been approved by DAFF (AQIS) for testing product destined for the USA at the time this project commenced.

The FSIS will commence a carcase baseline study later in 2012 to gain information on their own industry. It is possible that the approach to Big 6 STEC testing will be modified as the FSIS learns more about these strains in the beef supply chain.

1.2 Background on STEC

1.2.1 What are STEC?

There is a lot of confusing terminology concerning different *E. coli* strains, so we will explain the terminology that we use in this report, and terms that are being used by regulators and customers.

All of the *E. coli* strains we are concerned about produce Shiga toxins and are called STEC, which is the abbreviation commonly used for Shiga Toxin producing *E. coli*. STEC have either one or both of the genes *stx*1 and *stx*2 - only one gene is required to call *E. coli* an STEC. But, importantly, not all STEC cause disease in humans; some don't seem to cause any problems at all – that is, not all STEC are pathogenic.

1.2.2 What are pathogenic STEC?

An important (additional) factor in causing illness in humans is the one that allows *E. coli* to attach to the wall of the gut. Pathogenic STEC harbor a gene called *eae* which encodes the intimin protein that assists in the attachment of pSTEC to the gut wall. Strains of *E. coli* that have

the *eae* and an *stx* gene are often called EHEC, enterohaemorrhagic *E. Coli,* because they cause bloody diarrhoea. Note: there are also *E. coli* which have the *eae* gene but are not Shiga toxin producing.

In general terms, pathogenic STEC (pSTECs) refers to those *E. coli* that, in addition to being a Shiga toxin producing *E. coli*, have the *eae* gene which make them able to cause an adverse human health reaction. Without the *eae* gene, the STEC may be present but essentially pass through the human body and would be unlikely to make someone ill. We know that there are a number of other genes that are important in causing human disease, but we don't yet understand this well enough to develop this knowledge into simple tests.

E. coli O157:H7 strains usually have *stx* and *eae* genes, and are the most important EHEC - though presence of the *eae* gene is not part of the FSIS definition for O157 strains. In the USA, *E. coli* O157 that have an *stx* gene alone are considered to be adulterants.

There are many serotypes of STEC including O157 and those collectively called non-O157. While many of these can be carried by food animals, only a small number of serotypes commonly cause human illness. The O157 serotype was declared an adulterant in the USA as at the time it was the most common STEC serotype causing serious illness in that country.

Recently, another 6 strains of *E. coli* have been declared to be adulterants. These are strains of certain serotypes that have both an *stx* and an *eae* gene. The serotypes of concern in the USA are O26, O45, O103, O111, O121, and O145 (Big 6) because these six serotypes cause the majority of serious illnesses (for example bloody diarrhoea and HUS, which stands for haemolytic uraemic syndrome) that are not caused by O157.

In this report we have used the term pathogenic STEC (pSTEC), to mean the seven serotypes of *E. coli* (O157 plus the Big 6) that cause the majority of disease in the United States because they carry an *stx* and an *eae* gene.

In this project we have only looked at (tested for) the Big 6 pSTEC.

1.2.3 Overview of testing process for pSTEC

Testing for this group of organisms involves a complex process of detecting then isolating the target organism from others that could be in or on the sample. A major difference in testing for these organisms versus the more familiar culture ('agar plate') methods is that the organisms may or may not have certain genes which allow it to be pathogenic. Similarly, there are other species of bacteria, not just *E. coli*, which may have these "O" antigens. To give an example, assuming one detected the serotype *E. coli* O26, it may not have any or both of the necessary *stx* or *eae* genes to cause it to be pathogenic.

Each aspect of the strain needs to be confirmed and this requires different approaches to testing for each. To be confirmed as positive, the test process has to confirm, for each isolate, that it:

- is *E. coli*; and
- has one of the pSTEC 'O' antigens; and
- has an *stx* gene, and
- has the *eae* gene

When there is only one target, e.g. *E. coli* O157, the process is simpler. Now, because there are six more targets to look for in each sample, the testing process may need to continue and be repeated several times using the above detection and isolation steps until the result can be narrowed down to one serotype. Only then can that one serotype be checked to confirm it has the genes.

The testing process therefore requires a mix of detection and isolation methods including immunomagnetic separation (IMS), culturing on selective media (plates), biochemical methods, and genetic (DNA) testing using a method called polymerase chain reaction (PCR). The procedures within these steps are important because there are many things that can interfere with getting a correct result.

The FSIS MLG 5B.01 procedure includes definitions for the status of a test result at various stages of the process. These same definitions were used in the project[1] written here in a more explanatory form. They are:

Potential positive

A sample with positive PCR results for *stx*, *eae* and *wzx* [for one of the top 6 STEC serotypes] in the enrichment broth is considered a potential positive.

Presumptive positive

Any sample [colonies from mRBA] with (latex[2]) positive colonies for a Big 6 serotype is a presumptive positive for non-O157 STEC.

Confirmed positive

If the isolate is (latex) positive for top six STEC serotypes, PCR positive for *stx*, and *eae*, *wzx* genes and biochemically identified as *E. coli*, the sample is positive for non-O157 STEC.

The FSIS acknowledges there may be other methods or sequence of steps that commercial diagnostics companies could use and therefore they are free to design their own procedures and methods to achieve the same outcome.

[1] Refer to Figure 2 and Appendix 3 for further details on the confirmation testing process.

[2] Latex agglutination kits were not available for this project. Serotypes were determined using PCR.

2 Project objectives

The objectives of this project were to:

1. Provide an initial estimate of the prevalence of the Big 6 STEC serotypes in Australian manufacturing beef, including an understanding of the prevalence of positive results at the screening test stage (potential positive stage) and subsequent likelihood of confirmation; and

2. Gain an understanding of the performance of commercially available screening tests for the Big 6 STEC and subsequent confirmatory testing

It is important to note that this study did not validate any method according to FSIS requirements. There have been no attempts to determine the sensitivity of the methods, nor whether the methods will detect all strains of interest. Rather it is a comparative study of the performance of the methods using typical Australian meat samples destined for grinding in the USA.

3 Methods

The methods chosen as screening tests for this project were from diagnostics companies that are active in the market and claim to have validated methods (at least internal studies for sensitivity) for detecting the Big 6 STEC. The systems included were:

- BioControl Assurance GDS®
- IEH
- Pall Genedisc Technology
- Dupont Bax

A fifth system, AUSDiagnostics, was still in development at the time the project commenced and will be further evaluated outside the scope of this project.

An important issue is that the commercial systems or methods used for each system have their own definitions of what is ‘screen test positive’ and ‘confirmed positive’ and all have definitions that are different from those stated in the FSIS MLG 5B.01.

The general process, timeframes involved and point at which as result is achieved is as follows:

Day	Process	Result
0	Commence Enrichment	
1	Screening	Potential positive (screening positive)
2	Isolation - IMS beads and plating onto modified Rainbow agar	
3	Purification – Sheep Blood Agar	
4	PCR of pure colonies for *Stx* and *eae*	Presumptive positive
5	PCR for Big 6 "O" antigen and biochemical confirmation as *E. coli*	Confirmed positive

The timing shown above is 'ideal', assuming that screening tests and confirmatory tests are conducted in different laboratories. Timing could vary depending upon transport times and the operating hours of the laboratories.

A more detailed outline of the methods process involved in the project is provided in Appendix 1, Figure A1.

3.1 Overview of screening methods used

This section of the report provides a basic overview of the diagnostic systems evaluated in this project. Appendix 1 contains a full description of the method specified by each.

Screening tests for Big 6 serotypes rely on the detection of genes for Shiga toxin (*stx*1 and *stx*2), intimin (*eae*, for binding to the gut wall) and for the O-specific serotype (*wzx* genes). The genes are amplified (multiplied) in the test using PCR which is a way of increasing the number of copies of the genes of interest. To get these genes (inside the *E. coli* cell) to a high enough concentration to be detected easily, it is also necessary to grow the *E. coli* in an enrichment broth as a first step in all cases. Each method uses a different media for enrichment.

BioControl Assurance GDS® BioControl produce a number of test kits for the detection of STEC; we used the Assurance GDS MPX Top7 STEC kit.

The Assurance GDS MPX Top 7 STEC uses an IMS step prior to PCR. This sample preparation procedure aims to capture organisms belonging to 7 specific O serotypes (Big 6 plus O157) onto magnetic beads, which are then subjected to PCR. The intention is that any PCR reactions for *stx* or *eae* will come from *E. coli* of the serotypes of interest and reduce the number of screening test results that need further testing to be confirmed. The magnetic beads used for IMS and all the reagents necessary for PCR are contained in the kit. The GDS software provides separate results for *eae*, *stx1*, *stx2* and *E. coli* O157:H7 from a single test.

3.1.1 Dupont Qualicon Bax

The screening assay detects STEC virulence genes (*stx* and *eae*). If necessary (i.e. if the first stage is positive for both targets) this is followed by another two assays to determine if the sample is positive for one of the Big 6 STEC.

The equipment and procedure involved a simple DNA extraction - no separate cell concentration step - followed by PCR with all of the reagents packaged in individual PCR closed tubes to prevent contamination. Results (positive, negative) are read from the computer screen.

3.1.2 IEH

The IEH method is conducted in an IEH laboratory.

Enrichment broths are tested for *eae* and *stx*1 and *stx*2 and positive broths are subject to a secondary set of reactions with and without IMS prior to confirmatory PCR. The confirmatory PCR includes three new PCR multiplex assays. Two of the assays confirm the presence of an STEC. The remaining assay includes genetic targets which allow specific differentiation between each of the "Big Six" serotypes. An additional aliquot is subjected to lateral flow analyses to determine the presence of *E. coli* O157 as well as the top 6 non-O157 antigens. The last step (which is also performed during the screening) is for redundancy. In other words the "O" group

determination is done twice one using a multiplex PCR and a second time using lateral flow methodology.

3.1.3 Pall Genedisc Technology

After enrichment, a GeneDisc plate for STEC identifies the presence or absence of virulence factors that indicate the potential presence of pathogenic *E. coli* (*stx* and *eae*). A second GeneDisc (EHEC) enables testing of the same sample for the array of *E. coli* serotypes that may be considered adulterants by the FSIS.

Samples of the enrichment broth are lysed to allow DNA extraction, and the DNA is "extracted" through heat treatment. Extracted DNA samples are then loaded onto the GeneDisc, along with a liquid reagent ("master mix"), one tube of reagent per disc. The GeneDisc is pre-loaded with reagents (probes and primers) that include the appropriate controls for each sample. The test details are recorded and results are displayed on a computer screen. An individual report can be printed.

3.2 Confirmation procedures

Confirmation testing commenced as soon as possible after the potential positive result was obtained.

All enrichment broths were tested independently, replicating the workflow outlined in FSIS MLG5B.01. IMS beads were plated onto modified Rainbow Agar (mRBA) with and without acid treatment as per MLG5B.01 and at least one of each morphologically distinct (separated) colonies were subsequently tested by PCR using the PCR primers specified in MLG5B.01. Commercially available beads were used for serotypes O26, O103, O111 and O145.

Confirmation of GDS positive samples had the IMS step done using GDS Top7 IMS reagent as supplied. The bead suspension was plated onto Rainbow agar as per MLG5B.01.

Beads for serotypes O45 and O121 were not commercially available at the commencement of this project[3]. Consequently, samples that were presumptive positive for O45 and/or O121 were:

1. Spread plated onto mRBA agars without prior IMS followed by the selection of 10 colonies that were then be tested for virulence markers using a conventional multiplex PCR.

2. Stored in glycerol for subsequent investigation. The investigation involved IMS of these samples once the beads became available followed by picking of colonies and PCR confirmation in the same manner as described below.

Latex agglutination kits were not commercially available for this project. In their absence, colonies were selected from mRBA plates following incubation and plated onto sheep blood agar

[3] Late in the project Biocontrol provided beads that included these serotypes.

for further analysis. Following overnight incubation, isolates were tested by PCR for the serotype of interest, *stx* and *eae*.

Samples were considered negative for Big 6 serotypes if none of the morphologically distinct colonies tested are positive for *stx*, *eae* and a Big 6 antigen. All isolates recovered from samples shown to contain any or all of the genetic markers (i.e *stx*, *eae* and serotype) have been stored in the CSIRO culture collection and will be available for use in future studies.

3.3 Timeframe for testing

Figure A1 in Appendix 1 provides an overview of respective timeframes involved for each test method including the confirmation test process for the project. The FSIS designations for potential, presumptive and confirmed positives are shown in bold. These designations are also the stage at which a negative result can be reported. The timing is 'ideal' timing assuming that screening tests and confirmatory tests are conducted in different laboratories. Timing could be shortened once latex agglutination reagents become commercially available for confirming O types of colonies directly from mRBA plates. Timing could be shorter or longer depending upon transport times and the operating hours of the laboratories.

Specific points regarding the timing for screen testing system for each method are:

- GDS method IMS step is only a matter of minutes and therefore the screen result should be known in the morning of Day 1 .i.e. there is only one PCR step required
- BAX and Pall GeneDisc Technology have two PCR steps, a screen test followed by an identification test, with the second PCR step only required if the first is positive. Therefore the screen result could be known in the morning of Day 1 but may take around an additional two hours if the *stx*/*eae* step is positive.
- IEH could report initial reactive negatives on Day 1, however, the 'confirmed' result (by the method definition not FSIS definition) was not available until Day 2 in almost all cases in the project. The IEH test is ideally completed in 20 hrs from the time the samples are received by the laboratory. The reason for taking longer in this surveillance project was reported to be that the lab capacity was exceeded.
- For all methods there is time taken in the screening laboratory to check any anomalies. These include QC-related issues, indeterminate, invalid and other 'no result' findings at various steps in the process. Some tests may need to be re-run. However, the time taken for these was not quantified during the project.

3.4 Project operational approach

3.4.1 Sample Collection

The project collected samples of manufacturing beef using a similar approach to the AQIS notice 2011/04 for *E. coli* O157 sample collection except on chilled product in the Boning Room and a 1500g sample was taken – the 1500g was divided into 4 x 375g samples by the laboratories (Symbio or Silliker) and was then put through the four different test methods being trialled.

1. Samples collected in the boning room from cartons of trim (i.e. chilled not frozen).
2. No more than two samples were collected per boning chain per shift.
3. Ideally samples were selected from no less than 12 cartons, preferably at random
4. Each sample comprised 1500g of surface slices or small grab pieces (5-10g each) from across the cartons selected.
5. The sample was collected using sanitised instruments under sanitary conditions.
6. Each sample was placed in a large enough, e.g. 2-3L plastic bag, sealed and sent by overnight dispatch to the allocated laboratory.

Note: There was NO requirement for lot identification or lot retention as the samples are for research purpose only using non-approved methods and individual establishment results are not available.

Samples were sent by overnight courier service to the testing laboratory and packed with freezer bricks to ensure that the temperature on arrival at the laboratory was ≤7°C.

Screening laboratories mixed the sample as well as possible by hand; the aim was to mix the pieces, not to massage the pieces together or to distribute any weep. Inclusion of weep in subsamples was avoided where possible.

The sample was divided into 4 x 375g sub-samples and placed in separate bags for freezing.

The bags were allocated for each enrichment method in random order.

The sub-samples were then frozen to -18°C.

3.4.2 Testing

Frozen samples were thawed in the laboratory at 18-27°C for up to 3 hours before commencing the test (Australian Standard AS 5013.11.2-2006) or if required, were thawed over longer period under refrigeration (as per Australian Standard AS 5013.11.2-2006).

The sub-samples were tested according the prescribed test system's methods as outlined in Appendix 1.

If any broth from one of the 375g sub-samples gave a screening positive (FSIS potential positive) then all enrichment broths associated with that sample from all laboratories had 10mL aliquots of enrichment broth dispensed and sent chilled to CSIRO Brisbane for confirmation testing.

4 Results

4.1 Prevalence of Big 6 STEC in Australian Beef

The prevalence of Big 6 STEC is Australian beef is very low (Table 1). In this survey, 1197 samples were tested by each method (1196 for IEH) and only one positive screening test (potential positive) was confirmed (*E. coli* O26). There were a number of screening test (potential) positives and a small number of presumptive positives. Two further positives were confirmed in broths which initially screened negative.

Table 1: Potential, Presumptive and Confirmed Positives for Big 6 STEC (N=4320) in Australian Beef Trim

Screening assay	Valid screen test results*	Potential Positive	Presumptive Positive	Confirmed Positives**
Bax	871	12 (1.4%)	2 (0.2%)	0
GDS	1194	7 (0.6%)	2 (0.2%)	0
Pall	1063	63 (5.9%)	8 (0.8%)	1 (0.1%)
IEH	1196	15 (1.3%)	3 (0.3%)	0
Total (%)	4324	97 (2.2%)	13 (0.35%)	1 (0.02%)

Notes

* There were a number of indeterminate, invalid and/or inhibited and no amplification results which were excluded from these prevalence data (see the Appendix section 7.3 for further details).

** There were also two isolates confirmed from screen test negative broths. This occurrence would not be picked up in routine testing and therefore not shown in Table 1 (see Table 5.2 for further details).

4.2 Initial screening test results

Table 2 summarises the results for the initial screening tests, including negative, positive and 'no result' outcomes. Each screening method uses a different terminology to describe a "No Result" outcome. The Bax method refers to it as "indeterminate", GDS refers to it as "No Amplification", while Pall refers to it as "Invalid and/or inhibited". The IEH method, since it was conducted in an IEH laboratory, did not report any "No Result" outcome. The confounding factor for interpretation of these data is the nature and reasons for the indeterminate, invalid and/or inhibited and no amplification results differed for each method, as did the manner in which each No Result outcome can be resolved.

A key question for laboratory technicians when they read the results of the screening test is how to treat the No Result outcomes. Importantly, if these kinds of results were to occur during routine screening, the laboratory would need to either:

- Repeat the test using the same procedure
- Modify the procedure in some way e.g. dilute samples
- Consult the supplier of the test, who may provide advice
- Send the sample for confirmation testing with or without repeating the test
- Possibly notify customers of a delay, depending on the time involved.

During the trial period, there was insufficient time to resolve all issues with the No Result Outcomes. The diagnostic companies were provided their data so they could investigate the cause of these issues. Subsequent corrections were made and additional tests conducted to verify the changes were successful in achieving a screening test result. Refer to the Appendix Section 7.3 further details.

Table 2: Breakdown of Initial Screen Test Results by Test Method

	Bax	GDS	Pall	IEH	Total
Negative	859	1187	1000	1181	4227
Indeterminate*	326	0	0	0	326
Invalid and/or inhibited	0	0	134	0	134
No amplification	0	3	0	0	3
Potential positive	12	7	63	15	97
Total	1197	1197	1197	1196	4787

* There were a number of indeterminate, invalid and 'No Result' which were excluded from these prevalence data (see the Appendix section 7.3 for further details).

An analysis was made of the degree to which the four methods agreed/disagreed on samples which were deemed to have a result of negative or positive (i.e. excluding the 'no result' samples). This analysis is presented in Table A2 in the Appendix section 7.2.

4.3 Presumptive and confirmed positives – confirmation test results

Tables 3a to 4b summarise the presumptive positive results of the confirmation testing. Tables 3a and 3b show the results for confirmation of screen test positives (FSIS potential positives) and Tables 4a and 4b show the results for confirmation of screen test negatives that were also tested due to being derived from the same original meat sample. There was one confirmed positive found from screen test positives (Table 3b). There were two confirmed positive isolates of O26 found in screen test negative samples (Table 4b). 'No Result' screening tests were not sent for confirmation.

A total of 17 presumptive positive colonies from positive screen test broths were subjected to PCR confirmation of pSTEC virulence markers (Table 3b). Isolates 7 and 8 in Table 3b represent two different types of O103 colonies recovered from the same sample. All remaining isolates were recovered from independent samples.

E. coli of serotype O26 which harboured *eae* but lacked *stx* were the most common isolate recovered from positive screen test broth (Table 3b).

Discussion on the rate of conversion from potential positives to presumptive and confirmed positives is provided in Section 5.2.

Table 3a Presumptive positives from potential positive broths by O type and test system

	Screening test was positive on broth originating from							
	Bax		GDS		Pall		IEH	
	Potential + ve	Presumptive + ve	Potential + ve	Presumptive + ve	Potential + ve	Presumptive + ve	Potential + ve	Presumptive + ve
O26	3	2			19	5	2	2
O45	6	0			25	1	3	0
O103	2	0			33	2	4	0
O111	0	0			2	0	0	0
O121	2	0			12	0	2	0
O145	1	0			37	0	3	1
Top7			7	2				
Total*	14	2	7	2	128	8	14	3

Note:

* The data in this table represents the total number of O types found in positive screen test broths. The number is higher than the total number of positive screen test broths shown in Tables 1 and 2, due to the fact there was more than one O type found in some of the broths.

Table 3b Virulence gene results for the presumptive positives in Table 3a

Isolate No.	Screen test method	serotype	stx	eae	Confirmed + ve (Y or N)
1	IEH	O145	-	+	N
2	Bax	O26	-	+	N
3	Bax	O26	-	+	N
4	GDS	O26	-	+	N
5	Pall	O26	-	+	N
6	Pall	O26	-	+	N
7	Pall	O103	+	-	N
8	Pall	O103	-	-	N
9	Pall	O103	-	-	N
10	Pall	O45	-	-	N
11	IEH	O26	-	+	N
12	IEH	O26	-	+	N
13	Pall	O26	+	+	Y
14	Pall	O26	-	+	N
15	GDS	O103	-	+	N
16	Pal	O26	-	+	N
17	IEH	O45	-	-	N

Table 4a: Presumptive positives from screen negative broths from same sample group as potential positive broths by O type and test system

Screened on another test as positive for	Negative screen test was found to be presumptive positive on broth originating from							
	Bax		GDS		Pall		IEH	
	Potential + ve	Presumptive + ve	Potential + ve	Presumptive + ve	Potential + ve	Presumptive + ve	Potential + ve	Presumptive + ve
O26	21	1	25	2	5	1	22	2
O45	26	0	33	0	8	0	30	1
O103	34	0	35	1	2	0	33	0
O111	2	0	2	0	0	0	2	0
O121	13	0	15	0	3	0	13	0
O145	39	0	40	0	3	0	37	0
Top7	6	2			7	1	7	2
Total	141	3	150	3	28	2	143	5

A total of 15 colonies from the 13 presumptive positive plates from originally negative screen test broths (shown in Table 4a) were subjected to PCR confirmation of pSTEC virulence markers (Table 4b). Isolates 3, 4, 5, 6 and 7 were found to be presumptive positive for O26 in all four methods' broths from the original sample then all negative at final confirmation.

Isolate 2 (Table 4b) was found to be potential positive (Pall) then subsequently confirmed negative, however, an *E. coli* O26 confirmed positive isolate was found in the broth which had screened negative by another method (GDS).

Isolates 12 and 13 (Table 4b) were found to be Potential Positive in two of broths (GDS, and Pall) from the original sample and then subsequently confirmed negative for those broths, yet an *E.coli* O26 confirmed positive isolate was found in a separate broth which had screened negative (IEH).

Table 4b: Virulence gene results for the presumptive positives in Table 4a

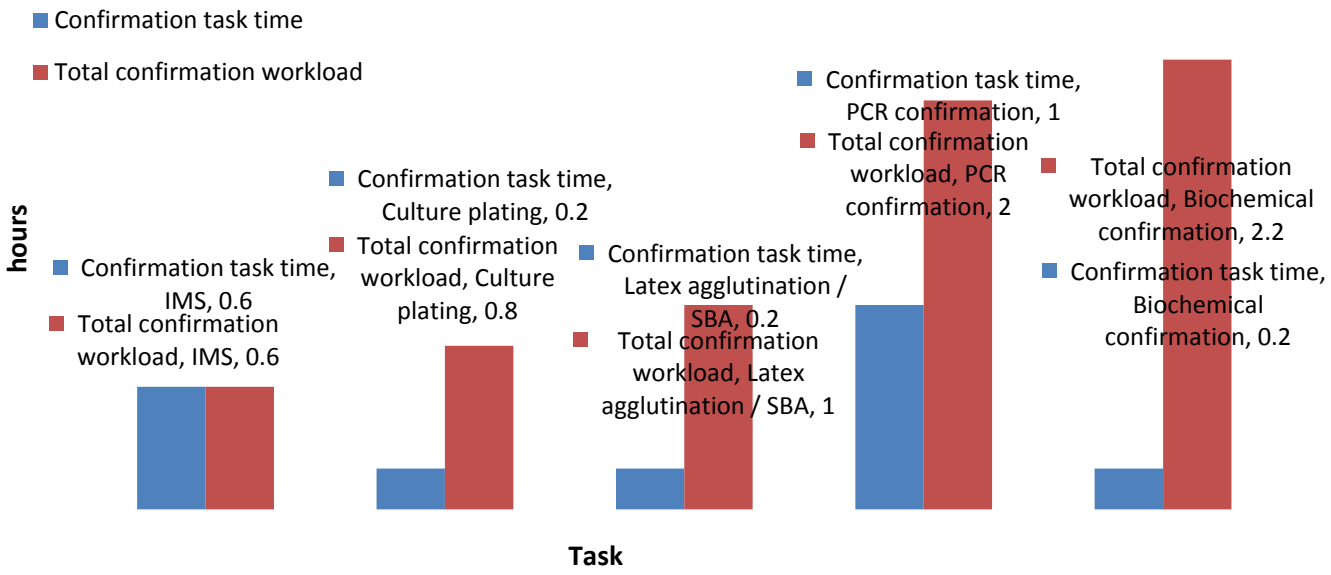
Isolate	Positive Screen test	Negative screen test was found to be presumptive positive on broth originating from…	Serotype	stx	eae	Confirmed + ve (Y or N)
1	Bax	GDS	O26	-	+	N
2	Pall	GDS	O26	+	+	Y
3	GDS	Bax	O26	-	+	N
4	GDS	Pall	O26	-	+	N
5	GDS	IEH	O26	-	+	N
6	Pall	BAX	O26	-	+	N
7	Pall	IEH	O26	-	+	N
8	Pall	GDS	O103	-	-	N
9	IEH	Pall	O26	-	+	N
10	Pall	IEH	O103	-	-	N
11	GDS	Bax	O103	-	+	N
12	GDS	IEH	O26	+	+	Y
13	Pall	IEH	O26	+	+	Y
14	Pall	Bax	O121	-	-	N
15	Pall	IEH	O121	-	-	N

4.4 Confirmation workload

The confirmation phase of the survey proceeded, where possible, as per MLG 5B.01. The MLG 5B.01 procedure outlines five distinct processes within the confirmation process: IMS; culture plating, latex agglutination; PCR confirmation of virulence markers; and biochemical confirmation (refer Figure A1 in the Appendix for further details). Significantly, the baseline survey was conducted without the use of latex agglutination kits as they were not yet commercially available. Latex agglutination kits enable a more rapid assessment of the sample to occur and will

substantially decrease the downstream confirmation timeframe and workload for samples that do not contain a Big 6 STEC. Figure 1 outlines the personnel hours required for each confirmation process and the total confirmation workload required for one sample.

Figure 1: Confirmation workload in personnel hours for individual tasks and the overall confirmation process.



Time estimates are based on a sample that yields at least one Big 6 isolate and are appropriate for the testing of 10 distinct colonies selected from the culture plating.

The total labour hours required to confirm that a sample contains a Big 6 STEC is estimated to be 2.25 hours. It has been the experience of this project that samples that are potentially positive may demonstrate approximately 10 distinct colony types across the four mRBA plates. The time estimates are based around the selection of 10 distinct colony phenotypes from mRBA. Increasing or decreasing the number of colonies to be tested by 50% (i.e. 15 or 5 colonies) will result in a variation in total confirmation workload of ±10%. Once available, the incorporation of latex agglutination kits will allow for a sample to be declared negative following the latex agglutination process. Negative samples can therefore require just 1 hour of personnel time and can be completed within 24 hours of sample receipt by the confirming laboratory.

Importantly, there is no ‘economy of scale’ to the confirmation process and therefore a sample that is presumptively positive for two of the six Big 6 serotypes will require double the personnel effort.

5 Discussion

5.1 Prevalence of Big 6 pSTEC in Australian Manufacturing Beef

The average rate of screening test positives (FSIS potential positives) was 2.2% for manufacturing beef, although one method (Pall Genedisc Technology) accounted for 63 (65%) of the 97 potential positives. As shown in Table 1, the potential positive rates ranged from 0.6% (GDS) to 5.9% (Pall).

From these potential positives, the rate of presumptive positives according to the FSIS definition was 0.35% and the rate of confirmed positives was 0.02% (Table 1). The confirmed positive serotype was O26. Because broths from negative screen tests from the same original meat sample were also tested in this project (i.e. the cohorts for potential positives), two further isolations of O26 occurred. This would not happen during normal testing.

The low detection levels of this baseline study may be compared with a CSIRO study where 300 faecal samples had 30 (10%) potential positives and no Big 6 STEC (Barlow and Mellor, 2010).

The low rate of confirmation could be due to the presence of many non-toxin producing strains in Australian cattle (Barlow and Mellor, 2010 and Tables 3b and 4b). The use of selective supplements in the mRBA plates would also be likely to reduce the number of strains able to be isolated. Barlow and Mellor (2010) had more success in isolating strains from potentially positive samples than in this present study. If FSIS modifies the isolation medium to use lower concentrations of selective supplements, then it is likely that the number of presumptive positive samples will increase. If IMS beads were available for O45 and O121, then the number of presumptive positives may also have increased[4]. However, a higher number of presumptive positives would not necessarily be translated into a proportionately higher rate of confirmed positives due to the non-toxin producing nature of most strains.

Another possible limitation is that a more rigorous culture confirmation step (selecting a larger number of colonies from mRBA plates) may have detected a higher number of positives. With both O157 and non-O157 culture confirmation it is often necessary to pick and examine several sets of colonies to find a positive colony.

5.2 Conversion of potential positives to presumptive positives

Overall, the rate of conversion of potential positives to presumptive positives was low (less than 10%). Potential positives for serotype O26 were the only samples that differed from the overall trend with 9/24 (38%) yielding a presumptive positive (Table 3a). The O26 potential positives also accounted for all the pSTEC isolated (confirmed) during the survey. The low conversion rate of potential positives to presumptive positives may suggest that confirmation is problematic and the likelihood of missing pSTEC is a realistic concern. Whilst the possibility that pSTEC may not

[4] IMS beads became available for those serotypes towards the end of this project but the procedures were not altered.

be detected is real, as it is with any pathogen detection testing protocol, there are several reasons for a low ratio of potential positives to presumptive positives.

The most important consideration is that the genetic targets used to identify potential positives are not exclusive to pSTEC. The genetic targets can be found in generic *E. coli*, shiga-toxigenic *E. coli* other than pSTEC, other pathogenic types of *E. coli* that are not targets, and in other species of bacteria. In enrichment broths containing diverse bacterial populations (i.e manufacturing beef samples) it appears that the majority of the potential positive samples harbour a range of organisms that between them contain the appropriate genetic targets. This scenario is distinct from one where the enrichment broth contains a bacterium that harbours all of the genetic targets of interest.

This study appears to have this scenario where multiple organisms give rise to the necessary positive genetic tests to create a potential positive at the screening stage more often than the scenario where a single bacterium harbouring all genetic targets is present.

Similarly, finding a presumptive positive in one of the screen test negative broths (Tables 4a and 4b) does not necessarily indicate a false negative in a screening test because they may have deemed the sample negative for stx and/or eae in the first PCR step and therefore been called negative for that reason, even though the sample may have had several of one or more of the O type targets present.

Despite this, the inherent limit of detection associated with PCR and IMS, background bacteria on the meat sample prior to enrichment, and the incorporation of antibiotics in media used in the confirmation phase may also contribute to the low conversion rate of potential positives to presumptive positives. However, these factors are unlikely to be the dominant factors affecting the low conversion rate observed in studies such as this and furthermore should be consistent across the testing systems in place throughout the world. Testing methodologies will continue to evolve and the specificity of screening tests with respect to pSTEC will improve and the conversion rate of potential positives to presumptive positives could be expected to subsequently increase. In the short term, it is expected that the conversion rate will be consistent with that observed in this study.

5.3 Comparing performance of test methods

The screening tests are all designed to detect the presence of genes in an enrichment broth and the present survey was set up to compare the performance of four methods using Australian manufacturing meat intended for export to the USA for grinding.

The FSIS definitions are based on the same genes being present in a single bacterial cell. Thus, the decrease in positive results from potential positives to presumptive positives to confirmed positives (Table 1) is to be expected. The fact that there is a low rate of potential positives to presumptive positives and then a very low rate to confirmed positives should not be viewed as an indication of a ‘false positive’ rate. The FSIS definition is complex (in that it requires several characteristics to be met by a single cell) which cannot be met easily (i.e. not within a single step process) by current technologies. The diagnostic tests inevitably cast their net wide at the early stage, and then rely on confirmation procedures to deal with screening test detections. This is evidenced by the eventually determined genetic profile of presumptive positive colonies from both positive and negative screen test broths (Tables 3b and 4b respectively).

Nevertheless, the fact remains there was a wide range of screen test potential positive results across the four methods evaluated which resulted in only one confirmed positive from the total of 97 potential positives for the project (Table 1).

A feature of the present study was the number and type of 'No Result' outcomes (Table 2). These were resolved eventually and the Appendix Section 7.3 provides further details on the issues associated with the different types of 'No Result' outcomes that occurred during the initial screen testing in this project.

5.4 Challenges for meat processors

It is understandable that meat companies might gravitate towards methods that produce the lowest rate of potential positives in order to save time, cost and inconvenience when there is a low likelihood of confirmed positives being found in any given method.

A key consideration for meat companies intending to perform screen testing on-site is the frequency with which potential positives are picked up, resulting in:

- Potential disruption to shipping arrangements
- Added costs of testing at the confirming laboratory
- Additional administrative procedures at the on-site or off-site freezer facility
- Unacceptability of the lot to those customers who will not accept screen-positive lots

This is inevitably going to result in more product being held up due to time delays waiting for test results. The time delays are due to the need for isolating and detecting an additional six target organisms in the one sample, with each target requiring the present of multiple attributes (genes) that may or may not be present in any or all of the organisms present. The testing process is longer because of the need to replicate organisms to a detectible level (enrichment) which is longer than for O157 and then to isolate the targets from other non-target organisms which may have some of the genes.

Figure A1 shows the overall process of confirmatory testing and the time taken to give results at presumptive and confirmed stages in an ideal situation. Meat companies could expect a similar rate of presumptive positives which need to go through the full confirmation process as shown in this project.

5.5 Challenges for diagnostic companies

This study did not validate any method according to FSIS requirements. There have been no attempts to determine the sensitivity of the methods, nor whether the methods will detect all strains of interest. Diagnostics companies need to validate methods as being equivalent to the FSIS method in order to gain approval by DAFF (AQIS) and/or NATA (if required) in Australia. The biggest challenge for diagnostics companies is to ensure they continue to improve the methods to suit the nature testing in the meat processing industry.

5.6 Likely changes to methods

The FSIS is continuing to develop their method. At present, FSIS would use different enrichment broths for O157 and non-O157 STEC. Seeking to combine methods may lead to changes in the

FSIS enrichment broth, and possibly other methods. It is also possible that FSIS will change other aspects of their method. Given that there is an expectation that methods used by industry should be equivalent to FSIS methods, this places a requirement on diagnostic companies to continue to revalidate their method against the latest FSIS method.

The method used in this project for confirmation followed the FSIS method as far as possible, but

- IMS for O45 and O121 was only conducted on frozen samples; and
- Latex agglutination reagents for O typing of colonies directly from mRBA plates were not available at the time of the project.

Availability of latex agglutination reagents would have saved time and cost.

6 References

Barlow, R. & Mellor, G. 2010. Prevalence of enterohemorrhagic *Escherichia coli* serotypes in Australian beef cattle. *Foodborne Pathogens and Disease*, vol. 7, no. 10, pp. 1239-1245.

FSIS. 2011. Shiga Toxin-Producing *Escherichia coli* in Certain Raw Beef Products. Docket Number FSIS-2010-0023. Federal Register 76(182) 58157

Paton, A. W. & Paton, J. C. 2010. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for *stx1*, *stx2*, *eaeA*, Enterohemorrhagic *E. coli hlyA*, rfb_{O111}, and rfb_{O157}. *Journal of Clinical Microbiology*, vol. 36, no. 2, pp. 598-602.

7 Appendices

7.1 Appendix 1 Detailed diagnostic methods

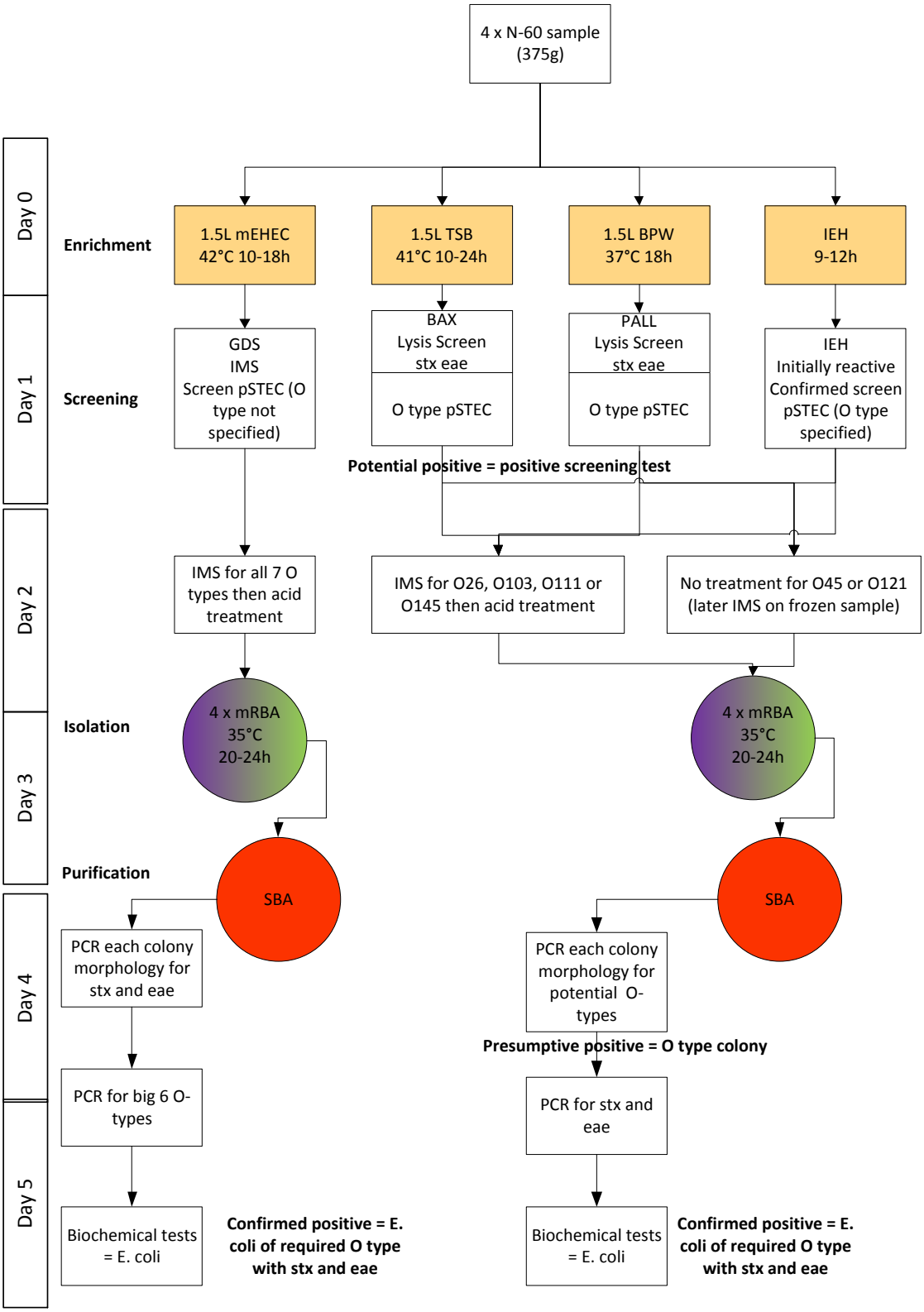


Figure A1 - Flow diagram summarising the testing conducted in this project.

The FSIS designations are shown in bold in Figure A1. The timing is 'ideal' timing assuming that screening tests and confirmatory tests are conducted in different laboratories. Timing could be shorter or longer depending upon transport times and the operating hours of the laboratories.

7.1.1 BioControl Assurance GDS

Sample Preparation

Beef Samples – aseptically weigh 375g test portion into 1,500 mL pre-warmed (42°C) mEHEC®. Masticate or homogenize sample by hand for 2 minutes.

Incubate for 10-18 hours at 42°C.

Test Procedure

Transfer 20 µL Concentration Reagent to each of the required number of Assurance GDS sample wells. Add 1.0 mL of incubated sample to each sample well. Vortex. Using PickPen, collect magnetic coated particles from sample. Transfer to Wash Solution and release. Retrieve particles with PickPen and transfer to resuspension plate containing Resuspension Buffer.

Transfer 30 uL of sample from the resuspsension plate wells into each GDS Amplification Tube. Place Amplification Tube into Assurance GDS Rotor-Gene®.

Upon completion of the run each sample will be identified as **Positive** or **Negative** for *E. coli* O157:H7, and Positive or Negative for Top STEC, or **No Amp.** The individual gene results (*eae*, *stx*1, *stx*2) are also presented.

E. coli O157:H7 Results:

Positive: Samples are positive for *E. coli* O157:H7

Negative: Samples are negative for *E. coli* O157:H7

No Amp: Amplification did not occur. Repeat the test beginning with the incubated mEHEC broth for that sample

Top STEC (*eae*/*stx*) Results:

Positive: Samples are positive for *E. coli* that belong to O serotypes O103, O111, O121, O145, O26, and O45 and contain the *eae* gene and one or both of the shiga toxin genes *stx*1 or *stx*2.

Negative: Samples are negative for *E. coli* that belong to O serotypes O103, O111, O121, O145, O26, and O45 and contain the *eae* gene and one or both of the shiga toxin genes *stx*1 or *stx*2.

No Amp: Amplification did not occur. Repeat the test beginning with the incubated mEHEC broth for that sample.

7.1.2 IEH

Beef samples will be tested for the presence of:

(1) *E. coli* O157
(2) Non-O157 *E. coli* including O26, O45, O103, O111, O121, and O145

Screening for *E. coli* will be performed using the IEH Multiplex Polymerase Chain Reaction Test System (IEH Test System) which detects *E. coli* O157 and Non-O157 Shiga toxin producing *E. coli* including O26, O45, O103, O111, O121, and O145.

Sample Preparation

Samples will be enriched in media at 42°C for 9 – 12 hrs.

Test method overview

During initial screening, the IEH Test System applies a 6-band multiplex Polymerase Chain Reaction (PCR) assay to aliquots of the sample enrichment. Three of the PCR assay targets screen for gene segments known to be associated with *stx*-producing *E. coli* (STEC) with intimin, while a fourth targets the specific gene associated with the O157 antigen. The pathogenic targets are intimin and Shiga-like toxin genes. The two remaining targets are gene segments specifically associated with *Salmonella* spp.

An additional aliquot will be subject to lateral flow analyses to determine the presence of *E. coli* O157 as well as the top 6 non-O157 antigens.

Enrichments that are positive for *eae* and *stx* 1 and/or 2 will be considered Initial Reactive (IR) for non-O157 STEC and will be subject to the secondary sets of reactions (Molecular Confirmation). Enrichments that are positive for *eae* and *stx* as well as O157 specific targets will be considered Initial Reactive (IR) for *E. coli* O157 and will be subject to the secondary sets of reactions (Molecular Confirmation).

Molecular confirmation includes three new PCR multiplex assays. Two of the assays confirm that the initial reactive is an STEC or specifically O157. The remaining assay includes genetic targets which allow specific differentiation between each of the “Big Six” serotypes. A positive result obtained by molecular confirmation is a “presumptive positive”.

7.1.3 Pall GeneDisc Technology

Sample Preparation

Weigh 375g sample (beef trim) into a filter stomacher bag

Add 1.5L BPW (prewarmed at 41.5 ± 1°C) and Stomach for 2 min.

Incubate 37 ± 1°C for 16 to 20 hours, (See Note 1)[5]

[5] In this project the screening laboratories incubated Pall samples at 37^{o}C ± 1^{o}C as instructed by Pall personnel. Pall Genedisc Technology also provides a 10 hour enrichment method.

Transfer 50 microlitres into the lysis tube.

Heat for 10min @ 100°C.

The processed sample is added to the appropriate GeneDisc plate or PCR tube (6 microlitres per well).Add an equivalent volume of “master mix”. Vacuum the sample into the wells. Place 2-3 drops of mineral oil into the GeneDisc, vacuum the oil to the wells.

Note: If samples are not tested immediately, store extracted sample on ice, or at 5°C ± 3°C, until PCR Analysis. For storage longer than 6h, freeze sample at -20°C ± 2°C.

Test Procedure

Run GeneDisc as per GeneDisc operating instructions

Results under “General” on the computer are for *E. coli* 0157 and pathogenic STEC

All GeneDisc positive samples are then run for the detection of pathogenic *E. coli* 0157 and the Big 6 non-O157 STEC (O26, O103, O111, O145, O121, O45)

Note 1: After the enrichment step a sample can be stored up to 72 hrs at 4 °C.

Note 2: Use Sterile powder free gloves and change gloves before loading GeneDisc plate

Note 3: If samples are not tested immediately store extracted samples at 5 ± 3°C until analysis. For storage longer than 6hs, freeze sample at -20 ± 2°C.

7.1.4 Bax

Sample Preparation

Add 1.5L Tryptic soy broth (TSB) (prewarmed to 46°C)

Incubate at 41°C for 10-24 hours

Transfer 200µl prepared lysis reagent to each cluster tube

Transfer **20µl** enriched sample to each cluster tube

Heat at 37 ± 2°C for 20 minutes

Transfer to 95 ± 2°C for 10 minutes

Cool for 5 minutes in cooling block

Hydrate PCR tube with **30 µl** of lysate, then seal with flat optical cap

(PCR tablets must be hydrated and re-sealed with 10 minutes after removing

the caps from the PCR tubes).

* Remaining lysate is then sealed and stored for additional testing if necessary.

Run BAX amplification and detection system as per current BAX user guide for Screening Assay Results (*stx* and *eae*)

Review of results for STEC Screen :

Positive **(+)** : A red well icon with a “plus” sign indicates that both *eae* and *stx* are present in that sample. The amplification plot shows a rise in the *stx (*blue) and *eae* (green) targets.

Confirmation to be carried out using the Panel 1 and 2 assays

Negative (-): A green well icon with a “minus” sign indicates that the combination of the *stx* and *eae* is not present in that sample. If only one of the *stx* or *eae* targets is present, the sample is considered negative.

Indeterminate (?) Targets and Internal positive control are negative.

Signal error (-) See BAX User Guide for details .

Review of results for Panel 1 and 2:

Panel 1 Assay Results: (*E. coli* O26,O111,O121)

Positive **(+)** : A red well icon with a “plus” sign indicates that one or more of the Panel 1 targets are present in that sample. **Confirmation to be carried out.**

- *E. coli* O26- the amplification plot shows a rise in the O26 (gold) target

- *E. coli* O111- the amplification plot shows a rise in the O111 (grey) target

- *E. coli* O121- the amplification plot shows a rise in the O121 (purple) target

Negative (-): A green well icon with a “minus” sign indicates that none of the Panel 1 targets are present in that sample

Panel 2 Assay Results: (*E. coli* O45,O103,O145)

Positive **(+)** : A red well icon with a “plus” sign indicates that one or more of the Panel 2 targets are present in that sample. **Confirmation to be carried out.**

- *E. coli* O45- the amplification plot shows a rise in the O45 (magenta) target

- *E. coli* O103- the amplification plot shows a rise in the O103 (brown) target

- *E. coli* O145- the amplification plot shows a rise in the O145 (turquoise) target

Negative (-): A green well icon with a “minus” sign indicates that none of the Panel 2 targets are present in that sample

7.1.5 Confirmation methods

FSIS MLG 5B.01 was followed for IMS and culture plating. Colonies were picked and streaked on Sheep Blood Agar prior to PCR for confirmation. The PCR step for O-types utilised the same primers as specified for screening in the FSIS MLG 5B.01 method. PCR for *stx* and *eae* were performed according to the method of Paton and Paton. 1998). Biochemical confirmation was performed using the Microbact 12E kit.

7.1.6 Control strains

The quality control procedures for the detection and isolation of pSTEC were consistent with those currently in place for *E. coli* O157 testing. Sample batches included a positive growth control, a negative control sample and an uninoculated media control. Laboratories typically use an attenuated *E. coli* O157 strain (e.g FSIS strain 465-97) as the positive growth control and *E. coli* ATCC 25922 as the negative control. These strains are also used as DNA extraction and PCR controls. In addition to the abovementioned controls, laboratories may require PCR positive controls for the pSTEC serotypes. Listed below are the suggested PCR controls for screening and confirmatory PCR.

PCR Controls

a. ***stx/eae* screen PCR**

- DNA template from bioluminescent *E. coli* O157:H7 (DNA extraction positive control)
- DNA template from *E. coli* ATCC 25922 (DNA extraction negative control)
- DNA template from a cocktail of top six STEC cultures (PCR positive control)
- No Template Control (NTC)

b. ***wzx* screen PCR**

- DNA template from a cocktail of top six STEC cultures (PCR positive control)
- NTC

c. ***stx/eae* confirmatory PCR**

- DNA template from a cocktail of top six STEC cultures (PCR positive control)
- NTC

d. ***wzx* confirmatory PCR**

- DNA template from a cocktail of top six STEC cultures (PCR positive control)
- NTC

The FSIS guidebook MLG5B.01 details the preparation of DNA template from a cocktail of the top six pSTEC cultures which can be prepared in bulk and stored at <-20°C for up to one year. This provides an option for laboratories not wanting to handle additional pSTEC cultures to have DNA prepared offsite and shipped to them as required. Laboratories conducting confirmation will need to have a complete set of pSTEC controls for use as plating controls. However, for safety reasons, toxin-negative or toxin-attenuated strains can be used provided they have an appearance on mRBA typical of pSTEC.

Screening systems that employ an IMS step prior to testing for *stx* and *eae* do not need additional controls to those listed above. Users should note that the DNA extraction negative control should not be put through the IMS step as it will not be picked up by the IMS beads and will result in an invalid screening test result. Similarly users must ensure that the DNA extraction positive control can be recovered with the IMS beads they are using. In cases where beads for O157 and the additional Big 6 serotypes are used than the controls listed above are satisfactory. However, if the system in use only targets the Big 6 serotypes then the DNA extraction positive

control must reflect this. Users of these systems may also choose to use a toxigenic O157 (*stx+/eae+*) as the DNA extraction positive control and the PCR positive control for the *stx/eae* screening test. This approach would satisfy the control requirements for the *stx*/*eae* screening PCR but laboratory personnel safety and possible cross-contamination scenarios should be considered prior to implementing this approach.

7.2 Appendix 2: Agreement between methods

Table A2. Observed distribution of agreement for four assays screening for Big 6 pSTEC in Australian beef trim

This table excludes data from any cohort of samples where at least one of the tests yielded “No result”

Category of result	Result pattern Bax/GDS/Pall/IEH	Number with category	Number in category	Pattern (%)	Category (%)
Complete agreement negative	NNNN	705	705	90.9%	90.9%
Complete agreement positive	YYYY	0	0	0.0%	0.0%
Balanced disagreement	YYNN	0	9	0.0%	1.2%
	YNYN	0		0.0%	
	YNNY	1		0.1%	
	NNYY	5		0.6%	
	NYNY	0		0.0%	
	NYYN	3		0.4%	
Minority disagreement A (mostly results negative)	YNNN	10	60	1.3%	7.7%
	NYNN	1		0.1%	
	NNYN	43		5.5%	
	NNNY	6		0.8%	
Minority disagreement B (mostly results positive)	NYYY	2	2	0.3%	0.3%

	YNYY	0		0.0%	
	YYNY	0		0.0%	
	YYYN	0		0.0%	
Total		776	776	100%	100.0%

Notes

Y = Screening test positive/FSIS potential positive

N = Screen test negative

7.3 Appendix 3: Resolving the 'No Result' Outcomes in Screen Test

As outlined above in this report, there were a number of 'No Result' outcomes that occurred in the initial screen test for this project. These 'No Result' outcomes were a mix of indeterminate, invalid and/or inhibited, and no amplification results depending on the nature of the issue and the reporting terminology of the method. Table 2 outlines the number of these occurrences by method, which in summary were:

- 27% indeterminate results by the Bax system
- 0.2% no amplification by the GDS system
- 11.2% invalid and/or inhibited or no result by the Pall system
- No information provided for the IEH system

It is important to note that

7.3.1 Resolution and supplementary testing for the Bax method

As reported, during the initial trial 27% of the samples tested by the Bax system gave an indeterminate result. This rate of indeterminates was almost identical at the two screening laboratories that performed the tests. A number of investigations were undertaken, but Bax were unable to resolve the issue during the initial trial. However, subsequently Qualicon (Bax) personnel identified an equipment fault in the machines at both laboratories, repaired that fault and checked the solution worked. They then engaged Symbio to conduct a supplementary trial of 100 samples using the same procedures as for the initial project. The subsequent trial resulted in nil indeterminate results and a rate of screening test positive consistent with the initial trial (refer to Table 2 and Table A3 below). The potential positive O45 was eventually found to be negative by CSIRO.

Therefore, it would be reasonable to assume the indeterminate results found during the initial trial had been due the problem with the equipment as determined by Qualicon (Bax).

Qualicon (Bax) provided the following comment on these results and requested its inclusion in this report:

"After reviewing the preliminary data from the study, we were concerned about the quality of the results, which showed atypical amplification plots and indeterminate results at rates that were grossly inconsistent with historical customer experience. We began an in-depth investigation to determine the root cause of these unexpected results. First we tested a variety of BAX(R) System assays on the same Q7 instruments, where similar patterns suggested that the issue was related to hardware rather than chemistry of the assays. An inspection of the two instruments used in this study showed that neither contained the correct Q7-customized tube holder. After installing the correct Q7 tube holders in both units, another 100 samples were tested, with zero indeterminate results, and one potential positive result for STEC O45, which was in line with the expected rate."

Table A3: Screening Test Results for Bax Method in Main Trial (n=1197) and Supplementary Trial (n=100)

Screen Test Result	Bax Results in Main Trail	Bax Results in Supplementary Testing
Negative	859 (77%)	99 (99.0%)
Indeterminate	326 (27%)	0
Invalid and/or inhibited, no result	0	0
No amplification	0	0
Potential positive	12 (1.0%)	1 (1.0%)
Total	1197	100

7.3.2 Resolution of results for the GDS Method

As reported, during the initial trial 0.2% of the samples tested by the GDS system were recorded as no amplification (“No Amp”). The GDS Directions for Use states that “No Amp” should be rerun. At one of the screening laboratories, the GDS tests were repeated for two samples and a result obtained for these in both cases. The 0.2% represents 3 samples that had not been repeated at the other screening laboratory due to time constraints and potential interruption to the overall project where concurrent testing was undertaken. Based on the experience at the first screening lab, it is reasonable to assume that if those three samples were repeated, a result would have been obtained.

7.3.3 Resolution and supplementary testing for the Pall method

As reported, during the initial trial 11.2% of the samples tested by the Pall system were recorded as an invalid and/or inhibited or no result by the Pall system. The 11.2% represents samples that had not been repeated at one of the screening laboratories only. At the other screening laboratory all 38 Pall tests that came up as invalid and/or inhibited or no result were repeated and a result obtained for these in all cases. Repeat testing involved dilution of the sample. In the other laboratory invalid and/or inhibited results were only repeated during the early stages of the project before repeating ceased and they were noted as “Invalid and/or inhibited” due to project time restrictions, practical considerations, limited kits and the cost of either repeating or sending for confirmation.

Pall, the manufacturers of the GeneDisc Technology, have investigated the results as to why there are a higher than expected number of inhibited and invalid results. They have reported that the parameters set here to analyze PCR results are not the actual ones. Pall set new parameters that improve the number of positives and the rate of inhibition but it was not possible to identify all the data files necessary to allow the results to be recalculated and presented here.

7.4 Appendix 4 FSIS MLG 5B.01