# final report

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# Pathogenic E.coli in the red meat industry

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# Contents

# Executive Summary

## Where were we in 2007

By 2007 there was a substantial amount of information available relating to the presence of *E. coli* O157 in red meat production and processing systems. Researchers had developed sensitive and specific methods for the detection, isolation and enumeration of *E. coli* O157 from ruminants such as cattle but also from the environments that the animals encounter during production and slaughter. The methods enabled studies to be conducted that aimed to measure the impact of various production parameters on the food safety risk caused by *E. coli* O157. Key areas identified for future research in relation to *E. coli* O157 at the time included investigating the mechanisms of survival and persistence of *E. coli* O157, the impact of processing practices in relation to the transfer of *E. coli* O157 from animal to carcase, and the influence of weather conditions on contamination of animals and their environments. Better understanding of these areas would assist in developing management strategies to control the persistence and spread of *E. coli* O157.

At the same time, evidence was mounting to suggest that pathogenic *E. coli* strains of serotypes other than O157 could be present in red meat production animals and potential existed for red meat to become contaminated with these other pathogenic *E. coli* strains. The growing awareness raised concerns about the implications of producing Australian meat exports that harboured such isolates. As many countries to which Australia  exports red meat were already testing for the presence of *E. coli* O157 in imported meat, the possibility that they may begin to test for other pathogenic *E. coli* strains such as non-O157 enterohaemorrhagic *E. coli* (EHEC) was considered likely.

Epidemiological information from human clinical cases that could not be attributed to *E. coli* O157, suggested that a range of non-O157 EHEC serotypes such as O26, O111, O45, O103, O121, O145 and O91 were more associated with human clinical disease than the majority of other *E. coli* serotypes. Furthermore, it was believed that the vehicle of transmission in some of these cases may have been foods derived from animals and in particular cattle. Little information was available on the prevalence and types on non-O157 EHEC in foods of animal origin. The absence of

this information was primarily due to the lack of reliable methods for the detection and isolation of these organisms. Consequently it was determined that a review of methods available for the detection and isolation of non-O157 EHEC was required. The review would identify appropriate methodologies which would then be implemented, validated and used to generate data on the prevalence and concentrations of non-O157 EHEC in cattle. Once such information had been generated the mechanisms used by these non-O157 EHEC to survive and be transmitted through processing environments was to be determined, along with identification and development of control strategies.

During the course of the project it became clear that the low prevalence of non-O157 EHEC in animals meant there would be few isolates available for determining survival mechanisms and transmission routes. The project direction was changed in consultation with MLA to provide a greater understanding of the virulence factors associated with pathogenic *E. coli*. As EHEC harbour virulence genes (*eae* and *stx*) that are found in enteropathogenic *E. coli* and Shiga-toxigenic *E. coli*, a survey of non-O157 EHEC will provide insights into the prevalence of these pathotypes in beef cattle and may pose further questions worthy of investigation. In addition to generating data on other *E. coli* pathotypes, this study will continue to develop knowledge on EHEC virulence. It is still unknown why some EHEC strains are more likely to cause human disease then others. Genetic characterisation and subsequent comparison of EHEC isolates from this study and from human sources may identify genes that are highly correlated with disease-causing EHEC. Data on the prevalence of additional *E. coli* pathotypes and the genetic relationships of EHEC will assist in managing these food safety risks.

## Where we are in 2010

A review of methods for the detection and isolation of non-O157 EHEC determined that a multi-step protocol would provide an holistic approach to the detection, isolation and characterisation of non-O157 EHEC in red meat systems. The approach initially determines which samples harbour a combination of genes (*stx* and *eae*) commonly found in EHEC isolates before determining whether samples containing this combination of genes also contain any of the *E. coli* serotypes of interest. Isolation of the target organism follows and samples from which a non-O157 EHEC

isolate is recovered are enumerated using a combinatorial polymerase chain reaction (PCR) and most probable number (MPN) technique. This approach is consistent with methods proposed by the Food Safety and Inspection Service (FSIS) and the European Food Safety Authority (EFSA) for non-O157 EHEC.

The methods developed in this project were used to conduct an investigation to determine the prevalence of non-O157 EHEC in Australian beef cattle. Eight EHEC serotypes encompassing the epidemiologically significant serotypes of the United States and Europe were included in the survey. Molecular screening of enriched faecal samples determined that 128 of 300 (42.6%) contained *stx* and 152 of 300 (50.6%) contained *eae*. A total of 78 of 300 (26%) samples had *stx* and *eae* present in the same sample. *E. coli* of nearly all target serotypes were recovered, however there was a lack of EHEC virulence markers in most isolates. The results indicated that the prevalence of EHEC serotypes other than *E. coli* O157 in the Australian beef cattle population is low thereby reinforcing Australia's position as a source of quality meat products. The complexity associated with non-O157 methods was highlighted during this study. A large proportion of samples were shown to contain *eae*, however this failed to translate into the isolation of EHEC isolates. Isolates harbouring *eae* could be isolated from beef cattle but they lacked additional EHEC virulence markers and accessory genes. This finding, in combination with the isolation of STEC from a number of samples reinforces the need for non-O157 methods to result in the isolation of an EHEC. As the isolation phase of the method is considered the least optimised component, the need for enhanced isolation protocols is evident.

In addition to the increased attention being placed on non-O157 EHEC, there remains a need to further understand *E. coli* O157. There is increasing evidence that specific genotypes of *E. coli* O157 are more likely to cause human disease than others. Genetic analysis of a collection of Australian O157 isolates from animals and humans determined that there were no obvious differences in the presence of virulence-associated genes in these isolates. However, *E. coli* O157 could easily be differentiated from other pathotypes of *E. coli* based on the presence or absence of virulence-associated genes. As the interrogation of *E. coli* O157 strains that are associated with outbreaks of human disease continues, it is probable that a genetic signature(s) associated with increased pathogenicity will be identified.

## Where to from here

Australia will continue to export quality Australian beef to overseas countries and point of entry testing will remain a concern to all beef producers. The United States have suggested that non-O157 EHEC are likely to be declared an adulterant in beef products in the near future. Although strategies and methods for the detection and isolation of non-O157 EHEC have been developed and implemented at a research level, their implementation at an industry level is currently a challenge from both a technical and practical viewpoint. Demonstrating the equivalence of methods worldwide is problematic because of a lack of commercially available test kits and consumables. It is likely that the methods may be refined to enable to be outside of research settings. Australia should continue to monitor the impact of any changes to the methodology so that current applicable industry data is available for trade negotiations.

Studies on non-O157 EHEC have centred on a specific set of serotypes that have been identified via epidemiological investigations. This approach is the best available at present using the information at hand. However, EHEC encompass a range of serotypes much broader than those included in current non-O157 EHEC methods. In addition it remains unclear as to why some EHEC strains are commonly associated with outbreaks of human disease whereas others are not. Investigations are continuing into the factors that yield enhanced pathogenicity in EHEC strains. The results of these studies should identify specific genetic markers that could be incorporated into alternative non-O157 EHEC methods thereby developing a method which targets EHEC that are likely to cause human disease irrespective of serotype. Such an approach would reduce the complexity of testing and should enable uptake by the industry if required.

Future studies on non-O157 EHEC should target the following areas:

- Increase sensitivity and specificity of methods for the detection and isolation of non-O157 EHEC via adoption of enhanced isolation protocolsDetermine which non-O157 EHEC serotypes of interest are most likely to be associated with Australian beef cattle and identify additional animal reservoirs of non-O157 EHEC (e.g sheep and goats)

- Determine if the prevalence of non-O157 EHEC in beef cattle faeces is indicative of what is present at the end of slaughter i.e. do the contamination dynamics of beef carcasses and boxed beef reflect what has been observed with *E. coli* O157?

- Identify genetic and/or phenotypic markers that are common to EHEC most likely to cause human disease. Incorporate these markers into a rapid screening protocol for implementation at the abattoir level

# General Project Summary

## Milestone 1 – Review the current methods used to study pathogenic *E. coli*

There is uncertainty as to what defines non-O157 EHEC that have the potential to cause human disease and therefore methods for their detection, isolation and enumeration have been lacking. The two general approaches for detection of non-O157 EHEC are to target all Shiga-toxigenic *E. coli* (STEC) initially, then define their potential pathogenicity by serotyping and virulence gene typing or alternatively target only those specific serotypes most often associated with human disease (e.g O26, O111, O145 etc). The first approach suffers in that it casts a broad net requiring significant effort which will capture isolates that are of little human health concern whereas the second approach which focuses on specific serotypes may potentially miss non-O157 EHEC that may be of increasing human health concern.

The methods available to detect Shiga toxins are many and varied and as such the ability to detect Shiga toxin or the genes that encode the toxins is considered routine even for commercial testing laboratories. The use of immunomagnetic separation (IMS) for the detection of O157 EHEC has long been used and the development of similar IMS beads for other STEC serotypes is seen as a natural progression with commercially available IMS beads already available for O26, O111, O145 and O103. The use of polymerase chain reaction( PCR; conventional or real-time) provides a way to rapidly screen samples for the presence of defined serotypes and in the case of real-time PCR, should provide an opportunity to enumerate a specific serotype within a sample. The isolation and characterisation of *E. coli* belonging to particular serotypes is crucial as not all strains within a serotype may carry *stx*.

Isolation with specific plating media is more problematic as there are no specific phenotypic markers present in all non-O157 EHEC that can be used for differentiation from other *E. coli* present in a sample. The use of colony hybridisation either with Hydrophobic Grid Membrane Filtration or nylon membrane and molecular probes targeting either *stx* or O serotypes is one of relatively few methods used for the isolation of STEC. However this approach is seldom used outside of research laboratories. The enumeration of STEC using *stx* as the target is also problematic as

strains of STEC can carry multiple copies of *stx* and consequently it is not possible to directly relate *stx* levels to the number of STEC in a sample. Nevertheless enumeration based on gene target copy number provides meaningful and comparable data from which valid conclusions can be drawn and is applicable to the enumeration of *stx* in red meat systems.

A multi-faceted approach to the detection, isolation and characterisation of non-O157 EHEC is required (Figure 1). This multi-faceted approach will encompass the above mentioned approaches and will target both a defined serotype group of O26, O111, O145, O103, O91, O45 and O121 as well as all STEC by virtue of *stx* interrogation of samples. Detection of target serotypes or *stx* will be performed using PCR thereby providing rapid point estimates of prevalence. Combinations of PCR (conventional and real-time) and the most probable number (MPN) technique will form the basis of enumeration with colony hybridisation and IMS central to isolation methodologies. Isolates will be characterised by PCR for EHEC associated virulence factors and in addition for the major pathogenic *E. coli* virulence factors. The latter characterisation is included primarily to compliment the serotype specific approach as it is probable that *E. coli* of the target serotypes will be isolated that are not EHEC. The development and implementation of the above mentioned multi-faceted approach would provide a holistic approach to the detection, isolation and characterisation of non-O157 EHEC in red meat systems and should generate data that is directly comparable to what will be generated through similar studies in the EU and US.

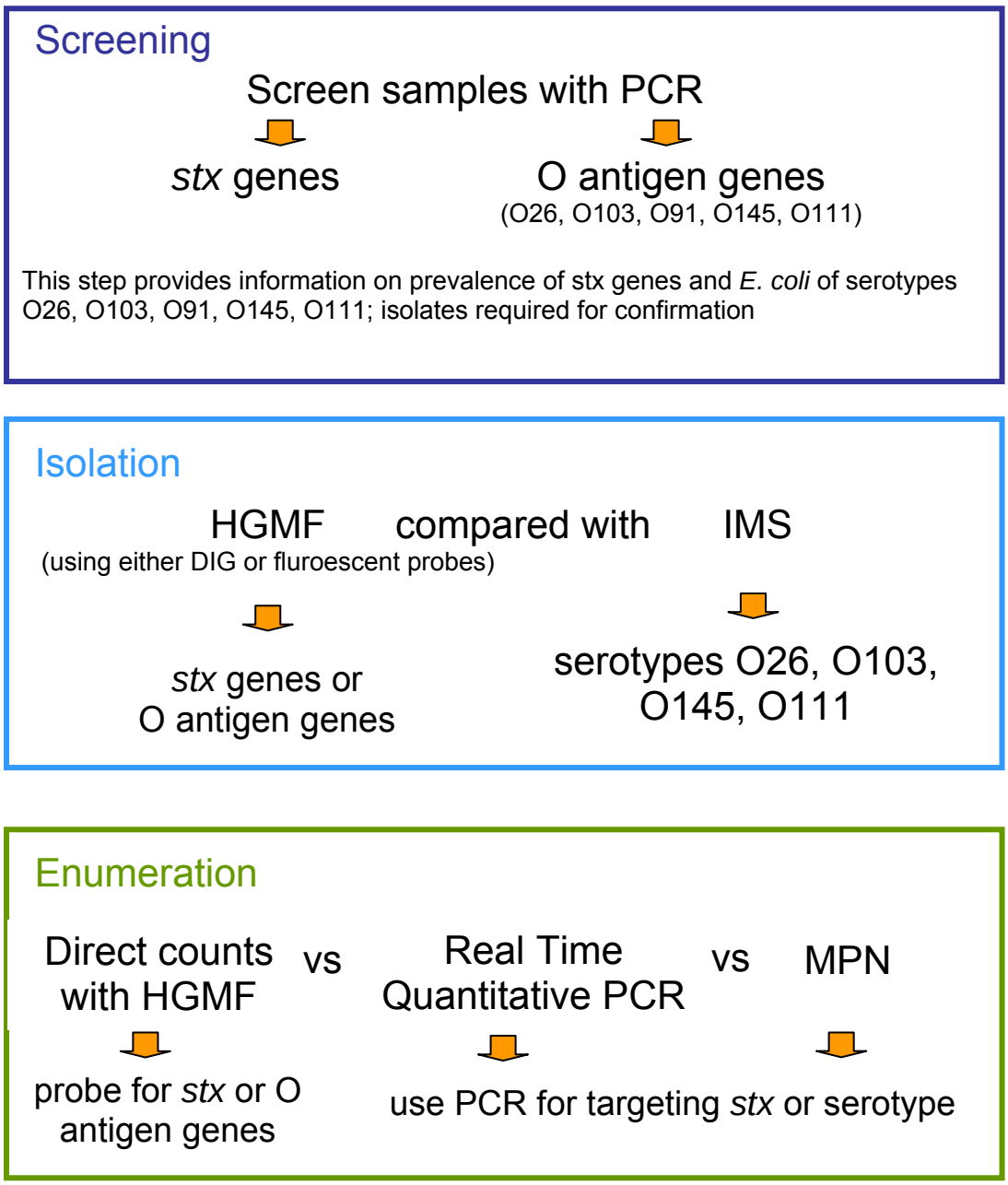


**Figure 1 General approach to screening, isolation and enumeration of non-O157 STEC. Screening methods will be developed/applied for targeting *stx* and O antigen genes. Isolation methods will compare colony hybridisation to IMS for detection of non-O157 STEC. Enumeration will be evaluated using 3 different approaches, direct counts using colony hybridisation, quantitative PCR and an MPN method where tubes will be tested with PCR.**

# Milestone 2 – Development and validation of methods for the detection, isolation and characterisation of non-O157 EHEC

At the commencement of this project there was no clear consensus about the appropriate targets, growth conditions, detection methods and isolation procedures for non-O157 EHEC. However, both the US (Food Safety and Inspection Service) and EU (European Food Safety Authority) produced position papers on this topic during the methodology development phase. Although some subtle differences were apparent, the overall approach was consistent with the multi-faceted approach proposed following a review of methodology literature.

The methods for detection and isolation of non-O157 EHEC were trialled on a series of spiked samples and they demonstrated the capacity to detect, isolate and quantify non-O157 serotypes and *stx*. Detection and isolation of non-O157 serotypes (O91 excluded) and *stx* is likely if the levels of the target organism exceed 4.00 $\log_{10}$ CFU/mL following enrichment. The limit of detection for O91 was slightly higher and was probably a result of commercially available Immunomagnetic separation (IMS) beads not yet being available for this serotype. Nevertheless, the methods used for detection and isolation were shown to be capable of isolating target organisms from samples that originally contained < 20 CFU/g.

Methods for the quantitation of target organisms were also developed. The combination of real-time PCR and the MPN approach allowed for the quantitation of non-O157 serotypes and *stx* at levels ranging from 3 to $10^8$ CFU/g. The multi-faceted method developed appears to be comparable with methods proposed by FSIS and EFSA. Indeed the in-built flexibility of this method brought about by the use of HGMFs with colony hybridisation signifies that this approach should remain best practice until a unique phenotypic marker is identified amongst all target organisms from which new isolation methodologies can be created.

## Milestone 3 – Investigate the prevalence of non-O157 EHEC in grass- and grain-fed cattle

This study investigated the prevalence of the EHEC (*stx* and *eae* containing *E. coli*) serotypes O26, O45, O91, O103, O111, O121, O145 and O157 in 300 (140 grain-fed and 160 grass-fed) beef cattle faecal samples using a sequential real-time PCR approach. Samples enriched in tryptone soya broth were tested for $stx_1$ and $stx_2$ and subsequently for *eae*. Samples that tested positive for *stx* and *eae* were then tested for the target serotypes. Isolation was performed on any sample testing positive to *stx, eae* and a target serotype using immuno-magnetic separation (IMS) or colony hybridisation. Resulting isolates were characterised for the presence of $stx_1$, $stx_2$, *eae*, and *ehxA* using a multiplex PCR.

Virulence gene profiles of the faecal samples determined that $stx_1$ was more prevalent in grain-fed faecal samples (19.3%) than grass-fed faecal samples (14.4%). Conversely, $stx_2$ was more prevalent in grass-fed cattle samples (34.4%) than grain-fed cattle samples (30.7%). However, differences in *stx* prevalence were shown not to be significant with respect to feed type. Comparisons of *eae* prevalence between groups determined that grain-fed cattle samples (70.7%) were significantly more likely to contain *eae* than grass-fed cattle samples (33.1%). Furthermore, grain-fed cattle samples (33.6%) were significantly more likely to contain *stx* and *eae* than grass-fed cattle samples (22.6%). Overall, samples harbouring *stx* and *eae* occurred on 78 (26%) occasions with 30 (38.4%) of these samples subsequently testing positive to at least one EHEC serotype. A total of 45 *stx, eae,* and EHEC serotype combinations were present in the 30 positive samples identified. Of the 30 samples containing *stx, eae*, and an EHEC serotype, 17 (57%) were positive for one serotype, 11 (37%) for two serotypes, and 2 (7%) for three serotypes. *E. coli* O91 (50%) and O45 (30%) were the most commonly detected serotypes in *stx* and *eae* positive samples with all remaining serotypes detected in no more than five (≤17%) *stx* and *eae* positive samples.

Isolation of a target serotype was achieved from 20 of the 30 *stx* and *eae* positive samples which represented an overall isolation rate of 56% from the 45 *stx, eae,* and EHEC serotype combinations. With the exception of *E. coli* O145, isolation of all target serotypes was achieved from at least one sample with recovery from grass-fed

cattle samples more easily achieved than from grain-fed cattle samples. Isolation of *E. coli* O145 was not achieved from any of the five positive samples tested. *E. coli* O157 was isolated from a total of six samples, however two of these samples did not test positive by PCR to *stx* and *eae* and were isolated as a result of the O157 IMS testing of all samples. A total of 27 *E. coli* of EHEC serotypes were isolated during the study. All isolates were confirmed as *E. coli* but only one *E. coli* O91, one *E. coli* O26 and five *E. coli* O157 tested positive for the presence of any EHEC virulence markers. The O91 isolate carried $stx_2$ but lacked $stx_1$, *eae* or *ehxA* whereas the O26 isolate carried *eae* but lacked the additional EHEC virulence markers. Of the six O157 isolates tested, four harboured $stx_1$, $stx_2$, *eae* and *ehxA*, one harboured $stx_2$, *eae* and *ehxA*, whilst the remaining isolate was shown to be sorbitol fermenting and did not carry any of the EHEC virulence markers. As the definition of EHEC applied in this study requires an isolate to be of a specific serotype and carry *eae* in addition to *stx*, the prevalence of EHEC in cattle faeces was 1.7%. Furthermore, all samples were deemed to be negative for non-O157 EHEC of serotype O26, O45, O91, O103, O111, O121, and O145.

This study represents the first attempt to determine the prevalence of EHEC serotypes other than O157 in Australian beef cattle. The use of the sequential PCR based approach combined with appropriate isolation methodologies is a suitable systematic approach for EHEC surveillance. The results indicate that the prevalence of EHEC serotypes other than *E. coli* O157 in the Australian beef cattle population is low. This is consistent with the low prevalence of these serotypes observed in previous epidemiological surveys of STEC in Australia.

## Milestone 4 & 5 – Identify, isolate and characterise *eae*-containing organisms and STEC from Australian beef cattle

Pathotypes of *E. coli* are defined, amongst other things, by the virulence markers that they harbour. During the last decade there has been an increase in the reporting of *E. coli* isolates that harbour *eae* but lack the additional virulence markers commonly associated with EPEC or EHEC. In the national survey for non-O157 EHEC in Australian beef cattle the prevalence of *eae* in beef cattle faeces samples was 51% (152 of 300), however this did not correlate with the presence of non-O157 EHEC.

The presence of *eae*-containing organisms in beef cattle faecal enrichments was investigated.

*Eae*-containing *E. coli* were isolated from the national survey samples and also from 50 additional freshly collected beef faecal samples at 8% (12/152) and 33% (6/18) respectively. Overall isolation rates of *eae*-containing *E. coli* from the national survey samples and freshly collected beef faecal samples were 4% and 12% respectively. The reduced isolation rate from the national survey samples may be a result of cell death during the frozen storage of these samples. *Eae*-containing *E. coli* were then tested for the presence of additional EHEC (*stx* genes) and EPEC (*bfpA*) genes but all isolates were negative for these additional markers. Thirteen isolates from six samples tested positive for non-O157 EHEC serotypes and were shown to be either O26 (2 samples) or O128 (4 samples).

The relevance of *eae*-containing *E. coli* that don't fit the traditional EHEC or EPEC definitions continues to be debated. It has been suggested that these organisms may be 'EHEC in waiting' or EHEC that have lost *stx* phage during the isolation process. Genetic screening of the isolates was conducted to determine if the *eae*-containing *E. coli* harboured additional genes that had previously been shown to be specific to sequenced EHEC strains. The isolates rarely harboured genes associated with EHEC such as *espR1* and *ureC* and were instead more likely to harbour genes common to a range of *E. coli* pathotypes. The results indicate that the *eae*-containing *E. coli* isolates recovered in the study should be included in the atypical EPEC pathotype (ATEC).

In addition to the isolation of ATEC strains this study also attempted to isolate all STEC from the national survey samples. Detection and isolation of STEC was performed without regard for specific *E. coli* serotypes. STEC were isolated from 14% of samples and all isolates were *eae* negative which is consistent with the findings of the national survey for non-O157 EHEC. Characterisation of the virulence markers determined that the majority (83%) of isolates carry $stx_2$ in association with $stx_1$ and/or *ehx.* Isolation of STEC from grass-fed beef faecal samples was more frequent than from grain-fed beef faecal samples. This finding is contrary to previous studies where grain-fed samples have typically yielded more isolates. Interrogation of beef faecal samples collected as part of a national survey has revealed that these

samples can contain *E. coli* belonging to the pathotypes STEC and ATEC but typically do no harbour non-O157 EHEC or EPEC strains.

## Milestone 6 – Genetically characterise the virulence attributes of Australian STEC

Pathogenic *E. coli* are divided into pathotypes primarily based on expressed virulence factors, their adhesion patterns to cultured cells and the type of disease they cause. Food-producing animals can be reservoirs of pathogenic *E. coli* strains which may cause disease in animals and humans. However, it is not always clear why some pathogenic *E. coli* (eg. enterohaemorrhagic *E. coli* O157) cause disease and others do not. It is proposed that the public health risk of O157 and non-O157 EHEC can be assessed by identifying the presence or absence of combinations of genes that are known to be associated with increased virulence. There is little published data on the virulence profiles of Australian pathogenic *E. coli* isolates. The aim of this study was to screen a large number of Australian *E. coli* from clinical and animal sources to identify the presence or absence of specific genes that may be present in human clinical isolates yet absent in those isolates with reduced clinical significance.

A total of 308 *E. coli* from Australia were tested comprising *E. coli* O157 from humans (47) and animals (149), non-O157 Shiga-toxigenic *E. coli* (STEC) from humans (31), animals (24) and meat (39) and atypical enteropathogenic *E. coli* (ATEC) from animals (18). All isolates were tested by PCR for the presence of 20 gene targets known to be associated with increased virulence in *E. coli*. Some differences in the prevalence of individual genes were identified between the human and animal *E. coli* O157 isolates, but these were relatively minor and in the main all *E. coli* O157 isolates carried the majority of gene targets screened for regardless of the source of isolate. A combination of virulence markers likely to indicate increased virulence (i.e present in human isolates but absent in animal isolates) in *E. coli* O157 was not identified in this study. The majority of *E. coli* O157 isolates from animals and humans share a common core of non-LEE effector (*nle*) genes that have previously been shown to be positive indicators for EHEC capable of causing life-threatening illnesses such as haemorrhagic colitis (HC) or haemolytic uraemic syndrome (HUS). STEC and ATEC isolates were significantly less likely to carry the majority of target

genes and did not display the *nle* profile of the O157 isolates. Genes encoding the STEC auto-agglutinating adhesin (*saa*) and iron regulation (*fyuA* and *irp2*) were the only target genes more prevalent in STEC and ATEC isolates than *E. coli* O157 isolates.

Molecular risk assessments are an increasingly useful approach to the characterisation of pathogenic bacteria. Despite this, they represent just one aspect of the complex interplay that occurs between bacteria, the environment and the human host. The results of this assessment indicate that there are no obvious differences in the presence of virulence genes in EHEC O157 isolated from animals and humans. *E. coli* O157 isolates were able to be differentiated from ATEC and non-O157 STEC as they contained sets of genes predominantly absent from STEC and ATEC isolates. This finding is consistent with published studies from countries where the disease rate from EHEC O157 is much higher than that in Australia. Clearly there are a number of other factors that need to be considered in determining the virulence potential of Australian *E. coli*. The PCR method used in this study determined the presence or absence of specific genes and provided no information on the sequences of the virulence genes. Small sequence variations (i.e sequence polymorphisms) can lead to mutations which may impair or modify the function of the gene or its encoded protein and sequencing would be necessary to determine whether or not the targeted virulence genes were mutated. Other factors associated with increased virulence may include the presence or absence of genes which were not included in this study as well as the degree to which certain genes are expressed. Studies such as this highlight the complexity associated with understanding why some bacteria are able to cause disease.

# Communications resulting from A.MFS.0128

The following are a list of publications and presentations where the data from A.MFS.O128 has been presented.

## Publications

Barlow, R.S and Mellor, G.E. (2010) Prevalence of Enterohemorrhagic *Escherichia coli* Serotypes in Australian Beef Cattle. *Foodborne Pathogens and Disease*. In press, DOI: 10.1089/fpd.2010.0574

## Conference presentations

Barlow, R.S, Mellor, G.E and Fegan, N. (2009) Prevalence of non-O157 EHEC serotypes in Australian beef cattle. 7$^{th}$ International Symposium on Shiga Toxin (Verocytotoxin) producing *Escherichia coli* Infections, Buenos Aires, Argentina. May 10-13, 2009. Poster presentation.

Mellor, G.E and Barlow, R.S. (2010) Presence and characterisation of ATEC in Australian beef cattle. Australian Society for Microbiology, Sydney. July 4-8, 2010. Microbiology Australia. Poster presentation.