final report

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E. coli subtyping: data collection

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Abstract

There is strong evidence to suggest that *E. coli* O157 genetic variants (genotypes) are differentially associated with animal and human hosts. The geographic distribution of these genotypes, particularly between countries with different rates of *E. coli* O157 related disease, is less well understood. Here, we employed molecular typing methods to investigate the extent of genetic variation between large diverse collections of *E. coli* O157 isolates from countries representing two geographically separate regions, Australia and the United States. This study demonstrates that Australian *E. coli* O157 populations largely segregate into groups distinct from those seen in the United States. Comparing these genotypes to previously published studies suggest that Australian isolates may be more closely related to *E. coli* O157 found largely in U.S. cattle rather than those associated with human disease. In addition, the production of Shiga toxin (an important factor associated with human disease) by Australian *E. coli* O157 was lower than isolates from the United States. This information provides the Australian Meat Industry and government bodies involved in trade negotiations such as DAFF with evidence to support the notion that *E. coli* O157 isolates in this country may be of lesser threat to public health than isolates from other countries. Future benefits may also arise from the application of this knowledge in commercial and diagnostic laboratories for improved targeting of isolates associated with increased public health risk.

Executive summary

Why the work was done

The U.S. is the second largest market for Australian beef exports and is valued at about $1 billion AUD[1]. In order to maintain access to this market the Australian beef industry must satisfy requirements around testing U.S. destined product for *E. coli* O157. Cattle are the major reservoir of *E. coli* O157 and beef products have, in the past, been a source of large outbreaks of *E. coli* O157 in the United States. *E. coli* O157 is present in Australian cattle and can occasionally be found on beef destined for export. Even though this pathogen is present in Australia, the incidence of human disease associated with *E. coli* O157 is much lower than many other countries including the U.S., Scotland and Argentina. Since the prevalence of *E. coli* O157 in cattle is similar worldwide[2,3,4,5], observed prevalence's, do not, on their own, explain differences in disease rates observed between countries. Instead, factors such as disease surveillance systems, food handling practices, supply chains and host susceptibility may impact on disease rates.

Another factor which may be associated with the observed differences in disease rates between countries is the genetic makeup of the *E. coli* O157. Although *E. coli* O157 from different countries share many genetic traits, recent research has suggested that genetic differences exist between isolates which may impact on their potential to cause human disease. The developing ideas in this area are that the population of *E. coli* O157 in cattle is genetically diverse, but only certain genetic types (genotypes) are able to cause severe human disease. Such observations have arisen as particular genotypes of *E. coli* O157 have been associated with an increased incidence and severity of disease in humans. The genetic types of *E. coli* O157 present in cattle are referred to as "bovine biased genotypes" while the genetic types found in humans are referred to as "clinical genotypes".

It is unclear how Australian *E. coli* O157 isolates compare to those from other countries, particularly in relation to the distribution of genotypes and the association of such genotypes with bovine biased or clinical sources. Information accumulating from previous CSIRO funded research on Australian isolates indicates they differ genetically from those in other countries. Understanding more about the differences between *E. coli* O157 from Australia

[1] http://www.mla.com.au/Prices-and-markets/Overseas-markets/North-America/Beef
[2] **Garcia A, Fox JG, Besser TE.** 2010. Zoonotic Enterohemorrhagic *Escherichia coli*: A One Health Perspective. Ilar J. **51:**221-232
[3] **Elder RO, Keen JE, Siragusa GR, Barkocy-Gallagher GA, Koohmaraie M, Laegreid WW.** 2000. Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. Proc. Natl. Acad. Sci. U. S. A. **97:**2999-3003.
[4] **Masana MO, Leotta GA, Del Castillo LL, D'Astek BA, Palladino PM, Galli L, Vilacoba E, Carbonari C, Rodriguez HR, Rivas M.** 2010. Prevalence, characterization, and genotypic analysis of *Escherichia coli* O157:H7/NM from selected beef exporting abattoirs of Argentina. J. Food Prot. **73:**649-656.
[5] **Fegan N, Vanderlinde P, Higgs G, Desmarchelier P.** 2004. The prevalence and concentration of *Escherichia coli* O157 in faeces of cattle from different production systems at slaughter. J. Appl. Microbiol. **97:**362-370.

and other countries may provide evidence to support the hypothesis that Australian strains are bovine biased and less able to cause severe disease in humans than *E. coli* O157 isolates from other countries, particularly those from the U.S. where large outbreaks and severe disease have occurred. This study was designed to examine the genetic relatedness of a large set (n=606) of Australian and U.S. *E. coli* O157 of both cattle and human (clinical) origin. The information gathered from this analysis will provide insight into the diversity that exists between Australian and U.S. *E. coli* O157 isolates, and whether this diversity may in part be responsible for the different human disease incidence observed between the two regions.

What was achieved

Four genotyping methods were used to look at the relationship between the 606 *E. coli* O157 isolates from Australia and United States. Analysis of the data provided strong evidence of segregation of the *E. coli* O157 populations based on their country of origin. These data also demonstrate that most of the Australian cattle and human *E. coli* O157 isolates were more closely related to cattle biased isolates from the U.S. rather than human isolates from the United States. This suggests that the Australian population of *E. coli* O157 may have evolved from a bovine biased subpopulation which now represents the predominant genotype isolated from Australian cattle and human sources. One of the findings from this work was associated with the Shiga toxin gene and where this gene is inserted within the *E. coli* O157 genome. The majority of Australian isolates (59% of 79 human and 88% of 205 cattle isolates) were shown to possess profiles that are typically overrepresented in U.S. cattle isolates (bovine biased genotypes) than U.S. clinical isolates (clinical genotypes) (Table 1). In addition, a greater proportion of Australian isolates (96% cattle and 85% human) possessed the Shiga toxin 2c subtype (stx_{2c}) compared to U.S. isolates (46% cattle and 16% human). In contrast, only a limited number of Australian isolates (1% cattle and 9% human) carried the Shiga toxin 2 subtype (stx_2) that was commonly associated with isolates from the U.S. (54% cattle and 87% U.S.). The stx_2 subtype is also common in isolates that cause large outbreaks and severe disease and mostly inserts in one of two regions of the *E. coli* O157 genome (*argW* or *wrbA*). Data from this study suggests that, instead of carrying stx_2 in the *argW* region, Australian isolates carry the potentially less toxic stx_1 gene[6] in this position.

A previously published genotyping technique, known as single nucleotide polymorphism (SNP) analysis, which identifies single base changes in the genome, was used for further comparative analysis of *E. coli* O157 from each of the different countries. This SNP method

[6] Fuller CA, Pellino CA, Flagler MJ, Strasser JE, Weiss AA. 2011. Shiga toxin subtypes display dramatic differences in potency. Infect. Immun. 79:1329-1337.

has previously been used by researchers in the U.S. to identify cattle and human groups of *E. coli* O157. This technique was used to analyse a small randomized set of 98 *E. coli* O157 isolates representing each country (Australia and U.S.) and each source (human and cattle). Using this approach Australian and U.S. isolates were largely clustered into different country and source groups which were defined as subclades. Of a possible 11 subclades previously identified in the U.S., nine were identified in this study of which two were represented by isolates from both Australia (14%) and the United States (71%). A single subclade which represented 31% of the Australian *E. coli* O157 isolates was not observed in any of the 530 U.S. isolates previously examined[7], suggesting this lineage is probably unique to Australia. Overall, this study provides compelling evidence that Australian *E. coli* O157 isolates are different based on the presence of unique subclades. Where similarities exist between isolates from each country, Australian strains tend to be aligned with subclades that are sparsely represented in the U.S. and thus host specific associations have not been tested on these subclades due to the low numbers of isolates. These isolates were then assessed for the production of Shiga toxin using a commercially available ELISA assay. Overall, isolates from Australian cattle and clinical sources produced less toxin than U.S. cattle and clinical isolates. A trend was also observed between the carriage of the stx_2 subtype (common in the U.S.) and higher total Stx expression amongst the *E. coli* O157 isolates tested.

While this study was not designed to investigate specific genetic factors underlying the differential virulence of Australian and U.S. isolates, it does provide some evidence for specific genes which may play a role in virulence. Specifically, the much higher frequency of stx_2 positive strains in the U.S. [8,9] and the occupancy of stx_1 at *argW* in Australia may be two factors that impact on the severity of symptoms caused by *E. coli* O157 isolates associated with human disease. The lower overall Stx production in Australian isolates also provides additional support for this. However, factors such as disease surveillance, food handling, supply chains and host susceptibility or alternative, unidentified genetic factors may also play a role in the differential rates of disease.

When and how industry can benefit from the work

This study was primarily designed to compare the diversity of *E. coli* O157 genotypes in Australia to those found in the United States. Using a combined genotyping approach we provide industry with compelling evidence that Australian isolates can be segregated from the majority of those isolated in the United States. Furthermore, using an individual typing

[7] Jung, W. K., J. L. Bono, et al. (2013). Lineage and Genogroup-Defining Single Nucleotide Polymorphisms of *Escherichia coli* O157:H7. Appl Environ Microbiol **79**(22): 7036-41.
[8] Shringi, S., C. Schmidt, et al. (2012). Carriage of *stx2a* differentiates clinical and bovine-biased strains of *Escherichia coli* O157. PLoS ONE **7**(12): e51572.
[9] Manning, S. D., A. S. Motiwala, et al. (2008). Variation in virulence among clades of *Escherichia coli* O157:H7 associated with disease outbreaks. Proc. Natl. Acad. Sci. U.S.A. **105**(12): 4868-73.

technique that targets Shiga toxin genes and their insertion sites we also identified a difference in the distribution of genotypes between Australian isolates and their U.S. counterparts. These differences infer that Australian *E. coli* O157 isolates group with isolates that are overrepresented from bovine sources in the United States. Interestingly, Australian isolates predominantly belong to a single bovine biased group that represents a relatively small proportion of the total population of U.S. genotypes previously reported[10]. While bovine biased genotypes can also cause human disease, evidence is mounting that these bovine biased genotypes do not account for the majority of human infections and are less likely to cause severe human disease or result in large outbreaks in the United States. This information provides the Australian Meat Industry with evidence that *E. coli* O157 populations in this country may be of lesser threat to public health than isolates from other countries.

This study has also identified some candidate genes that may play a role in the differential virulence in each country. The advancement of genotyping techniques may eventually lead to the identification of specific genes or SNPs, rather than organisms, for assessing the health risk of foods. This study provides preliminary information that this approach may be valid at some point in the future and advancements in this field may lead to improved targets for identifying isolates with greater clinical significance. We believe any future attempt to risk assess the clinical significance of *E. coli* O157 would result in the majority of Australian cattle strains being categorised into low risk groups.

Who can benefit from the results

Exporters of Australian beef will benefit from the results of this work as it provides them with material that supports Australia's clean, green image and the production of safe food. DAFF can use the information in discussions with U.S. regulators as it provides a scientific evidence base for *E. coli* O157 from Australia to be potentially less virulent than those from other countries. Scientists working in this area will benefit from the generation of new knowledge relating to the ecology of an important food borne pathogen which adds support to the notion that geographic divergence occurs amongst food borne pathogens. In addition to these immediate benefits, future benefits from these results may include:

- Enhancement of diagnostic technologies through the identification of more specific gene targets for identifying and targeting *E. coli* O157 more likely to cause severe human disease

[10] Shringi, S., C. Schmidt, et al. (2012). Carriage of *stx2a* differentiates clinical and bovine-biased strains of *Escherichia coli* O157. PLoS ONE **7**(12): e51572.

- Establishment of evidence that confirms identified genetic differences as an underlying mechanism for enhanced virulence and enables the generation of risk-based frameworks around the presence of *E. coli* O157 in beef products

Communications resulting from A.MFS.2036

Conference presentations

Mellor, G., Smith, H., Jennison, A., Gobius, K., Fegan, N. (2012) **Comparison of Australian cattle and clinical *Escherichia coli* O157 based on motility, *stx* type and pulsed field gel electrophoresis.** 8th International Symposium on Shiga Toxin (Verototoxin) Producing *Escherichia coli* Infections. Amsterdam, Netherlands 6th-9th May 2012. Proffered paper.

Mellor G.E., Gobius K.S., Fegan N., Smith H.V., Jennison A.V., Eckmann, M.K., Davis M., Besser T. (2012) **Lineage specific polymorphism assay shows a geographical bias in *E. coli* O157 isolates from Australia and the USA.** Australian Society for Microbiology Annual Scientific Meeting. Brisbane, Australia 1st – 4th July, 2012. Proffered paper.

Doyle, C.J., Mellor, G.E., Fegan, N., Gobius, K.S., Smith, H.V., Jennison, A.V. (2012) **Multi-locus number tandem repeat analysis of Australian *Escherichia coli* O157 isolates from human and bovine sources.** Australian Society for Microbiology Annual Scientific Meeting. Poster presentation.

Glen E. Mellor, Thomas E. Besser, Margaret A. Davis, Brittany Beavis, WooKyung Jung, Helen V. Smith, Amy V. Jennison, Narelle Fegan, Kari S. Gobius. (2013) **Comparison of Shiga toxin subtypes and chromosomal insertion sites in *Escherichia coli* O157 isolated from Australia and the USA.** International Association for Food Protection Annual Meeting. Poster presentation.

Draft publications

Mellor, G.E., Besser, T.E., Davis, M.A., Beavis, B., Jung, W., Smith, H.V., Jennison, A.V., Doyle, C.J., Chandry, P.S., Gobius, K.S., Fegan, N. (2013) **Multilocus genotype analysis of *E. coli* O157 from Australia and the U.S.A. provides evidence of geographic divergence.** Applied and Environmental Microbiology, 79:5050-5058.

Mellor, G.E., Besser, T.E., Davis, M.A., Beavis, B., Jung, W., Smith, H.V., Jennison, A.V., Doyle, C.J., Chandry, P.S., Gobius, K.S., Fegan, N. **Australian and USA *E. coli* O157 isolates vary with respect to phylogeny and Stx expression** – in preparation.

Interpretation of milestones in the appendices

Some of the data in the appendices is considered confidential and requires approval from MLA/CSIRO prior to release. The information contained in individual milestones listed in the appendices developed throughout the course of the project as strain sets became available and more in-depth analyses were performed. Consequently, the reader should apply caution when comparing results between milestones, particularly with respect to Shiga toxin genotyping. Any reference to Shiga toxin genotypes in milestones 2 and 3 include combined *stx*$_2$ subtypes whereas later milestones (4, 5 and 6) refer to subtypes of *stx*$_2$; *stx*$_{2a}$ and *stx*$_{2c}$. Shiga toxin subtype *stx*$_{2a}$ is considered the traditional *stx*$_2$ toxin and as such is often internationally referred to as *stx*$_2$, which is the case for milestones 2 and 3 of this report. Thus prevalence data for *stx*$_2$ in early milestones (which refer to combined subtypes) may be confused with *stx*$_2$ references in later milestones (which refer to *stx*$_{2a}$). As minor amendments were made to the strain set throughout the project, prevalence data and statistical analyses may also differ slightly based on the total number of strains examined in each milestone.

Milestones 4 and 6 represent draft manuscripts only and may be altered based on reviewer comments following submission to a journal for publication. In milestone 6 the term "subclade" was used to describe each of the SNP groupings. This term has since been altered to "SNP lineage" in the final manuscript (Jung, W. K. et al. 2013. Appl Environ Microbiol **79**(22): 7036-41). In addition, SNP lineage Vc has since been combined with SNP lineage Vb and will not appear in the final manuscript by Jung, W.K. et al. (2013) but will appear in milestone 6. Appropriate amendments will be made to this milestone prior to submission for publication.

Appendices

Table 1

Distribution (percent) of BBG and CG within Australian and U.S.A. isolates

Country	Source	n	BBG[1]	CG[2]	Unclassified[3]
Australian	Human	79	47 (59%)	6 (7.6%)	26(32.9%)
	Cattle	205	180 (88%)	3 (1.5%)	22(12%)
	Total	284	227 (80%)	9(3.2%)	48(16.9%)
U.S.A.	Human	179	15 (8.4%)	140 (78.2%)	24 (13.4%)
	Cattle	143	60 (42%)	71 (49.6%)	12 (8.4%)
	Total	322	75 (23.3%)	211 (65.5%)	36 (11.2%)

[1]BBG refers to the Bovine Biased Groups identified from previous studies using Shiga toxin bacteriophage insertion (SBI) typing

[2]CG refers to the Clinical Groups identified in previous studies using SBI typing methods

[3]Unclassified refers to isolates with SBI types that were not statistically associated with cattle or human sources

Milestone 2

Milestone report

MLA project code:	**A.MFS. 2036**
MLA project title:	***Escherichia coli* O157 subtyping**
Project leader:	**Dr. Narelle Fegan**
MLA project manager/coordinator:	**Ian Jenson**
Milestone number:	**2**
Date:	**31-05-2011**

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Milestone 3

Milestone report

MLA project code:	**A.MFS. 2036**
MLA project title:	***Escherichia coli* O157 subtyping**
Project leader:	**Dr. Narelle Fegan**
MLA project manager/coordinator:	**Ian Jenson**
Milestone number:	**3**
Date:	**14-11-2011**

Commercial-in-confidence

Milestone 4

Milestone report

MLA project code:	**A.MFS. 2036**
MLA project title:	***Escherichia coli* O157 subtyping**
Project leader:	**Dr. Narelle Fegan**
MLA project manager/coordinator:	**Ian Jenson**
Milestone number:	**4**
Date:	**03-05-2012**

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Milestone 5

Milestone report

MLA project code:	**A.MFS. 2036**
MLA project title:	***Escherichia coli* O157 subtyping**
Project leader:	**Dr. Narelle Fegan**
MLA project manager/coordinator:	**Ian Jenson**
Milestone number:	**5**
Date:	**28-05-2012**

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Milestone 6

Milestone report

MLA project code:	**A.MFS. 2036**
MLA project title:	***Escherichia coli* O157 subtyping**
Project leader:	**Dr. Narelle Fegan**
MLA project manager/coordinator:	**Ian Jenson**
Milestone number:	**6**
Date:	**07-12-2012**

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Milestone 4 - published manuscript

Applied and Environmental Microbiology

Multilocus Genotype Analysis of Escherichia coli O157 Isolates from Australia and the United States Provides Evidence of Geographic Divergence

Glen E. Mellor, Thomas E. Besser, Margaret A. Davis, Brittany Beavis, WooKyung Jung, Helen V. Smith, Amy V. Jennison, Christine J. Doyle, P. Scott Chandry, Kari S. Gobius and Narelle Fegan

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