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G.MFS.0286: The prevalence of pSTEC in cattle from different systems used for production of Australian beef
G.MFS.0285: Antimicrobial resistant bacteria in beef production in Australia

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Abstract

Pathogenic STEC (pSTEC) and antimicrobial resistance (AMR) have been identified as important factors when evaluating the perceived safety of beef products and the risk to human medicine. The aim of this study was to determine the prevalence of pSTEC and the AMR status of *Salmonella*, *E. coli*, and *Enterococcus* in Australian beef cattle. A total of 1500 faecal samples from 31 export abattoirs comprising three animal groups: beef cattle, dairy cattle and veal calves were tested for pSTEC. Overall, 44.7% of samples were deemed potentially positive for at least one pSTEC serotype of which 100 (6.7%) contained *E. coli* O157 and 19 (1.3%) contained *E. coli* O26 or O111. Young animals were significantly more likely to harbor pSTEC than older animals. Pathogenic STEC of serotypes O45, O103, O121 and O145 were not isolated from any sample. *Salmonella* was present in 216 (14.4%) samples and was more likely to be associated with dairy cattle than the other animal groups. *E. coli* (92.3%) and *Enterococcus* (86.4%) were readily isolated and subsets of 800 isolates were selected for AMR testing. In general, resistance to clinically significant antimicrobials was seldom observed and resistance to most antimicrobials was low by international comparisons. Despite this, a cluster of cephem resistant *Salmonella* and resistance to daptomycin and tigecycline in *Enterococcus* was identified and is of concern. However, the likely overall impact of cattle derived AMR on human medicine would appear to be low. Although the prevalence of pSTEC and AMR are low, there remains a challenge for Australian producers to maintain strict guidelines and procedures around processing and antimicrobial use to ensure Australia's reputation as a supplier of safe and healthy food is to be maintained.

Executive summary

Australia is one of the world's most efficient producers of cattle and third largest exporter of beef, exporting 67% of its total beef and veal production in 2012-2013. The supply of Australian beef products into world trade markets is dependent on the capacity of producers to export products that are, upon consumption, unlikely to cause disease or be detrimental to human medicine. Pathogenic STEC (pSTEC) and antimicrobial resistance have been identified as important factors when evaluating the perceived safety of beef products and the risk to human medicine. The group of *E. coli* collectively referred to as pSTEC include the prototype pSTEC serogroup O157 and six additional serogroups (known as the Big6) O26, O45, O103, O111, O121 and O145. In 2012, the USA introduced regulations that classified the seven serogroups of pSTEC as adulterants of raw non-intact beef products. Similarly, there has been recent pressure to classify specific strains of antimicrobial resistant *Salmonella* as adulterants in beef products. Australia does not have ongoing multi-focus surveillance programs capable of evaluating the presence of zoonotic pathogens and antimicrobial resistance (AMR) in food production systems and instead conducts relatively short-term intensive surveys to evaluate the industry status. This report details the prevalence of pSTEC and the AMR status of *Salmonella*, *E. coli*, and *Enterococcus* in Australian beef cattle.

A stratified sampling plan was employed to collect 1500 faecal samples from three animal groups: beef cattle, dairy cattle and veal calves. A total of 910 beef cattle, 290 dairy cattle and 300 veal calf samples were collected during the survey. Samples were collected across two main sampling windows which occurred in February and March 2013 (Window 1) and August and September 2013 (Window 2). A total of 31 export registered beef abattoirs participated in the survey representing >85% of annual export production. Information on the feed type, carcase weight and animal source was also collected and was used as the basis of subsequent analysis. World's best practice methodologies consistent with pSTEC testing programs and international AMR surveillance programs were utilised throughout the survey which enables the direct comparison of the survey results with available international data.

The prevalence of pathogenic STEC in beef cattle is poorly understood, both domestically and internationally. Beef cattle, dairy cattle and veal calf faecal samples were assessed for the presence of pSTEC using the BAX system followed by confirmation as outlined in the FSIS laboratory guidebooks 5.07 and 5B.04. In addition, *E. coli* O157 were further targeted by using a combined buffered peptone water – immunomagnetic separation technique on all samples. Prior to statistical analysis of pSTEC prevalence the animal groups were further broken down by carcase weight into

young animal and adult animal classes. Overall, 44.7% of samples were deemed potentially positive (contained *stx*, *eae* and an O antigen marker) for at least one pSTEC serotype. The most frequently occurring serotypes were O103 (33.5%), O45 (25.1%), O121 (23.4%), O26 (19.5%) and O157 (10.4%). In total, 115 (7.7%) samples yielded a pSTEC isolate, of which 100 (6.7%) contained *E. coli* O157 and 19 (1.3%) contained a Big6 isolate. Of the Big6 isolates 15 (1.0%) were *E. coli* O26 and four (0.3%) were *E. coli* O111. Four samples (0.3%) were shown to harbor two pSTEC strains of differing serogroups. Pathogenic STEC of serotypes O45, O103, O121 and O145 were not isolated from any sample, even though genes indicative of *E. coli* belonging to these serotypes were detected by PCR.

In this study we attempted to gain a greater understanding of the risks associated with different beef production systems by investigating the prevalence of pSTEC in five animal classes. The animal classes most likely to yield a pSTEC isolate, in order of prevalence, were veal (12.7%), young beef (9.8%), young dairy (7.0%), adult beef (5.1%) and adult dairy (3.9%). When animals were grouped into two classes (young and adult) significantly higher levels of *E. coli* O157 and non-O157 pSTEC serotypes were observed in younger animals. Analysis of pSTEC by carcase weight reinforced the effect of young animals as 80% of weight classes below 250 kg had a pSTEC prevalence that exceeded the overall prevalence for the survey (7.7%). Conversely, all weight classes greater than 250 kg had a prevalence of pSTEC less than the overall prevalence for all groups. One particular group of animals, with carcase weights between 50 and 150 kg, had a prevalence almost three fold greater than the mean. Comparisons of grass-fed beef cattle, grain-fed beef cattle and dairy cattle did not identify differences in pSTEC prevalence based on production system or feed.

Salmonella were isolated from 216 (14.4%) of all samples. Importantly, this survey included dairy cattle faecal samples and these were shown to be significantly more likely to harbor *Salmonella* than samples from beef cattle or veal calves. *E. coli* (92.3%) and *Enterococcus* (86.4%) were readily isolated from all samples with the clinically relevant *E. faecalis* and *E. faecium* identified in 6.4% and 8.0% of *Enterococcus* isolates, respectively. Sub-sampling of *E. coli* and *Enterococcus* was conducted to achieve a set of 800 *E. coli* and 800 *Enterococcus* isolates for AMR testing. Two samples that yielded *Salmonella* isolates were shown to harbor differing *Salmonella* serovars and consequently 218 *Salmonella* isolates were tested for AMR. The results of AMR testing suggest that resistance to clinically significant antimicrobials is generally low. Furthermore, greater than 84% of all *E. coli* and 79% of *Salmonella* were susceptible to all antimicrobials tested regardless of animal group. Resistance to lincomycin and flavomycin exceeded 80% for all *Enterococcus* isolates although resistance to ampicillin, vancomycin, gentamicin and linezolid was not observed. A cluster of cephem

resistant *Salmonella* in dairy cattle was identified and is of concern. Similarly, resistance in enterococci recovered from all animal groups to quinupristin / dalfopristin (44.6%), daptomycin (5.3%) and tigecycline (2.8%) is requiring of additional investigation. The clustered cephem resistance aside, there is minimal evidence that specific production practices are responsible for disproportionate contributions to AMR development and in general the lack of resistance to antimicrobials of significance in human medicine and favourable comparisons with international AMR surveillance programs is a pleasing outcome.

This study reports the prevalence of pSTEC and the AMR status of *Salmonella*, *E. coli* and *Enterococcus* from beef cattle groups slaughtered at Australian export registered abattoirs. The results indicate that *E. coli* O157 remains the dominant pSTEC in Australian cattle with *E. coli* O26 and *E. coli* O111 the only other pSTEC serogroups identified. The isolation of pSTEC serogroups O45, O103, O121 and O145 did not occur from any sample and is consistent with previous investigations and suggests that these serogroups are extremely rare in Australian cattle. Nonetheless, the presence of any pSTEC serogroups in cattle represents an ongoing challenge for producers who must continue to adhere to stringent processing guidelines and testing procedures to help ensure contaminated beef products do not enter commerce. Similarly, whilst the AMR data generated by this study suggests that in general beef cattle production practices are likely to have minimal effect on human clinical treatment outcomes there are data that warrant further investigations. It therefore remains necessary to maintain strict guidelines and controls around the use of antimicrobials in food-production animals in Australia and to continually monitor the effects of all antimicrobial use if Australia's reputation as a supplier of safe and healthy food is to be maintained.

Contents

Project objectives (G.MFS.0286 & G.MFS.0285)

- Determine the prevalence and enumeration of *E. coli* O157 in Australian beef cattle
- Determine the prevalence and enumeration of pSTEC in Australian beef cattle
- Establish whether or not there is any variation in the prevalence of pSTEC across seasons
- Determine the overall prevalence of cattle carriage of key human pathogenic bacteria in differing cattle groups
- Determine the incidence of antimicrobial resistance in key human pathogenic bacteria in the faeces of beef destined for processing, culled dairy cows and veal calves
- Estimate the impact that an introduction of a US food safety program targeting specific AMR *Salmonella* serovars may have to the Australian beef industry

Success in achieving project objectives

The project objectives of G.MFS.0286 and G.MFS.0285 have been successfully completed. A total of 31 export abattoirs representing >85% of total beef exports participated in the study. A total of 1500 faecal samples were collected from three animal groups (beef cattle, dairy cattle and veal calves) and were analysed for the prevalence of pSTEC. In addition, samples were tested for the presence of *Salmonella*, *E. coli* and *Enterococcus*. All *Salmonella* and a subset of *E. coli* and *Enterococcus* were tested for antimicrobial resistance. This document is the compilation of a series of reports and associated appendices compiled following this studies successful completion. The document includes:

- Report: Prevalence, concentration and characterization of Shiga toxin producing *Escherichia coli* serotypes O26, O45, O103, O111, O121, O145 and O157 in Australian beef cattle faeces
- Report: Antimicrobial resistance status of *Salmonella*, *Escherichia coli* and *Enterococcus* from Australian cattle populations at slaughter.
- Report: Comparison of two methods for the isolation of Shiga toxin producing *Escherichia coli* O157 from Australian beef cattle faeces.
- Appendix: Comparison of Australian animal isolates AMR surveys
- Appendix: Survey learnings

Prevalence, concentration and characterization of Shiga toxin producing *Escherichia coli* serotypes O26, O45, O103, O111, O121, O145 and O157 in Australian beef cattle faeces

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Prevalence of pSTEC serotypes in Australian cattle

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Abstract

Escherichia coli O157 and six non-O157 Shiga toxin (*stx*)-producing *E. coli* (STEC) serotypes (O26, O45, O103, O111, O121, O145) have been classified as adulterants in U.S. beef due to their dominant association with clinical disease. STEC that cause severe human disease have been termed pathogenic STEC (pSTEC). In this study we define pSTEC as containing intimin (*eae*) and belonging to any of the aforementioned STEC serotypes. While beef cattle are a known reservoir for the most extensively studied pSTEC serotype, *E. coli* O157, little is known about the dissemination of non-O157 pSTEC serotypes in cattle. Here, we report on the prevalence and concentration of pSTEC serotypes in 1500 faecal samples collected from beef cattle, dairy cattle and veal calves at slaughter. These were further broken down by carcase weight into young animal and adult animal classes. PCR was used to screen enriched faecal samples for O antigens, *stx* and *eae* markers. Overall, 44.7% of samples were deemed potentially positive (contained *stx*, *eae* and an O antigen marker) for at least one pSTEC serotype. The presence of pSTEC serotypes, in order of frequency was; O103 (33.5%), O45 (25.1%), O121 (23.4%), O26 (19.5%), O157 (10.4%), O111 (5.7%) and O145 (5.3%). In total, 115 (7.7%) samples yielded a pSTEC isolate, of which 100 (6.7%) contained *E. coli* O157 and 19 (1.3%) contained a Big 6 pSTEC. Fifteen (1.0%) of the 19 samples positive for a Big 6 pSTEC contained *E. coli* O26, while four (0.3%) contained *E. coli* O111. Pathogenic STEC of serotypes O45, O103, O121 and O145 were not isolated from any sample, even though genes indicative of *E. coli* belonging to these serotypes were detected by PCR. Young animals were associated with significantly higher prevalence and concentrations of pSTEC than adult animals ($P < 0.05$). In contrast to *E. coli* O157, and consistent with previous findings, this study reports a relatively low prevalence of non-O157 pSTEC serotypes in Australian cattle populations. While Australian animals do not appear to be a major reservoir for many non-O157 pSTEC serotypes, prevalence estimates rely on detection and isolation methodologies that continue to present challenges.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are important food-borne pathogens capable of causing a variety of disease symptoms in humans. STEC are distinguished from non pathogenic *E. coli*, which comprise the normal intestinal flora of healthy mammals, including humans, by the production of Shiga toxins (Stx). Clinical symptoms can manifest as mild gastroenteritis or acute bloody diarrhoea resulting from haemorrhagic colitis. In severe cases, patients can develop a life threatening sequela known as haemolytic uraemic syndrome. This condition is characterised by the development of haemolytic anaemia and acute renal failure and can sometimes result in death.

E. coli O157 has been the most extensively studied STEC serotype. This serotype first emerged as a food-borne hazard associated with ground beef products in the early 1980's and has since been implicated in numerous food-borne outbreaks worldwide. It was later established that healthy ruminants are the major reservoir of *E. coli* O157. Since then, numerous studies have reported the prevalence of *E. coli* O157 in cattle across the globe (Elder, Keen et al. 2000; Omisakin, MacRae et al. 2003; Fegan, Vanderlinde et al. 2004; Garcia, Fox et al. 2010). While a variety of food types have been implicated in outbreaks, a large proportion of cases continue to be associated with the consumption of contaminated beef products, typically undercooked ground beef (Mainil and Daube 2005). Furthermore, secondary contamination of food products such as fresh produce with cattle faeces has been identified as a growing source of transmission of *E. coli* O157 into the human food chain (Mainil and Daube 2005). Consequently, cattle as a source of *E. coli* O157 continue to attract interest from industry and regulatory bodies.

While *E. coli* O157 is an important, much publicised food-borne pathogen, it is not the only STEC serotype capable of causing disease in humans. Greater than 470 STEC serotypes have been recovered from humans, many of which are also reported to occur in beef cattle (Gyles 2007). These serotypes are not isolated from humans in equal proportions and the majority are infrequently associated with clinical disease (Gyles 2007). In a study conducted by the Centers for Disease Control and Prevention, six STEC serotypes (O26, O45, O103, O111, O121 and O145) were identified as the most common cause of non-O157 illness in the United States. Collectively, these serotypes represented 71% of all non-O157 STEC isolates submitted to 42 public health laboratories between 1983 and 2002 (Brooks, Sowers et al. 2005). Since a variety of factors contribute to an isolates capacity to cause disease, distinguishing pathogenic STEC from non-pathogenic STEC may be impossible (Gyles, Johnson et al. 1998). Supporting this is a general lack of consensus in the scientific literature regarding the precise virulence factors that enable an STEC to cause human disease.

Despite being inconclusive, there are some combinations of virulence genes that are highly correlated with severe human disease. One specific virulence factor (intimin), responsible for intimate adhesion of STEC to human epithelial cells, was commonly identified in clinical isolates, and strongly associated with bloody diarrhea, in North America (Brooks, Sowers et al. 2005). The association of intimin with a particular subtype of *stx* (stx_2) has also been correlated with severe disease in humans (Boerlin, McEwen et al. 1999). In a risk profile for non-O157 STEC published by the U.S. Food Safety and Inspection Service (FSIS), pathogenic STEC are defined as any STEC capable of causing severe human illness (U.S. Department of Agriculture 2012). Similarly, Bosilevac and Koohmaraie (Bosilevac and Koohmaraie 2011) classified pathogenic STEC based on the detection of intimin or subtilase and genetic markers indicating the presence of at least one virulence-related O islands (OI 36, 57, 71, or 122) . In this study we have chosen to define pathogenic STEC (pSTEC) as *E. coli* isolates that possess *stx*, intimin (*eae*) and belong to *E. coli* O157 or one of the big 6 U.S. clinical serotypes (O26, O45, O103, O111, O121, O145). However, it is important to note that *E. coli* isolates possessing *stx* and *eae* are also commonly referred to as Enterohaemorrhagic *E. coli*, regardless of the serotype to which they belong.

In the mid 1990's, the U.S. Food Safety and Inspection Service (FSIS) declared *E. coli* O157 an adulterant of raw non-intact beef products and product components in the U.S (Food Safety and Inspection Service 1996). In 2012, the FSIS extended this definition to include the additional six pSTEC serotypes identified as clinically significant by the CDC (U.S. Department of Agriculture 2012). In addition to *E. coli* O157, regulatory testing of raw non-intact beef products for non-O157 serogroups O26, O45, O103, O111, O121 and O145 began in the U.S.in mid 2012. Australian exporters of beef to the U.S. have been testing for *E. coli* O157 in raw non-intact beef products for many years. In response to the growing importance of non-O157 pSTEC to public health and global trade, Australian exporters have implemented equivalent procedures for the detection of non-O157 pSTEC serotypes in raw non-intact beef products destined for the U.S.

Although non-O157 pSTEC have been isolated from animals and animal products, including ground beef, it is not clear if cattle are the major reservoir of all of these serotypes. In a recent risk profile published by FSIS, several gaps in the knowledge of pSTEC in beef production were identified, in particular, baseline information on the prevalence of these organisms in cattle used to produce ground beef (U.S. Department of Agriculture 2012). Likewise, very little is understood about the prevalence of pSTEC in Australian animals used in beef production. Therefore, a detailed understanding of cattle as a possible reservoir for non-O157 pSTEC will help guide future regulatory

decisions and help establish if current industry practices are likely to result in positive public health outcomes such as a decreased transmission of these serotypes into the food production system. Thus the primary objective of this study was to investigate the prevalence of pathogenic STEC serotypes O26, O45, O103, O111, O121, O145 and O157 in 1500 beef cattle faecal samples collected from Australian beef export abattoirs. In addition, sampling data for each animal was used to assess if relationships existed between pSTEC and animal class (beef, dairy, veal), feed type (grass or grain) or generic *E. coli* counts. The levels of the pathogens in faecal samples that yielded pSTEC isolates were estimated and pSTEC isolates were characterised using previously published subtyping techniques.

Materials and methods

Sample collection and preparation

A sampling plan was developed for three animal groups: Australian beef cattle, dairy cattle, and veal calves with a collection target of 900, 300 and 300 samples respectively for the three groups. A total of 31 abattoirs representing >85% of total beef exports agreed to participate in the survey (Table 1). The number of cattle to be sampled at each abattoir was stratified based on production type (beef, dairy, veal) and slaughter volumes. Systematic random sampling was used to collect the samples across a consecutive two day period in each of the sampling windows. The sampling window occurred over an eight week period with the first window occurring in February and March, 2013 and the second sampling window occurring in August and September, 2013. Faecal samples were collected post-evisceration by cutting the intestine 15-30 cm from the rectal end and squeezing at least 40 g of material into a sterile jar. Samples were kept chilled and returned to the laboratory by overnight courier for processing.

Two faecal slurries were prepared per sample. The first faecal slurry was prepared by diluting 20 g of faeces 1 in 10 in MP Media (DuPont Qualicon, UK). A 60 ml portion of each slurry was transferred to a sterile jar and subsequently stored <4°C for use in enumeration experiments if required. The faecal slurry was then enriched at 41 ± 1°C for 18 h without agitation. A second faecal slurry was prepared by diluting 10 g of faeces 1 in 10 in buffered peptone water (BPW; Oxoid, UK) and subsequently incubated at 42 ± 1°C for 6 h.

Classification of animal, feed, weight and plant groupings

Participating establishments were asked to provide details on product type (beef, dairy veal), feed type and carcase weight for all animals from which faecal samples were collected. Carcase weights were subsequently used to group animals into three age categories; veal calves, young animals and adult animals. Animals were defined as veal in accordance with the AUS-MEAT definition which states that veal carcases should weigh no greater than 150 kg (AUS-MEAT Limited 2011). Young animals were classified as those with carcase weights equal to or less than 250 kg but greater than 150 kg and animals were considered to be adults if they had a carcase weight that exceeded 250 kg. The estimated age of each animal was combined with the production type (beef, dairy, veal) to generate the following categories; veal, young beef, young dairy, adult beef and adult dairy. Any animal that had a carcase weight of less than 150 kg was considered veal regardless of the designation provided by abattoir personnel. A more detailed analysis of carcase weights was also

performed where nine weight classes were generated, each representing 50 kg increments. Carcase weights greater than 400 kg were combined into a single group to generate a suitable sample size.

Individual animal data provided by each establishment was used to separate animals into additional groupings based on feed type, carcase weight or plant production volumes. For the purpose of generating meaningful feed groups, sampled animals with missing or incomplete feed data and all veal calves were removed from the sample set. From the remaining set, animals listed as dairy were combined into a single feed type while beef cattle were divided into grass or grain fed groups based on feed data provided by abattoirs. Lastly, plants were separated into three groups based on weekly production estimates provided by each establishment. Plants were assigned to low, medium and high production groups if total weekly slaughter volumes were ≤ 2500 cattle, > 2500 & < 5000 cattle or ≥ 5000 cattle, respectively.

Detection of pSTEC

Faecal samples that were enriched in MP media were tested for the presence of *stx*, *eae* and the seven pSTEC serotypes (O26, O45, O103, O111, O121, O145 and O157) using the BAX System Real-Time PCR STEC Suite and the BAX System PCR Assay for *E. coli* O157:H7 MP (DuPont Qualicon, UK). A crude DNA extraction was performed on each enrichment as follows. A 1 ml volume of each MP enrichment was placed in a 1.5 ml eppendorf tube and centrifuged for 1 min at 13 000 rpm. The supernatant was gently removed and pellets were resuspended in 1 ml of Phosphate Buffered Solution (PBS, Sigma Aldrich) prior to a second centrifugation for 1 min at 13 000 rpm. After gently removing the supernatant, pellets were resuspended in 1 ml sterile distilled water. Suspensions were boiled for 10 min prior to commencing the BAX System lysis step. Preliminary experiments were also performed to determine a dilution factor that would further reduce the level of inhibition related to faecal content in the assays (data not shown). To achieve the desired dilution, an aliquot of 5 µl was added to 200 µl of BAX lysis solution as this resulted in much lower inhibition than the 20 µl recommended by BAX. Following this, assays were performed as per the manufacturer's recommendations.

Isolation and confirmation of pSTEC

Samples that tested positive for *stx*, *eae* and at least one of the pSTEC serotypes were deemed to be potentially positive for a pSTEC and subjected to the following isolation procedure.

Initial attempts were made using immunomagnetic separation (IMS) to isolate pSTEC from samples that were enriched in MP Media. IMS was performed using Assurance GDS Poly IMS – Top 7 STEC beads (BioControl, U.S.) and an automated bead retriever (Life Technologies, Australia). The resulting bead-bacteria complexes were plated onto Rainbow Agar O157 (Biolog, U.S.) supplemented with 5.0 mg/L sodium novobiocin, 0.05 mg/L cefixime trihydrate and 0.15 mg/L potassium tellurite, cefixime-tellurite sorbitol MacConkey agar (CT-SMAC; Oxoid, UK), USMARC chromogenic agar medium (Kalchayanand, Arthur et al. 2013) and WBAM (Sugiyama, Inoue et al. 2001). All agar plates were incubated at 37 ± 2°C for 20-24 h. Depending on the number of morphologically distinct colonies present following incubation, a minimum of six and a maximum of 20 representative colonies were picked from all plates, streaked onto SBA and incubated at 37 ± 2°C for 20-24 h. The resulting colonies were tested for the presence of *stx* and *eae* using a published multiplex PCR (Paton and Paton 1998). Colonies that tested positive for *stx* and/or *eae* were then tested for the Big 6 serotypes as per the FSIS Guidebook MLG5B.03 (U.S. Department of Agriculture and Inspection Service 2012) and for O157 using a previously published protocol (Perelle, Dilasser et al. 2004). All isolates were confirmed as *E. coli* and stored at -80°C.

Attempts were made to isolate *E. coli* O157 from all BPW enrichments using a previously published method (Fegan, Vanderlinde et al. 2004). The resulting O157 bead-bacteria complexes were plated onto CT-SMAC and incubated at 37 ± 2°C for 20-24 h. Following incubation, non sorbitol-fermenting colonies were tested using the O157 latex agglutination kit (Oxoid). Isolates that tested positive were plated onto SBA and incubated at 37 ± 2°C for 20-24 h. Colonies were tested for the presence of *stx* and *eae* by Paton multiplex, confirmed as *E. coli* using the Microbact 12E or 24E system (Oxoid) and stored at -80°C using MicroBank (Pro-Lab Diagnostics, U.S.).

Enumeration of pSTEC serotypes

The enumeration of pSTEC serotypes in samples from which pSTEC were recovered was performed using a combined most probable number (MPN) and real-time PCR approach. A total of 114 samples from which pSTEC were isolated were enumerated. The technique used a five dilution (1.0 to 0.0001g), three tube MPN to determine counts of <0.3 to 11,000 MPN/g (Speck 1976). The MPN tubes contained 10 ml of MP Media and were incubated for 18 ± 2 h at 37 ± 2°C. Each tube was tested for *E. coli* O157 (Perelle, Dilasser et al. 2004) and non-O157 pSTEC serotypes (U.S. Department of Agriculture and Inspection Service 2012) using previously published real-time PCR protocols. MPN counts were based on the number of tubes that tested positive for the pSTEC serotype though no attempt was made to isolate a pSTEC serotype from each tube. Where an MPN

count exceeded 11,000 MPN/g, a boiled cell lysate (BCL) was prepared using 1 ml of unenriched faeces, diluted 10^{-1} with MP broth. BCL's were centrifuged at 13 000 rpm for 3 min, resuspended in 500 µl of sterile water and boiled for 10 min at 95°C. Tubes were centrifuged for a further 3 min at 13 000 rpm and tested for pSTEC serotype markers using quantitative real-time PCR. A pSTEC concentration was determining by plotting the threshold value (Ct) of the sample onto a standard Ct curve produced from known cell densities of the target serotype.

Characterisation of pSTEC

Lineage specific polymorphism analysis (LSPA-6) was conducted on all *E. coli* O157 isolates using primers fluorescently labeled with FAM ™ and VIC ® dye technology (Applied Biosystems) (Yang, Kovar et al. 2004; Whitworth, Zhang et al. 2010). LSPA-6 alleles were amplified using the cycling conditions described by Yang et al. (Yang, Kovar et al. 2004) and separated using previously defined capillary electrophoresis parameters (Applied Biosystems) (Mellor, Besser et al. 2013). Interpretation of results was achieved with the aid of Peak Scanner software (Version 1.0; Applied Biosystems). Isolates with LSPA-6 profiles 211111 or 111111 were classified as lineage I/II (LI/II) and lineage I (LI), respectively while all other profiles were collectively classified as lineage II (LII).

All pSTEC isolates recovered in this study were screened for the presence of key virulence genes (*stx*$_1$, *stx*$_2$ and *eae*) using a conventional multiplex PCR (Paton and Paton 1998). For *E. coli* O157 isolates, *stx*$_2$ subtypes (*stx*$_{2a}$ and *stx*$_{2c}$) and *stx*-bacteriophage-chromosome junctions for common *stx*-bacteriophage insertion sites (*argW*, *sbcB*, *wrbA* and *yehV*) were examined following the method of Shringi et al. (Shringi, Schmidt et al. 2012). Interpretation and analysis of SBI genotyping results was performed as previously described (Mellor, Besser et al. 2013).

Prevalence and enumeration of generic E. coli

E. coli counts were estimated by plating 1 ml of serial dilutions of the unenriched faecal slurry onto 3M™ Petrifilm™ *E. coli*/Coliform Count Plate (3M; St. Paul, Minnesota, U.S.). The number of *E. coli* present was determined after incubation for 24 ± 2 h at 35°C as per AOAC official method: *Escherichia coli* Counts in Poultry, Meats, and Seafood, Dry Rehydratable Film Method (Petrifilm EC Plate Method). Negative samples were arbitrarily assigned a count of half the limit of detection for this method (1 log cfu/g faeces).

Statistical analysis

Statistical analyses were performed in Stata version 12.1 using a 2x2 contingency table and Fisher's exact test (StataCorp U.S.). *P* values were two-tailed and groups were considered significantly different if *P* values were <0.05. Group comparisons were performed using a one-way ANOVA with Bonferroni correction to *P* values to account for multiple comparisons.

Results

Establishment participation and sample classifications

Of the 31 plants that participated in this survey, 27 provided samples on both sampling occasions while four provided samples on only one sampling occasion. A total of 753 samples were provided by 29 establishments in sampling window 1 and 747 samples were provided by 29 establishments in sampling window 2.

All 1500 samples were classified into one of three production types (beef, dairy or veal) and three age classes (adult, young and veal) based on carcase weight to form five age/production classes; veal, young beef, young dairy, adult beef and adult dairy. The sample numbers and mean carcase weights for each animal class are listed in table 2. The number and types of samples (animal class, mean carcase weight and feed type) were not significantly different between sampling windows (*P* > 0.05). In total, samples were sourced from six states and one territory. The biggest beef producing States; Queensland (46.7%), New South Wales (24.1%) and Victoria (15.9%), supplied the majority of samples. Of the 31 export plants that participated, 26 supplied samples from adult or young beef cattle. Fewer plants processed dairy cattle and veal calves in volumes sufficient to participate in this survey. As such, dairy and veal samples were supplied by eight and 11 plants, respectively. Of these, three plants supplied the majority of dairy samples while two plants supplied the majority of veal samples. The animals slaughtered at these facilities were, however, sourced from numerous spatially separated producers. Of the 1500 samples collected in this study, 1148 were classified into dairy, grass and grain fed groups, with each group comprising 271, 302 and 575 samples, respectively. Twenty nine plants were represented by at least one feed type while two plants, comprised solely of samples derived from veal calves or samples with missing or incomplete feed data, were consequently excluded from feed type analyses.

Presence of stx, eae and pSTEC serotype markers by PCR

Of the 1500 samples, *stx* was detected in 52.2%, *eae* in 51.8% and a pSTEC serotype in 71.9% (Table SI). The presence of *stx* and *eae* in the same sample occurred in 765 (51.0%) samples (Table 3). The

majority of samples that tested positive for *stx* and *eae* (51%) also tested positive for at least one pSTEC serotype with 671 of 1500 samples (44.7%) deemed potentially positive (presence of markers for *stx*, *eae* and at least one serotype) for pSTEC. On average, samples containing *stx* and *eae* tested positive for 2.7 pSTEC serotypes, giving a total of 1841 potential positives combinations across all samples. All seven pSTEC serotype markers were detected by PCR in this study and a breakdown is provided in Table 3. The most frequently occurring serotype markers were O103 (33.5%), O45 (25.1%), O121 (23.4%), O26 (19.5%) and O157 (10.4%). Serotypes O145 (5.3%) and O111 (5.7%) were present in similar ratios that were, with the exception of *E. coli* O157, distinctly lower than all other pSTEC serotypes. Faecal samples collected between February and March (49.8%) were more likely to test positive for a pSTEC markers than samples collected later in the year (39.5%).

Prevalence of pSTEC serotypes

A total of 671 of 1500 (44.7%) samples contained all the markers for a Top 7 pSTEC serotype and were subjected to the isolation procedure. Overall, pathogenic STEC were recovered from 7.7% of samples. Of these, 100 (6.7%) contained *E. coli* O157, while 19 samples (1.3%) contained a non-O157 pSTEC serotype. Pathogenic STEC of serotypes O157, O26 and O111 were isolated from at least one enrichment broth while an additional four pSTEC serotypes (O45, O103, O121 and O145) were not recovered from any potentially positive sample (Table 4). Although the majority of samples yielded a single pSTEC serotype, four samples were shown to harbor two different pSTEC serotypes (Table 5). Pathogenic STEC belonging to serotypes O157 and O26 were isolated from three samples while a single sample contained pSTEC of serotypes O157 and O111. An additional six samples yielded pSTEC of a single serotype that had two different virulence profiles (Table 5). The most likely animal classes to yield a pSTEC isolate, in order of prevalence, were veal (12.7%), young beef (9.8%), young dairy (7.0%), adult beef (5.1%) and adult dairy (3.9%). Veal calves were significantly more likely to yield pSTEC than adult beef or adult dairy cattle ($P < 0.05$) though no significant differences were observed for any of the other animal groups. When combined, young beef, young dairy or veal calves (67.8%) were significantly more ($P < 0.05$) likely to yield pSTEC than adult beef or dairy cattle (32.2%) (Table 4). This relationship was also observed when *E. coli* O157 and non-O157 isolates were analyzed separately. In addition, significantly more isolation's occurred during the warmer months of February and March (9.4%) than August and September (5.9%) ($P < 0.05$).

Of the 100 samples from which *E. coli* O157 were isolated, 37 (2.5%) were obtained using the BAX system while 90 (6.0%) were recovered from BPW enrichments. The prevalence of *E. coli* O157 in each of the animal groups was 10.5% for veal calves, 8.4% for young beef, 5.6% for young dairy, 4.9%

for adult beef and 3.1% for adult dairy. Veal calves were respectively 3.4 and 2.1 times more likely to yield *E. coli* O157 than adult dairy cattle or adult beef cattle. In contrast, no significant difference ($P > 0.05$) in the prevalence of *E. coli* O157 from grass-fed cattle (7.5%), dairy cattle (4.4%) or grain-fed cattle (4.0%) was identified. In total, Big 6 pSTEC were isolated from across 32.3% of the 31 abattoirs tested. Of the 19 samples that yielded a Big 6 pSTEC, 15 (1.0%) contained *E. coli* O26 and four (0.3%) contained *E. coli* O111 (Table 4). Non-O157 pSTEC occurred more frequently in younger animals with 52.6% of isolates recovered from young beef and dairy cattle, 36.8% recovered from veal calves and 10.5% isolated from adult animals (Table 4). In addition to the seven pSTEC serotypes isolated, a further 15 EHEC were isolated across all five animal classes. The serotypes of these isolates have not been determined but they do not belong to one of the seven pSTEC serotypes.

The carcase weight of each animal sampled was used to generate nine weight classes, in 50 kg increments, to further investigate the relationship between carcase weight and pSTEC (Table 6). The highest prevalence of pSTEC was observed in animals with a carcase weight between 50 and 100 kg (21.4%) while the lowest prevalence belonged to animals in the highest weight class (2.6%). Four of the five weight classes below 250 kg had a pSTEC prevalence that exceeded the overall prevalence for the survey (7.7%). Conversely, all weight classes greater than 250 kg had a prevalence of pSTEC less than the mean for all groups.

Variability in the prevalence of pSTEC was observed for individual plants (Fig 2; Table SI). A pSTEC belonging to one of the Top 7 serotypes were isolated from 25 (80.6%) plants while a further six plants failed to yield a pSTEC serotype. The isolation rate differed between each window, with 22 of 29 (75.9%) pSTEC positive plants identified in sampling window one compared to 13 of 29 (44.8%) positive plants in sampling window 2. Of the 27 plants that provided samples for both windows, 10 (37.0%) plants yielded at least one pSTEC positive on both sampling occasions and 13 (48.1%) plants yielded a pSTEC positive on only one sampling occasion. The highest prevalence and greatest range between sampling windows was observed in plant 7 in which 53.3% of samples yielded a pSTEC in the first window while 0.0% of samples were confirmed in the second window. Despite having the highest prevalence, samples collected from plant 7 represent a small proportion of the total number of samples tested in sampling window 1 (2.0%) and sampling window 2 (1.9%). Non-O157 pSTEC were isolated from 10 different establishments, with *E. coli* O26 and *E. coli* O111 isolates recovered from 9 and three establishments, respectively. Two establishments yielded multiple O26 isolates. One plant yielded four *E. coli* O26 isolates from a single sampling window while the other plant yielded four *E. coli* O26 from across both sampling windows; one from sampling window 1 and three

from sampling window 2. Isolation of different Big 6 pSTEC from samples belonging to a single abattoir occurred only twice with *E. coli* O26 and *E. coli* O111 recovered from different samples collected from the same abattoir during the same sampling window.

Plants were separated into three groups based on the weekly production volumes provided by each establishment (Table 7). A total of 11, 13 and seven plants were identified as low, medium and high volume processors, respectively. The mean carcase weight of animals slaughtered in low volume plants was less than the mean weight of medium and high volume plants. The types of animals processed within each production class were shown to vary considerably (Fig 3). Low volume plants processed the majority of young dairy cattle (87.4%), veal calves (78.1%) and adult dairy cattle (63.3%) sampled in this survey. In total, veal and dairy calves represented 65.8% of their production volume. Adult beef cattle and young beef cattle collectively represented 73.9% of medium and 98.0% of high volume plant production. Plants that were assigned to low, medium and high production groups exhibited differences in the total number of confirmed pSTEC isolates (Table 7). Overall, plants that processed low weekly volumes (≤ 2500 animals) were more likely to yield a pSTEC isolate (9.4%) than plants that processed medium (6.9%) and high (6.4%) weekly volumes. The primary animal classes processed in each of the production groups had a major impact on the number of pSTEC that were confirmed in each production category.

Enumeration of pSTEC

Enumeration of confirmed pSTEC serotypes in each sample was determined using a combined most probable number (MPN) and real-time PCR approach. The MPN used had an upper limit of 1.1×10^4 MPN/g of faeces. Counts of *E. coli* O157 ranged from <-0.52 to 6.89 $\log_{10}$MPN/g of faeces. Sixty seven (70.5%) of the 95 samples containing *E. coli* O157 had counts less than 3.00 $\log_{10}$MPN/g of faeces with 36 (37.9%) of the 95 samples at or below the limit of detection for the MPN procedure (-0.52 $\log_{10}$MPN/g of faeces). Differences were observed in the *E. coli* O157 count for each of the animal classes, with younger animals significantly more likely to be associated with counts exceeding 3.00 $\log_{10}$MPN/g of faeces (Fig 4). Of the 28 counts that exceeded 3.00 $\log_{10}$MPN/g of faeces, 14 were from veal calves, seven were from young beef, five were from adult beef and two were from young dairy. Twenty two of the 28 samples containing *E. coli* O157 had counts that exceeded 4.00 $\log_{10}$MPN/g of faeces and are consistent with counts observed in supershedding animals. A higher proportion of *E. coli* O157 isolated in sampling window 2 (34.2%) were considered to be from supershedders than window 1 (15.8%). Seventeen of the 22 samples that exceeded 4.00 $\log_{10}$MPN/g of faeces were associated with veal, young beef or young dairy while only five samples were

associated with adult cattle. The maximum count of *E. coli* O157 was greatest in samples sourced from veal (6.89 $\log_{10}$MPN/g of faeces) and young dairy cattle (5.98 $\log_{10}$MPN/g of faeces). Adult beef and young beef both had a maximum count of 4.38 $\log_{10}$MPN/g of faeces while adult dairy had a maximum count of <-0.52 $\log_{10}$MPN/g of faeces. The mean *E. coli* O157 count also varied for each animal class. Veal calves and young dairy cattle had the highest mean counts, with both exhibiting 2.4 $\log_{10}$MPN/g of faeces, respectively. Young beef, adult beef and adult dairy had lower respective mean counts of 1.5, 1.2 and -0.52 $\log_{10}$MPN/g of faeces.

Comparison of non-O157 serotype prevalence's and animal or feed type classes were constrained by the limited number of isolates recovered. *E. coli* O26 counts across animal classes ranged from <-0.52 to 4.38 $\log_{10}$MPN/g of faeces. The lowest count of *E. coli* O26 was observed in adult beef (0.96 $\log_{10}$MPN/g faeces) while the highest count (4.38 $\log_{10}$MPN/g of faeces) was detected in a veal calf. *E. coli* O111 was isolated from three young beef samples, all of which had counts <-0.52 $\log_{10}$MPN/g faeces, and one veal calf sample (2.63 $\log_{10}$MPN/g faeces).

Characterization of pSTEC isolates

Pathogenic STEC of serotype O157 were typed using a lineage specific polymorphism assay to gain additional insight into the genotypic structure of current cattle populations. In total, 5 unique LSPA-6 designations were identified. Lineage I/II (211111) accounted for the majority of isolate (80%) while LII (20%) accounted for the remainder. Isolates belonging to LI were not detected in this survey. LII isolates were represented by four LSPA-6 designations; 221212 (16.8%), 211112 (1.1%), 211212 (1.1%) and 232212 (1.1%).

All pSTEC isolates were also characterised into three groups based on virulence gene combinations (*stx*$_1$, *stx*$_2$ and *eae*) possessed by each pSTEC serotype (Table 8). A total of 10 samples yielded isolates of different serotypes or of the same serotype with different virulence profiles. When considering these additional isolates, 125 pSTEC were obtained from the 115 pSTEC positive samples (Table 5). Of the 125 pSTEC, 56.3% possessed *stx*$_1$, *stx*$_2$ and *eae*, 35% possessed *stx*$_2$ and *eae* and 15.1% possessed *stx*$_1$ and *eae*. The majority of *E. coli* O157 isolates (96.2%) were shown to possess *stx*$_2$ and *eae*, either in the presence (66.7%) or absence (33.3%) of *stx*$_1$. In contrast, isolates belonging to pSTEC serotypes O26 and O111 possessed *stx*$_1$ and *eae* in the absence of *stx*$_2$ (75%) while a further 25% of isolates were shown to carry *stx*$_2$ in the presence of *stx*$_1$ and *eae*.

A more detailed examination of Shiga toxin bacteriophage insertion sites and *stx* gene subtypes was also undertaken for *E. coli* O157 isolates. In total, 8 unique SBI genotypes were identified of which three accounted for greater than 92% of all isolates. Specifically, SBI types ASY12c, SY2c and AS12c represented 44.2%, 29.5% and 19.0% of isolates, respectively. The remaining five genotypes (ASY1, ASY2c, ASWY122c, AY1 and S2c) were represented by 7.4% of isolates.

Enumeration of generic E. coli

A total of 1385 of 1500 (92.3%) samples had counts above the limit of detection using *E. coli/*coliform count plates. Overall, the mean *E. coli* counts were not significantly different ($P > 0.05$) (Fig 5) between screen negative, potential positive or confirmed positive samples, although the mean count for confirmed positive samples was higher than screen negative or potentially positive samples. Generic *E. coli* counts were as low as 1 log cfu/g faeces and as high as 8.6, 8.7 and 7.9 log cfu/g faeces for screen negative, potential positive and confirmed positive samples, respectively.

No significant difference was observed between the prevalence ($P = 1.000$) or mean count ($P = 0.873$) of *E. coli* across sampling windows. However, some differences were observed between animal classes. The mean count of *E. coli* in veal calves was significantly greater than all other animal classes ($P < 0.05$). Likewise, the mean *E. coli* counts in both adult beef and young beef were significantly greater than adult dairy ($P < 0.05$) and young dairy ($P < 0.05$). However, no statistical difference was observed between the mean *E. coli* count of adult beef and young beef ($P > 0.05$) or adult dairy and young dairy ($P > 0.05$).

Discussion

The prevalence of pathogenic STEC in beef cattle is poorly understood, both domestically and internationally. Here, we attempt to address this knowledge gap by providing insight into the prevalence of pSTEC (*E. coli* O157 and the "Big 6") serotypes in cattle processed in Australian beef export abattoirs. This project was designed to sample animals in similar volumes, across two sampling windows; one of which occurred in February and March 2013 and the other in August and September 2013. This temporal variation in sampling was intended to provide a glimpse into how seasonal change can impact on the prevalence and isolation of pSTEC. However, we urge caution when interpreting this data as animals would need to be tested in large numbers across numerous seasons to gain an accurate depiction of seasonality. Cattle faeces was chosen as the most appropriate sample type for the detection and isolation of pSTEC because it is minimally exposed to process related sources of cross contamination. Faecal content was obtained from the large

intestine, sliced approximately 30 cm from the rectum, to further reduce the risk of contamination and ensure that prevalence figures relate solely to the animal being sampled. Overall, this study achieved a high plant participation rate, representing greater than 85% of annual export production. This level of participation ensured that animals sampled throughout this survey were representative of total Australian export production.

Here, the presence of individual pSTEC serotype markers ranged from 5.3% for *E. coli* O111 to 33.5% for *E. coli* O103 which is comparable to the range observed in a recent U.S. survey of cattle faeces (0.94% for *E. coli* O111 to 34.0% for *E. coli* O157)(Cernicchiaro, Cull et al. 2013). A similar overall prevalence (51.3%) and range (0% for *E. coli* O111 to 51.3% for *E. coli* O145) of markers was also reported to occur in France (Bibbal, Loukiadis et al. 2014). In contrast, the presence of *stx* and *eae* markers alone or in combination with pSTEC serotypes did not corroborate with results from our previous survey which reported significantly fewer potential positive samples (Barlow and Mellor 2010). While both of our surveys relied on sequential PCR based approaches to detection, the primers and PCR parameters used in each study differed substantially. The current study employed a test that is widely used by commercial abattoirs for screening raw ground beef or beef trim enrichments, however, little is known about the effectiveness of this method in complex matrices such as faecal enrichments and how this might impact on potential positive rates. Climatic differences were also observed between these two studies, with the earlier study occurring during drought conditions and the current survey occurring in periods of high rainfall. Smith et al. (Smith, Blackford et al. 2001) reported a higher prevalence of *E. coli* O157 in feedlot cattle during muddy pen conditions than in normal conditions. While not investigated, it is equally possible that the variability in prevalence estimates between surveys was indirectly associated with rainfall.

In agreement with our findings, multiple pSTEC serotypes have previously been detected in cattle faeces or ground beef samples by molecular screen tests or culture isolation (Barlow and Mellor 2010; Bosilevac and Koohmaraie 2011; Bosilevac and Koohmaraie 2012; Bibbal, Loukiadis et al. 2014). In direct contrast to the high proportion of samples that were potentially positive for multiple pSTEC serotypes, the majority of samples that were confirmed for pSTEC in the current study yielded a single serotype. This finding is consistent with previous survey results of ground beef (Bosilevac and Koohmaraie 2011) and suggests that while pSTEC can co-exist in beef cattle the majority of cattle are likely to only carry a single dominant pSTEC serotype. Our study is not the first to report a disconnection between the number of screen test positive samples and strain isolations (Barlow and Mellor 2010; Bosilevac and Koohmaraie 2011; Bibbal, Loukiadis et al. 2014). In addition to *E. coli* of

the Top 7 serotypes, *stx* and *eae* may also be harbored by *E. coli* of non-Top 7 serotypes along with other members of the Enterobacteriaceae family such as *Citrobacter, Enterobacter, Shigella and Salmonella* (Schmidt, Montag et al. 1993; Paton and Paton 1996; Karch, Bielaszewska et al. 1999; Gyles 2007; Chandry, Gladman et al. 2012). Furthermore, greater than 435 serotypes of STEC have been isolated from cattle (Gyles 2007). In the current study, potential positive samples that did not yield a pSTEC may have alternatively harbored isolates that, while not conforming to the pSTEC definition, collectively possessed the genes necessary to generate a positive result. On this basis, we suggest that current molecular based methods of screening upwardly distort the prevalence of pSTEC due to the simultaneous presence of *stx*, *eae* and serotype genes in a sample where they are not necessarily associated with a single isolate. A greater understanding of the enrichment broth composition, from a microbiological perspective, would elucidate factors that may hinder accurate determination of prevalence estimates. If molecular based screening techniques continue to be the method of choice for pSTEC detection, the identification of new targets that provide more accurate pSTEC prevalence estimates should be investigated.

Due to the high potential positive rate obtained in this study, almost half of all samples tested were sent through to confirmation. Since Poly-IMS beads were used to target pSTEC, each potential positive sample was confirmed for all Top 7 serotypes, regardless of the initial screen test result. In an effort to improve isolation rates, the resultant bead-bacterial complexes were plated onto four different media, one of which targeted O157 and three of which targeted non-O157. Despite the significant efforts made to isolate pSTEC, only three of the seven pSTEC serotypes were recovered from the samples tested, with O157 significantly more common than non-O157 serotypes. The use of CT-SMAC to isolate *E. coli* O157 has proven exceptionally effective in discriminating pathogenic O157 from non-pathogenic O157. The lack of equally discriminative agar for non-O157 isolation may be the reason for the low comparative isolation rates. While the prevalence of *E. coli* O157 in this study is comparable to that observed in other countries (Elder, Keen et al. 2000; Omisakin, MacRae et al. 2003; Garcia, Fox et al. 2010; Masana, Leotta et al. 2010) the rate reported here represents an increase from our previous 2008 survey (1.7%) (Barlow and Mellor 2010) but a reduction from values reported in 2004 (13%) (Fegan, Vanderlinde et al. 2004). Within-plant variation was also identified between sampling windows and overall sampling window prevalence's differed significantly. The greater than 2 fold difference in isolation between the current study and our 2004 study cannot be attributed to methodology as identical procedures were employed in each. While the exact cause of this variation is unclear from our results, previous studies have correlated prevalence with multiple

factors including seasons, herds, rainfall, production types and feed type (Rhoades, Duffy et al. 2009).

Although our 2008 survey failed to yield non-O157 pSTEC isolates from any faecal sample (Barlow and Mellor 2010), the difference in pSTEC isolation between the two surveys was not statistically significant ($P > 0.05$). Unfortunately, the limited data available on non-O157 pSTEC prevalence in cattle faeces makes broad comparisons to equivalent international studies difficult. Nonetheless, our results were comparable to some studies (Monaghan, Byrne et al. 2011; Hofer, Stephan et al. 2012) but significantly lower than others (Joris, Pierard et al. 2011; Bibbal, Loukiadis et al. 2014). Although not directly related to faeces, an additional study reported a low prevalence (0.05%) of non-O157 in ground beef samples using a sequential PCR based approach (Bosilevac and Koohmaraie 2012). Another study of ground beef identified 10 STEC isolates from 4133 samples (0.24%) that were likely to be pSTEC based on virulence attributes, of which only 7 were shown to possess *stx*, *eae* and a Big 6 serotype gene (Bosilevac and Koohmaraie 2011). While the prevalence of non-O157 pSTEC in cattle faeces has been shown to vary considerably, the prevalence of these serotypes appears to be substantially lower than internationally reported prevalence's of *E. coli* O157. As alluded to earlier, this may be related to differences in the relative sensitivities of *E. coli* O157 vs non-O157 STEC methods of isolation. Alternatively, it may be an indication of the likelihood that non-O157 serotypes are present most often in forms that are non-EHEC.

The infrequent occurrence of non-O157 serotypes in Australian cattle faeces might partly explain the low incidence of disease identified in Australia. According to Valley et al. six STEC serotypes; O157 (58%), O111 (13.7%), O26 (11.1%), O113 (3.6%), O55 (1.3%) and O86 (1.0%) were the most common STEC serotypes detected in Australian clinical cases between 2001 and 2009 (Vally, Hall et al. 2012). Only three of the Top 7 U.S. clinical serotypes (O157, O111 and O26) were commonly detected in Australian humans, two (O103 and O145) were uncommon (< 1%) and a further two (O121 and O45) were not detected in humans over this period (Vally, Hall et al. 2012). Since case definitions and isolation methods can impact on epidemiological data, it is hard to draw any firm conclusions as to whether beef cattle are a major source of human STEC disease. Nonetheless, three pSTEC serotypes isolated from cattle in this study (O157, O26 and O111) are also the most common Australian clinical serotypes. The corroboration between predominant clinical and cattle isolates indicates that contaminated beef products destined for human consumption may be facilitating the transmission of pSTEC serotypes O26, O111 and O157 into the human population. In contrast to this, a study by McPherson et al. (McPherson, Lalor et al. 2009) indicates that, among other things, the consumption

of food types other than beef is a risk factor associated with non-O157 infections. Likewise, the lack of pSTEC serotypes O45, O103, O121 and O145 in both surveys suggests that these are not present, uncommon or present in levels too low to detect in Australian cattle. This is strongly supported by human epidemiological data that suggests that these four serotypes are either not isolated or infrequently isolated from humans in Australia. From the limited data available, the distribution of serotypes in humans and cattle appears to vary geographically and elevated relevance of specific serotypes to public health and industry is evident in different continents, countries or regions within the same country. Of the six non-O157 serotypes commonly isolated from humans in the U.S. only two are common in humans in Australia while four are common in humans in Europe. From a clinical perspective, additional non-Big 6 serotypes are significant in Australia and Europe. As our knowledge of non-O157 pSTEC increases, greater focus will undoubtedly be placed on controlling geographically relevant serotypes that pose the greatest risk to human health.

In 2009, the European Food Safety Authority (EFSA) released technical specifications for the monitoring and reporting of verotoxigenic *Escherichia coli* (VTEC) on animals and food. In that document, the EFSA recommended increased industry surveillance of STEC in animals aged between three and 24 months (Anonymous 2009). In this study, we report a higher prevalence of pSTEC in younger animal than adult animals with the highest prevalence observed in veal calves. A previous Australian study detected STEC and EHEC at higher frequencies in 1-14-week old weaning calves than adult cows (Cobbold and Desmarchelier 2000). Other authors have also reported peak prevalence in calves aged between two and six months (Nielsen, Tegtmeier et al. 2002). Since veal in this study encompassed all animals with carcase weights equal to or less than 150 kg, direct comparisons to weanling calves are difficult to make without further analysis. As such, animals were divided into carcase weights with the assumption that a positive association exists between carcase weight and animal age. Using this approach, there is some evidence for peak pSTEC prevalence in animals with carcase weights between 50 and 100 kg. While not investigated, it is possible animals with 50 and 100 kg carcase weights may represent animals aged between two to six months and if so our finding would support these previous studies. On this basis, veal calves may represent a potentially high risk animal class with respect to pSTEC. Although it is possible that clustering has upwardly distorted the prevalence figures in this weight class, the compelling evidence for increased prevalence of pSTEC in young animals, particularly veal calves, warrants further investigation.

The categorization of animals into different risk groups with respect to pSTEC is not well examined in the literature, though some studies have indicated that dairy animals may present a higher risk of

carrying *E. coli* O157 and non-O157 STEC serotypes (Oporto, Esteban et al. 2008; Bosilevac 2012). In contrast, our study showed adult dairy cattle to be the least likely animal class to harbor pSTEC. Furthermore, adult dairy and adult beef cattle were the only two groups with significantly less pSTEC than veal calves. The lack of corroborative data suggests that further work is required, on a larger animal set, to reinforce these findings with respect to the potential lower risk that adult dairy cattle and beef cattle present to the Australian beef industry. Since contamination of carcasses during processing has been correlated with the prevalence and numbers of *E. coli* O157 in cattle faeces (Elder 2000) it is equally important to understand the concentration of *E. coli* O157 in each of the animal classes. In this survey, 77.2% of samples that had *E. coli* O157 counts exceeding 4.00 $\log_{10}$MPN/g of faeces were associated with veal, young beef or young dairy. Animals that shed isolates at this level have previously been referred to as supershedders (Chase-Topping, Gally et al. 2008) and pose a high risk to processors due to increased potential for cross contamination of carcases. While inconclusive, this study does suggest that young animals are more likely to harbor pSTEC in higher concentrations than adult animals. Therefore, industry may benefit from implementing additional control measures during periods in which high volumes of young animals are processed.

It is well established that the prevalence of *E. coli* O157 is strongly seasonal, with peak associations with cattle populations occurring during warmer months (Barkocy-Gallagher, Arthur et al. 2003; Smith, Moxley et al. 2005; Williams, Withee et al. 2010). There is some evidence, albeit minimal, to suggest that seasonal fluctuations in the carriage and shedding of pSTEC may have influenced the pSTEC prevalence in the present study. Specifically, the rate of potential and confirmed positive samples was significantly higher during sampling that occurred in the warmer months of February and March. Likewise, a significantly greater number of plants also tested positive for an overall higher prevalence of pSTEC during this sampling period. Consistent with international studies, an increase in Australian clinical cases of STEC has been reported to occur in summer months while lower numbers were reported in winter (Vally, Hall et al. 2012). The higher prevalence of pSTEC in cattle and humans in summer, along with the isolation of predominant serotypes (O157, O26 and O111) from both sources, points to cattle as a potential source of transmission to humans. However, caution should be applied when interpreting this data as animals would need to be sampled across a much larger timeframe to accurately measure the affect of seasonality on pSTEC prevalence.

To investigate the role of production systems on the prevalence of *E. coli* O157, 1148 animals were separated into dairy, grass and grain feed types. Due to the low number of isolates obtained,

comparisons between Big 6 pSTEC and feed type were not performed. The isolation rate of *E. coli* O157 did not differ significantly between dairy cattle, grass-fed cattle or grain-fed cattle suggesting that, on this occasion, feed type did not influence *E. coli* O157 carriage rates in Australian cattle. This result is consistent with earlier findings by Fegan et al. (Fegan, Vanderlinde et al. 2004) that did not report a significant difference between the prevalence of *E. coli* O157 between grass-fed or grain-fed cattle. Interestingly, grain fed-cattle had a substantially higher rate of potentially positive samples than dairy or grass-fed cattle. However, this did not convert to a higher rate of isolation from grain-fed cattle, with grass-fed cattle yielding the greatest number of isolates. In our earlier study, no significant difference in pSTEC screen test positives were identified with respect to feed type though a much higher frequency of serotype only isolates were recovered from grass than grain-fed cattle (Barlow and Mellor 2010). Due to the concentration of animals in feedlots and animal exposure to similar production processes, it is reasonable to assume that cattle herds in the same yard are more likely to develop similar flora profiles than their grass-fed counterparts. These similar flora profiles may frequently include organisms that, while not consistent with the definition of a pSTEC, still generate positive screen test results. Therefore, further assessment of faecal enrichment profiles from grain-fed cattle is necessary to elucidate any potential challenges of working with this sample type.

In this survey, we chose to enumerate generic *E. coli* in all samples to investigate the suitability of generic *E. coli* as an indicator of pSTEC. No significant differences in the mean *E. coli* counts were observed between screen test negative, potentially positive and confirmed samples, suggesting that higher concentrations of generic *E. coli* are not indicative of the presence of pSTEC in faeces. Furthermore, the similarly broad range of generic *E. coli* counts observed in screen negative, potential positive and confirmed positive samples indicate that pSTEC can be isolated from samples with generic *E. coli* counts at the limit of detection (1 log cfu/g faeces) or as high as 8.7 log cfu/g faeces. However, the mean count in pSTEC positive samples was shown to be closer to 6 log cfu/g faeces. While the mean counts of pSTEC in unenriched samples varied for each animal class, in all cases they were significantly lower than the mean generic *E. coli* counts reported for confirmed positive samples. These data suggest that for almost all pSTEC positive samples, the dominant *E. coli* isolate was not the pSTEC. Some studies suggest that the likelihood of isolating a pSTEC may be directly related to the concentration of pSTEC present in a sample. The large number of pSTEC that were isolated from samples that had pSTEC counts below the limit of detection highlights a need for utilising media that capably increases the levels of pSTEC to concentrations that can be readily isolated using existing methods.

A virulence profile of each pSTEC isolate was generated using molecular subtyping methods. The frequency of isolating pSTEC with *stx*$_1$, *stx*$_2$ and *eae* varied for each serotype. *E. coli* O157 and non-O157 pSTEC serotypes harbored *stx*$_2$ in 96.2% and 25% of isolates, respectively. All non-O157 pSTEC carried *stx*$_1$ as opposed to 66.7% of *E. coli* O157 isolates. Despite this, other studies have reported various combinations of *stx*$_1$ and or *stx*$_2$ in non-O157 pSTEC so it is possible that the small number of recovered pSTEC in the current study may have distorted the prevalence of *stx* genes. Further analysis of *E. coli* O157 isolates was conducted to determine how current bacterial populations relate to previous phylogenetic studies on Australian cattle isolates. The majority of isolates (92%) were shown to belong to three dominant Shiga toxin bacteriophage (SBI) genotypes (ASY12c, SY2c and AS12c). These three SBI genotypes were also the three predominant genotypes observed in our previous study of 205 Australian cattle *E. coli* O157 isolates (Mellor, Besser et al. 2013). Additional phylogenetic analysis of *E. coli* O157 identified a single lineage (LI/II) accounted for 80% of isolates, which is highly consistent with previous results from Australia (Mellor, Sim et al. 2012; Mellor, Besser et al. 2013). On the basis of these results, Australian *E. coli* O157 isolates appear to have remained stable over time. Despite the fact that isolates were recovered from a large geographic spread of cattle, limited diversity of lineages and SBI genotypes were identified. This finding suggests that *E. coli* O157 are highly conserved within cattle populations across Australia.

While cattle are a major reservoir for *E. coli* O157, insufficient data exists to conclude that Australian cattle are a main reservoir for all non-O157 pSTEC serotypes. In many cases it is not practical or feasible to attempt isolation on large numbers of potentially positive samples that, despite persistent and varied attempts, are unlikely to yield a pSTEC isolate. Faeces are the most appropriate samples for determining true prevalence estimates in cattle. However, the inherent problems associated with such complex matrices, means that further development of PCR based detection methodologies are necessary to improve prevalence estimates of pSTEC. Furthermore, inherent challenges associated with current confirmation methods may also hinder attempts to gain accurate prevalence estimates of non-O157 pSTEC in cattle. Nonetheless, using current technologies, this study suggests that cattle are a potential reservoir for non-O157 pSTEC serotypes O26 and O111. The lack of isolation of serotypes O45, O103, O121 and O145 suggests that these serotypes are not present or present in levels too low to detect in Australian cattle faeces.

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Table 1 Number of samples collected from each animal class by state and plant.

Plant No.	Adult beef	Young beef	Total beef	Adult dairy	Young dairy	Total dairy	Veal	Total beef, dairy, veal
1	0	14	14	0	0	0	86	100
2	7	9	16	1	1	2	10	28
3	0	0	0	43	102	145	15	160
4	0	0	0	0	0	0	20	20
5	9	0	9	7	6	13	26	48
6	0	0	0	30	21	51	0	51
7	0	0	0	0	0	0	29	29
8	28	10	38	0	0	0	0	38
9	54	0	54	0	0	0	0	54
10	7	4	11	1	0	1	0	12
11	29	32	61	0	0	0	0	61
12	13	29	42	0	0	0	0	42
13	3	17	20	0	0	0	0	20
14	32	16	48	0	0	0	0	48
15	37	0	37	0	0	0	0	37
16	0	1	1	0	0	0	125	126
17	30	14	44	0	0	0	1	45
18	19	0	19	0	0	0	0	19
19	98	38	136	8	0	8	0	144
20	17	6	23	0	0	0	0	23
21	37	8	45	0	0	0	0	45
22	0	0	0	31	11	42	1	43
23	9	7	16	0	0	0	0	16
24	26	18	44	0	0	0	0	44
25	55	22	77	0	0	0	1	78
26	6	3	9	7	2	9	0	18
27	19	14	33	0	0	0	0	33
28	46	5	51	0	0	0	0	51
29	17	7	24	0	0	0	0	24
30	22	5	27	0	0	0	1	28
31	8	7	15	0	0	0	0	15
Total	628	286	914	128	143	271	315	1,500

Table 2. Number of samples and mean carcase weights of animals tested in each sampling window

Class	Freq.	Window 1		Window 2	
		Percent of samples tested	Mean carcase weight in kg	Percent of samples tested	Mean carcase weight in kg
Age					
Young and veal	743	49.3	175.2	49.4	174.6
Adult	757	52.1	318.5	48.7	308.5
Production					
Veal	315	46.7	104.8	53.3	92.7
Beef	914	51.8	272.4	48.3	267.8
Dairy	271	49.1	252.7	50.9	254.4
Age/Production					
Veal	315	46.7	104.8	53.3	92.7
Young beef	286	51.8	219.9	48.3	219.2
Young dairy	143	49.7	201.0	50.4	212.0
Adult beef	628	51.8	324.9	48.3	316.3
Adult dairy	128	48.4	312.0	51.6	300.6
Feed type					
Grass-fed	575	51.0	277.3	49.0	268.9
Grain-fed	302	55.3	316.8	44.7	327.1

Table 3. Percent of samples in each animal class that tested potentially positive for a pSTEC serotype.

			Percent of samples that tested potentially positive for a pSTEC serotype								
class	n	*stx*/*eae*	O26	O45	O103	O111	O121	O145	O157	Big 6[a]	Top 7[b]
Adult beef	628	52.7	18.2	28.8	33.9	4.6	26.0	5.1	14.6	45.7	45.9
95% CI		43.1-62.1	11.9-26.7	19.7-40.1	24.7-44.5	2.5-8.3	17.4-36.8	3.5-7.5	8.6-23.9	35.2-56.6	35.4-56.6
Adult dairy	128	43.8	17.2	19.5	29.7	3.1	28.1	6.3	1.6	40.6	40.6
95% CI		30.0-58.6	9.3-29.5	10.4-33.6	17.7-45.3	0.9-10.3	18.5-40.3	1.7-20.9	0.5-5.3	27.2-55.6	27.2-55.6
Veal	315	53.0	23.5	24.4	36.2	7.9	20.0	6.3	7.6	45.7	46.0
95% CI		46.7-59.3	20.3-27.1	21.3-27.9	29.6-43.3	6.1-10.3	15.4-25.5	4.6-8.7	5.8-9.9	40.3-51.2	41.0-51.1
Young beef	286	53.1	18.5	22.7	30.8	1.7	21.0	5.6	9.8	44.4	45.1
95% CI		43.7-62.4	11.0-29.5	12.5-37.8	20.9-42.8	0.5-6.5	13.8-30.5	3.2-9.6	5.3-17.5	32.8-56.7	33.5-57.3
Young dairy	143	41.3	19.6	19.6	34.3	11.2	20.3	6.3	7.7	39.9	39.9
95% CI		39.7-42.9	16.2-23.4	17.3-22.1	30.8-37.9	8.3-15.0	18.0-22.8	3.5-10.9	4.7-12.3	38.4-41.3	38.4-41.3
Total	1500	51.0	19.4	25.1	33.5	5.3	23.4	5.7	10.5	44.5	44.7
95% CI		45.0-56.9	15.2-24.4	19.3-31.9	28.0-39.5	3.6-7.7	18.9-28.7	4.4-7.3	7.2-15.1	38.3-50.8	38.6-51.1

[a]Refers to samples that tested positive for any one of pSTEC serotypes O26, O45, O103, O111, O121 or O145.

[b]Refers to samples that tested positive for any one of pSTEC serotypes O26, O45, O103, O111, O121, O145 or O157.

Table 4. Distribution of pSTEC serotypes isolated from the faeces of beef cattle classes

		Percent[a] confirmed pSTEC samples				
Class[b]	n[c]	O26	O111	O157	Big 6[d]	Top 7[e]
Age						
Young and veal	744	1.7	0.5	8.7	2.3	10.5^{A}
Adult	756	0.3	0	4.6	0.3	4.9^{B}
Production						
Veal	315	1.9	0.3	10.5	2.2	12.7^{A}
Beef	914	0.5	3.3	6.0	0.9	6.6^{B}
Dairy	271	1.5	0	4.4	1.5	5.5^{B}
Age/production						
Veal	315	1.9	0.3	10.5	2.2	12.7^{A}
Young beef	286	1.4	1.0	8.4	2.4	9.8^{AB}
Young dairy	143	2.1	0	5.6	2.1	7.0^{AB}
Adult beef	628	0.2	0	4.9	0.2	5.1^{B}
Adult dairy	128	0.8	0	3.1	0.8	3.9^{B}
Total	**1500**	**1.0**	**0.3**	**6.7**	**1.3**	**7.7**

[a]Percent of samples in each class that were confirmed for a pSTEC serotype.

[b]Classes were defined as beef, dairy and veal and were subdivided into young or adult based on the weight of each animal.

[c]The number of samples tested in each age or production class.

[d]Big 6 refers to O26, O45, O103, O111, O121 and O145. Only Big 6 pSTEC of serotypes O26 and O111 were isolated.

[e]Top 7 refers to the Big 6 serotypes (O26, O45, O103, O111, O121 and O145) and O157. For each class, values that do not share a common superscript capital letter are considered significantly different ($P < 0.05$).

Table 5. Frequency of confirmed pathogenic STEC identified in cattle faeces.

Confirmed pSTEC serotype (virulence profile)	Freq.
O157(stx1,stx2,eae)	59
O157(stx2,eae)	28
O26(stx1,eae)	8
O157(stx1,eae)	3
O157(stx1,stx2,eae); O157(stx2,eae)	3
O26(stx1,stx2,eae)	3
O111(stx1,eae)	2
O111(stx1,stx2,eae)	1
O157(stx1,eae); O157(stx1,stx2,eae)	1
O157(stx1,stx2,eae); O26(stx1,eae)	1
O157(stx2,eae); O111(stx1,eae)	1
O157(stx2,eae); O157(stx1,stx2,eae)	1
O26(stx1,eae); O157(stx1,stx2,eae)	1
O26(stx1,eae); O157(stx2,eae)	1
O26(stx1,stx2,eae); O26(stx1,eae)	1
O157(stx2,eae)	1
Total	115

Table 6. Percent of confirmed pSTEC by carcase weight

Animal class	Weight class in kg	Number of animals	Percent confirmed Top 7 [a] pSTEC
Veal	<50	43	9.3
	50-100	84	21.4
	100-150	187	9.6
Young (beef and dairy)	150-200	110	7.3
	200-250	319	9.4
Adult (beef and dairy)	250-300	348	4.9
	300-350	223	6.3
	350-400	128	3.9
	>400	58	2.6
Total	NA	1,500	7.7

[a]Top 7 pSTEC refers to serotypes O26, O45, O103, O111, O121, O145 and O157 that carry *stx* and *eae* virulence markers.

Table 7. Percent of pSTEC isolated from low, medium and high production plants

Production volume [a]	Mean carcase weight	No. of plants	No. of animals	Percent of confirmed pSTEC
Low	194.8 kg	11	564	9.4
Medium	251.5 kg	13	479	6.9
High	292.3 kg	7	457	6.4
Total	242.6 kg	31	1500	7.7

[a]Plants were assigned to low, medium and high production groups if total weekly slaughter volumes were ≤ 2500 cattle, > 2500 & < 5000 cattle or ≥ 5000 cattle, respectively.

Table 8. Virulence gene profiles for confirmed pathogenic STEC isolates.

Confirmed pSTEC serotypes	Freq	Percent of each virulence profile [a]		
		stx$_1$, *eae*	*stx*$_2$, *eae*	*stx*$_1$, *stx*$_2$, *eae*
O157	105	3.8	33.3	62.9
O26	16	75	0	25.0
O111	4	75	0	25.0
Total	125	15.1	28.0	56.3

[a]Pathogenic STEC were isolated from 115 samples, 10 of which contained multiple pSTEC of different serotypes or of the same serotype with different Shiga toxin profiles and were included in the above table.

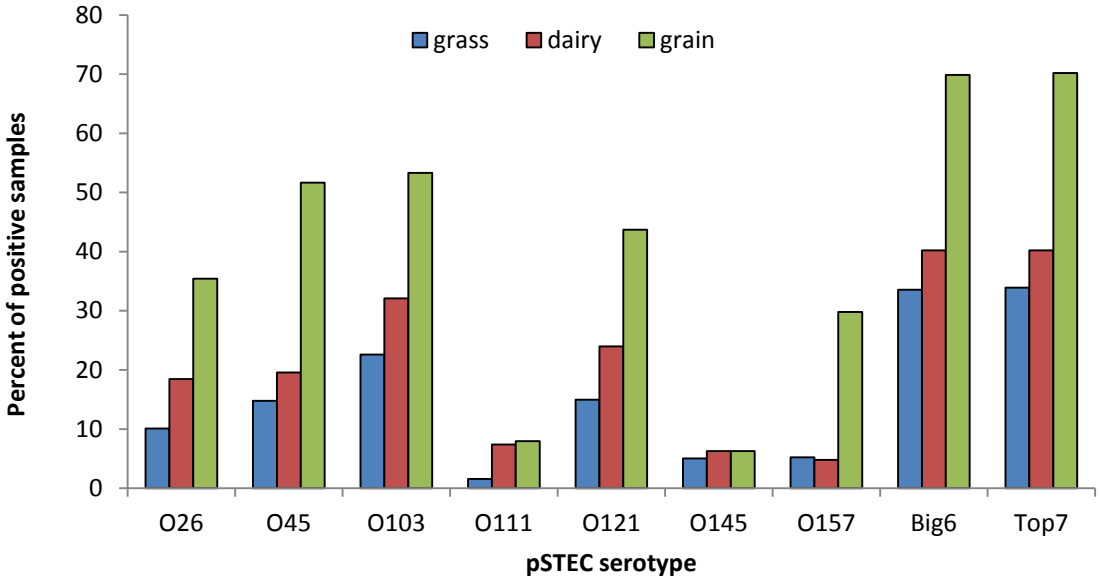

Fig 1. Percent of samples that tested potentially positive for pSTEC in each feed type. A total of 1148 samples were included in this analysis. Any animal with missing or incomplete feed data and all veal calves were removed from the sample set. From the remaining set, animals listed as dairy were combined into a single feed type while all other animals were divided into grass or grain fed groups based on feed data provided by abattoirs.

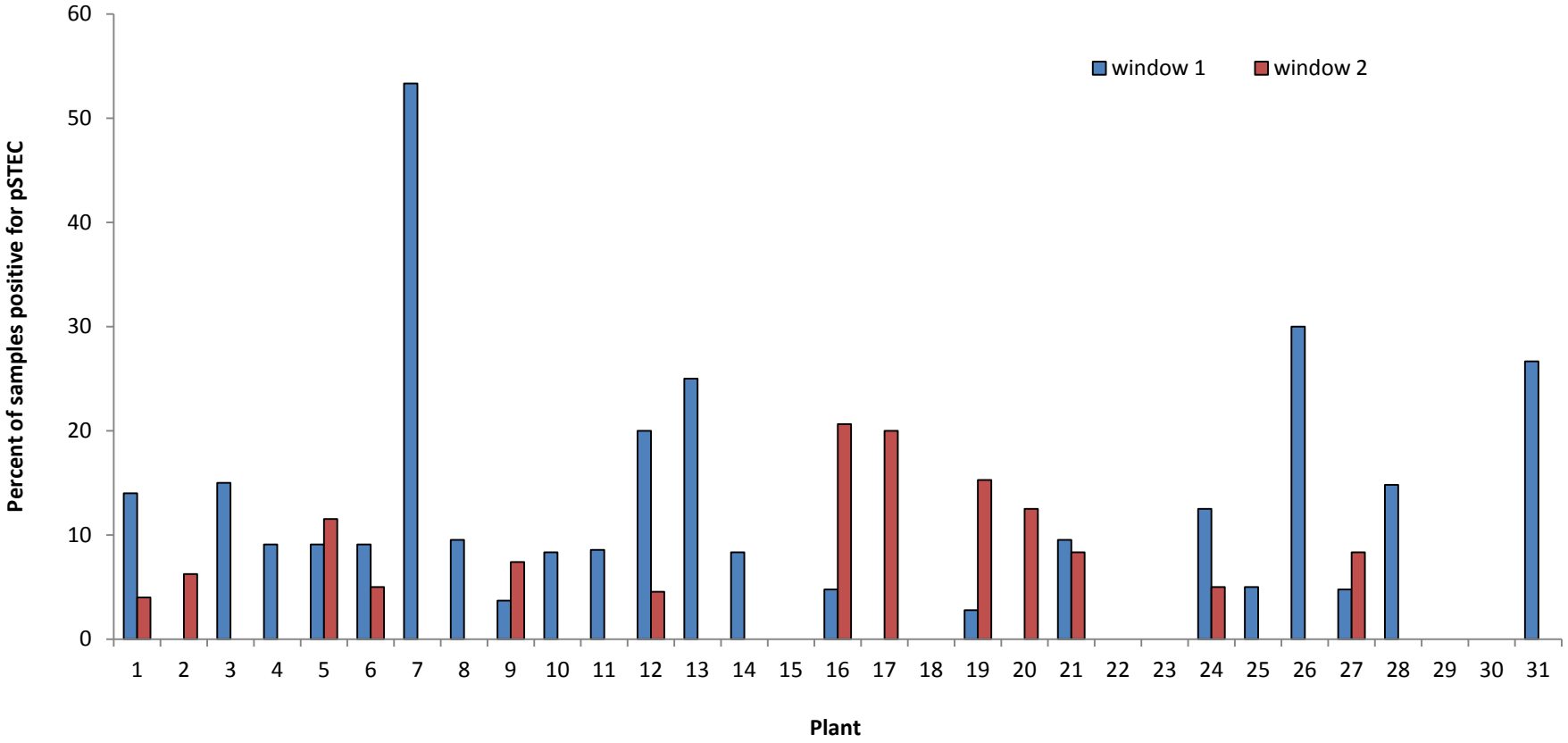

Fig 2. Distribution of Pathogenic STEC isolates across plants. Of the 31 plants that participated in this survey, 27 provided samples on both sampling occasions while four plants (10, 23, 29 and 31) provided samples on only one sampling occasions. In total, 29 establishments provided 753 samples in sampling window 1, while the same number of plants provided 747 samples in sampling window 2.

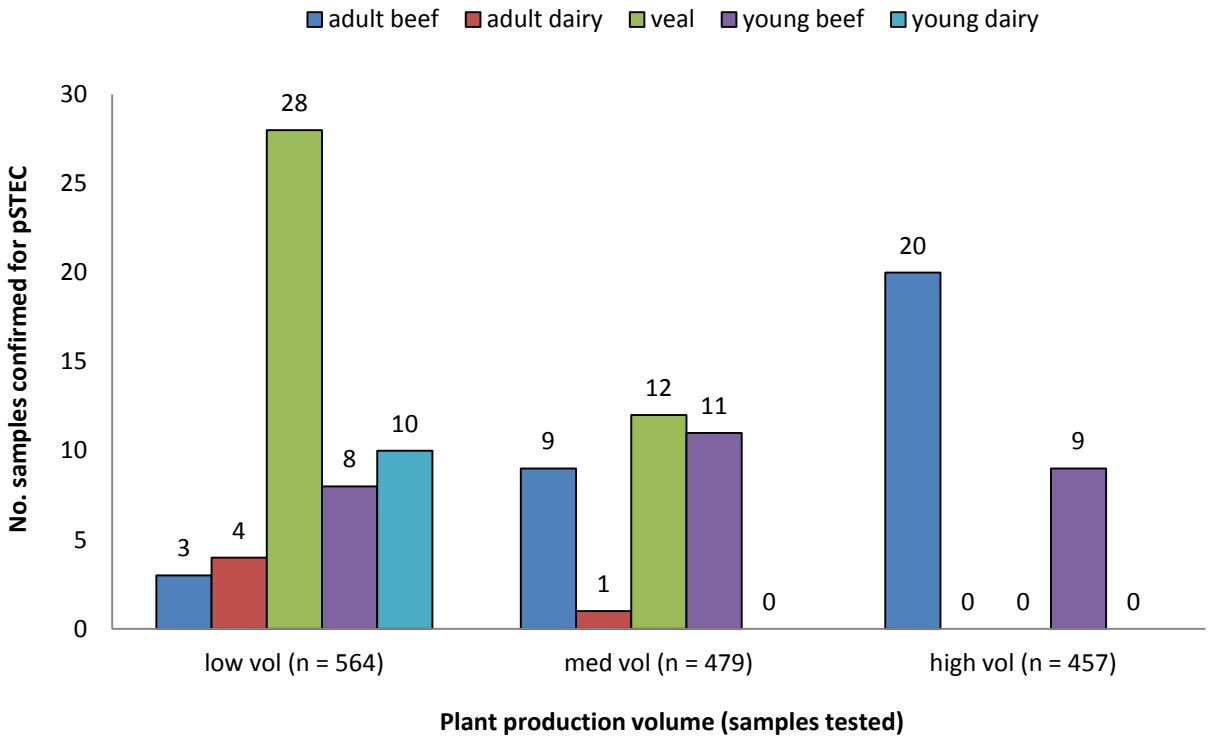

Fig 3. Frequency of pSTEC positive samples in each production volume group and animal class. Plants were separated into three groups based on weekly production estimates provided by each establishment. Each plant was assigned to low, medium or high production groups if total weekly volumes were ≤ 2500, > 2500 & < 5000 or ≥ 5000, respectively.

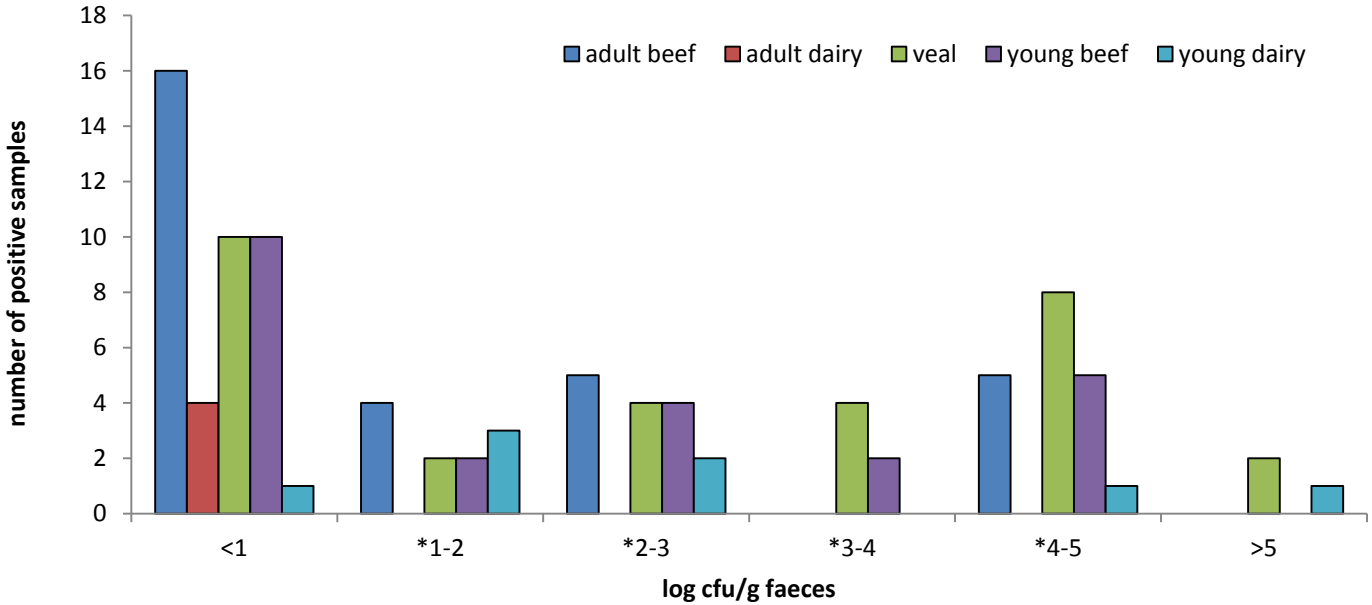

Fig 4. Concentration of *E. coli* O157 in cattle faeces at slaughter. Bacterial counts were determined using the most probable number method followed by PCR confirmation of *E. coli* O157. Ninety-five of the 100 *E. coli* O157 isolates recovered were enumerated. Samples are separated by animal class and counts are displayed as a log of colony forming units per gram of faeces.

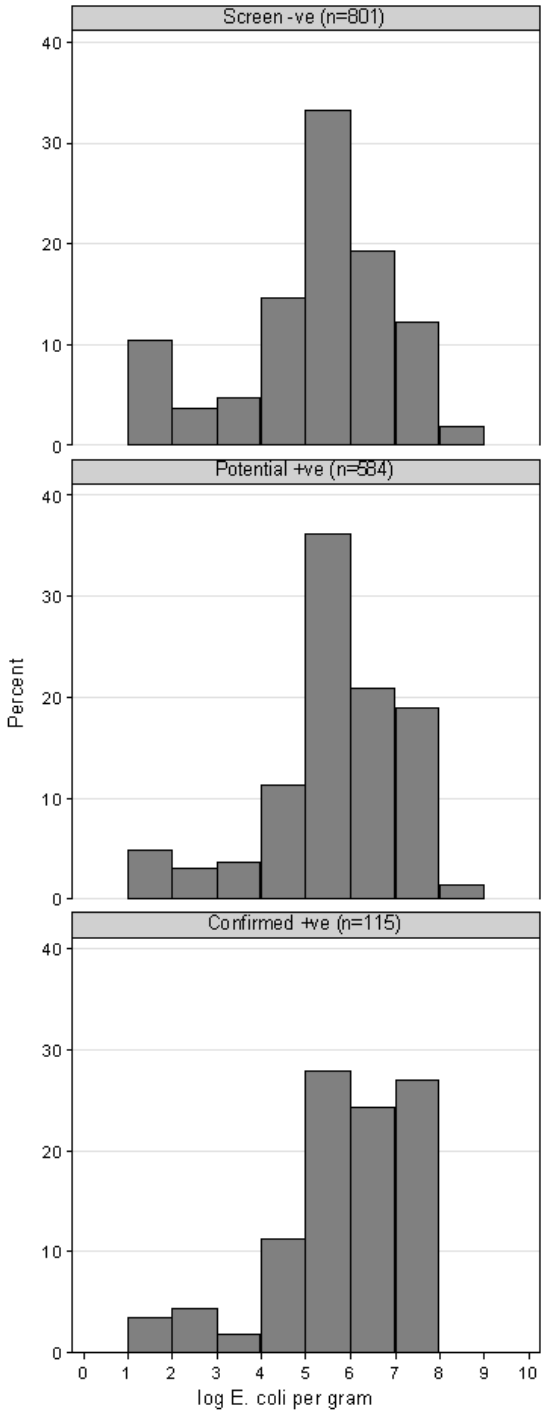

Fig. 5 Panel plot displaying the generic *E. coli* count (log/g faeces) of samples that were screen negative, potentially positive or confirmed for at least one of the pSTEC serotypes tested. Counts were binned on integer values of log count and presented as a percent of the total number of samples within each group.

Table SI Percent of screen positive or confirmed positive samples within each plant during a single sampling window

		Percent of BAX screen positive samples												Percent of confirmed pSTEC samples								
Plant No.	Sampling period	*stx*	*eae*	Serotype	O26	O45	O103	O111	O121	O145	O157	Big 6	Top 7	O26	O45	O103	O111	O121	O145	O157	Big 6	Top 7
1	1	62.0	62.0	80.0	54.0	64.0	58.0	18.0	44.0	10.0	22.0	56.0	58.0	2.0	0.0	0.0	0.0	0.0	0.0	12.0	2.0	14.0
1	2	26.0	26.0	60.0	10.0	18.0	44.0	0.0	6.0	4.0	2.0	20.0	20.0	0.0	0.0	0.0	0.0	0.0	0.0	4.0	0.0	4.0
2	1	58.3	58.3	58.3	16.7	33.3	50.0	8.3	16.7	0.0	0.0	41.7	41.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	2	37.5	37.5	56.3	6.3	12.5	18.8	0.0	18.8	0.0	0.0	18.8	18.8	0.0	0.0	0.0	0.0	0.0	0.0	6.3	0.0	6.3
3	1	51.3	51.3	80.0	43.8	30.0	66.3	23.8	40.0	6.3	13.8	51.3	51.3	5.0	0.0	0.0	0.0	0.0	0.0	11.3	5.0	15.0
3	2	26.3	26.3	65.0	17.5	21.3	37.5	2.5	27.5	5.0	5.0	23.8	23.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
4	1	72.7	72.7	81.8	45.5	36.4	36.4	0.0	18.2	9.1	18.2	63.6	63.6	0.0	0.0	0.0	0.0	0.0	0.0	9.1	0.0	9.1
4	2	44.4	44.4	55.6	11.1	22.2	22.2	0.0	22.2	0.0	0.0	22.2	22.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	1	68.2	68.2	77.3	18.2	18.2	68.2	0.0	31.8	9.1	0.0	59.1	59.1	4.5	0.0	0.0	0.0	0.0	0.0	4.5	4.5	9.1
5	2	73.1	76.9	84.6	38.5	38.5	73.1	7.7	34.6	19.2	11.5	57.7	57.7	0.0	0.0	0.0	0.0	0.0	0.0	11.5	0.0	11.5
6	1	45.5	45.5	54.5	18.2	18.2	45.5	18.2	18.2	9.1	0.0	45.5	45.5	0.0	0.0	0.0	0.0	0.0	0.0	9.1	0.0	9.1
6	2	52.5	52.5	77.5	42.5	40.0	60.0	12.5	50.0	20.0	5.0	52.5	52.5	0.0	0.0	0.0	0.0	0.0	0.0	5.0	0.0	5.0
7	1	66.7	66.7	86.7	40.0	46.7	73.3	13.3	60.0	6.7	13.3	66.7	66.7	0.0	0.0	0.0	0.0	0.0	0.0	53.3	0.0	53.3
7	2	35.7	35.7	64.3	21.4	35.7	35.7	0.0	42.9	7.1	0.0	35.7	35.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8	1	38.1	38.1	47.6	4.8	19.0	38.1	4.8	23.8	4.8	14.3	23.8	23.8	0.0	0.0	0.0	0.0	0.0	0.0	9.5	0.0	9.5
8	2	29.4	29.4	29.4	0.0	5.9	23.5	0.0	0.0	5.9	0.0	5.9	5.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9	1	85.2	85.2	96.3	29.6	85.2	59.3	0.0	55.6	0.0	44.4	85.2	85.2	0.0	0.0	0.0	0.0	0.0	0.0	3.7	0.0	3.7
9	2	51.9	51.9	81.5	14.8	55.6	37.0	0.0	51.9	3.7	33.3	44.4	44.4	0.0	0.0	0.0	0.0	0.0	0.0	7.4	0.0	7.4
10	1	41.7	41.7	83.3	25.0	41.7	66.7	8.3	25.0	16.7	16.7	41.7	41.7	0.0	0.0	0.0	0.0	0.0	0.0	8.3	0.0	8.3
11	1	82.9	82.9	94.3	60.0	88.6	71.4	5.7	40.0	8.6	25.7	80.0	80.0	2.9	0.0	0.0	0.0	0.0	0.0	5.7	2.9	8.6
11	2	73.1	73.1	80.8	19.2	42.3	65.4	0.0	26.9	11.5	0.0	65.4	65.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
12	1	50.0	50.0	40.0	5.0	15.0	25.0	0.0	15.0	0.0	0.0	15.0	15.0	0.0	0.0	0.0	0.0	0.0	0.0	20.0	0.0	20.0
12	2	27.3	27.3	45.5	13.6	18.2	18.2	0.0	4.5	22.7	0.0	27.3	27.3	0.0	0.0	0.0	0.0	0.0	0.0	4.5	0.0	4.5
13	1	83.3	83.3	83.3	58.3	58.3	66.7	8.3	66.7	0.0	50.0	83.3	83.3	0.0	0.0	0.0	0.0	0.0	0.0	25.0	0.0	25.0
13	2	75.0	75.0	100.0	37.5	62.5	75.0	0.0	87.5	12.5	25.0	75.0	75.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14	1	25.0	25.0	79.2	41.7	33.3	50.0	0.0	50.0	20.8	0.0	25.0	25.0	0.0	0.0	0.0	0.0	0.0	0.0	8.3	0.0	8.3
14	2	41.7	41.7	37.5	4.2	12.5	33.3	0.0	12.5	4.2	0.0	20.8	20.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15	1	94.1	94.1	94.1	76.5	94.1	94.1	17.6	82.4	11.8	64.7	88.2	88.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

15	2	95.0	95.0	100.0	45.0	55.0	95.0	25.0	70.0	10.0	45.0	95.0	95.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16	1	55.6	55.6	73.0	11.1	46.0	60.3	1.6	19.0	4.8	1.6	46.0	46.0	1.6	0.0	0.0	0.0	0.0	0.0	3.2	1.6	4.8
16	2	57.1	57.1	77.8	38.1	27.0	57.1	17.5	25.4	12.7	12.7	52.4	52.4	4.8	0.0	0.0	1.6	0.0	0.0	14.3	6.3	20.6
17	1	36.0	36.0	36.0	4.0	24.0	20.0	0.0	4.0	4.0	0.0	12.0	12.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
17	2	75.0	75.0	70.0	35.0	25.0	45.0	0.0	30.0	25.0	5.0	65.0	65.0	0.0	0.0	0.0	10.0	0.0	0.0	15.0	10.0	20.0
18	1	100.0	100.0	90.9	45.5	9.1	45.5	9.1	72.7	9.1	18.2	90.9	90.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
18	2	87.5	87.5	100.0	37.5	62.5	37.5	12.5	100.0	0.0	75.0	87.5	87.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
19	1	34.7	34.7	59.7	15.3	37.5	37.5	2.8	25.0	0.0	11.1	30.6	30.6	0.0	0.0	0.0	0.0	0.0	0.0	2.8	0.0	2.8
19	2	77.8	66.7	77.8	15.3	15.3	54.2	4.2	27.8	22.2	6.9	44.4	45.8	0.0	0.0	0.0	0.0	0.0	0.0	15.3	0.0	15.3
20	1	86.7	86.7	100.0	66.7	86.7	60.0	20.0	66.7	0.0	26.7	86.7	86.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20	2	87.5	87.5	100.0	25.0	62.5	87.5	12.5	62.5	0.0	50.0	87.5	87.5	0.0	0.0	0.0	0.0	0.0	0.0	12.5	0.0	12.5
21	1	52.4	52.4	90.5	52.4	66.7	52.4	4.8	28.6	14.3	19.0	52.4	52.4	4.8	0.0	0.0	0.0	0.0	0.0	9.5	4.8	9.5
21	2	75.0	75.0	95.8	20.8	41.7	54.2	8.3	45.8	12.5	41.7	70.8	75.0	0.0	0.0	0.0	0.0	0.0	0.0	8.3	0.0	8.3
22	1	34.8	34.8	78.3	0.0	39.1	60.9	0.0	56.5	0.0	0.0	30.4	30.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22	2	60.0	65.0	70.0	30.0	35.0	45.0	0.0	40.0	0.0	0.0	45.0	45.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
23	2	12.5	12.5	37.5	18.8	0.0	25.0	0.0	0.0	0.0	0.0	12.5	12.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24	1	66.7	66.7	95.8	50.0	62.5	58.3	12.5	41.7	4.2	25.0	66.7	66.7	0.0	0.0	0.0	0.0	0.0	0.0	12.5	0.0	12.5
24	2	70.0	70.0	95.0	35.0	50.0	75.0	0.0	60.0	5.0	20.0	70.0	70.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	0.0	5.0
25	1	45.0	45.0	55.0	2.5	2.5	37.5	0.0	12.5	5.0	2.5	37.5	37.5	0.0	0.0	0.0	0.0	0.0	0.0	5.0	0.0	5.0
25	2	26.3	26.3	39.5	5.3	10.5	18.4	0.0	15.8	2.6	0.0	13.2	13.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
26	1	70.0	70.0	80.0	40.0	30.0	60.0	0.0	50.0	10.0	10.0	50.0	50.0	10.0	0.0	0.0	0.0	0.0	0.0	20.0	10.0	30.0
26	2	25.0	25.0	37.5	0.0	0.0	37.5	0.0	12.5	0.0	0.0	25.0	25.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
27	1	52.4	52.4	66.7	19.0	28.6	28.6	0.0	33.3	19.0	9.5	38.1	42.9	0.0	0.0	0.0	0.0	0.0	0.0	4.8	0.0	4.8
27	2	50.0	50.0	50.0	8.3	33.3	33.3	0.0	8.3	0.0	25.0	33.3	33.3	8.3	0.0	0.0	0.0	0.0	0.0	0.0	8.3	8.3
28	1	48.1	48.1	81.5	37.0	40.7	59.3	7.4	40.7	7.4	18.5	48.1	48.1	0.0	0.0	0.0	0.0	0.0	0.0	14.8	0.0	14.8
28	2	16.7	16.7	83.3	25.0	45.8	54.2	0.0	12.5	4.2	20.8	16.7	16.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
29	2	16.7	16.7	50.0	0.0	16.7	29.2	0.0	16.7	0.0	0.0	12.5	12.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30	1	41.2	41.2	82.4	5.9	41.2	41.2	5.9	52.9	0.0	0.0	41.2	41.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30	2	27.3	27.3	63.6	0.0	36.4	27.3	0.0	27.3	0.0	0.0	9.1	9.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
31	1	73.3	73.3	93.3	20.0	66.7	46.7	20.0	20.0	6.7	13.3	66.7	66.7	6.7	0.0	0.0	6.7	0.0	0.0	20.0	13.3	26.7
N/A	1	55.6	55.6	75.0	29.9	43.3	53.3	7.7	35.6	6.2	13.9	49.5	49.8	1.5	0.0	0.0	0.1	0.0	0.0	8.2	1.6	9.4
N/A	2	48.7	47.9	68.7	20.5	27.8	46.2	4.3	28.8	9.2	10.2	39.4	39.6	0.5	0.0	0.0	0.4	0.0	0.0	5.1	0.9	5.9
N/A	Combined	52.2	51.8	71.9	25.2	35.6	49.7	6.0	32.2	7.7	12.1	44.5	44.7	1.0	0.0	0.0	0.3	0.0	0.0	6.7	1.3	7.7

Antimicrobial resistance status of *Salmonella*, *Escherichia coli* and *Enterococcus* from Australian cattle populations at slaughter

Running title: AMR status of Australian cattle populations.

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Keywords: antimicrobial resistance, *Salmonella*, *E. coli*, *Enterococcus*

Abstract

Antimicrobial agents are used in food production systems for the prevention and control of bacterial associated diseases and for growth promotion purposes. An inevitable consequence of their use is the development of antimicrobial resistance (AMR). Zoonotic bacteria that are resistant to antimicrobials are of increased concern to public health officials throughout the world as they may compromise the ability of various treatment regimes to control disease and infection in human medical settings. Australia is the world's third largest exporter of beef; however it does not have an ongoing surveillance system for AMR in cattle or foods derived from these animals. This study examined 910 beef cattle, 290 dairy cattle and 300 veal calf faecal samples collected at slaughter for the presence of *E. coli*, enterococci and *Salmonella* and determined the phenotypic AMR of 800 *E. coli*, 800 enterococci and 218 *Salmonella*. *E. coli* (92.3%) and enterococci (86.4%) were readily isolated from all samples whereas *Salmonella* was recovered from 14.4% of samples and was significantly more likely to be isolated from dairy cattle samples. The results of AMR testing corroborate previous Australian based animal and retail food surveys that have shown a low level of AMR. Nevertheless, the detection of a cluster of ceftiofur resistant *Salmonella* in dairy cattle (17.1%) is of concern. Similarly, resistance in enterococci recovered from all animal groups to quinupristin / dalfopristin (44.6%), daptomycin (5.3%) and tigecycline (2.8%) is requiring of additional investigation. Ceftiofur resistance aside, there is minimal evidence that specific production practices are responsible for disproportionate contributions to AMR development and in general resistance to antimicrobials of significance in human medicine was low regardless of the isolate source. Whilst comparisons with internationally available AMR data suggest AMR in bacteria from Australian cattle to clinically relevant antimicrobials is low, it is necessary to maintain strict guidelines and controls around the use of antimicrobials in food-production animals in Australia and to continually monitor the effects of all antimicrobial use if Australia's reputation as a supplier of safe and healthy food is to be maintained.

Introduction

Antimicrobial agents are used in food production systems for the prevention and control of bacterial associated diseases and for growth promotion purposes. An inevitable consequence of the use of antimicrobials is the potential for antimicrobial resistance (AMR) to develop in bacteria, including zoonotic pathogens which can be transferred to the human population via the food chain or by direct exposure to animals (Collignon and Angulo 2006; Heuer, Hammerum et al. 2006). Novel resistance phenotypes continue to emerge in zoonotic foodborne pathogens and commensal bacteria isolated from food production animals (Walsh and Fanning 2008; Szmolka and Nagy 2013). Consequently, understanding, assessing and mitigating the risks of non-human use of antimicrobials on human health outcomes remains a high priority. The World Health Organisation (WHO) has developed and maintains criteria and ranks antimicrobials based on their importance to human medicine (World Health Organisation 2011). These lists will help regulators and stakeholders identify appropriate antimicrobials for use in food animal production systems (Collignon, Powers et al. 2009).

Australia is one of the world's most efficient producers of cattle and third largest exporter of beef, exporting 67% of its total beef and veal production in 2012-13 (Meat & Livestock Australia 2013). Australia has taken a conservative approach to the registration of antimicrobials for use in food-producing animals. Antimicrobials that are of human clinical importance such as fluoroquinolones and gentamicin have never been registered for use in food-producing animals and only one 3rd or 4th generation cephalosporin (ceftiofur) has been registered (Barton, Pratt et al. 2003). There is currently no ongoing surveillance for AMR in Australia although there have been recent attempts to assess the AMR status of bacteria of food animal origin (Fegan, Vanderlinde et al. 2004; DAFF 2007; Barlow and Gobius 2008). Isolates of *Salmonella*, *E. coli*, and *Enterococcus* from cattle at slaughter and/or in retail beef products demonstrated that phenotypic resistance to all antimicrobials tested was generally low. More specifically the resistances observed were to antimicrobials of lesser importance to human medicine (DAFF 2007; Barlow and Gobius 2008). Similarly, genotypic investigations of AMR determined that resistance to fluoroquinolones or third-generation cephalosproins was absent in *Salmonella* from Australian cattle populations (Abraham, Groves et al. 2014). Furthermore, the presence of class 1 and class 2 integrons was not correlated with specific production practices such as feed-lotting and the gene cassettes harboured by the integrons mostly encoded resistance to antimicrobials of limited human clinical significance (Barlow, Pemberton et al. 2004; Barlow, Fegan et al. 2008).

In comparison to Australia, a number of countries do have established AMR surveillance programs in place. Whilst the main focus of these programs revolves around AMR in bacteria from humans there is considerable and increasing demand to enhance their impact by assessing AMR in bacteria from animals during production and from foods at the retail level. Multi-focus surveillance programs enable trends in AMR development to be further evaluated with respect to production practices, animal type and clinical use and are particularly useful in addressing concerns from regulators about the overall impact of antimicrobial use. Indeed, countries such as the United State through their NARMS program could evaluate the impact that a petition aimed at declaring specific strains of AMR *Salmonella* as adulterants in beef and poultry products might have (Center for Science in the Public Interest 2011). Countries that lack sophisticated multi-focus surveillance programs instead rely on relatively short-term intensive surveys to evaluate the prevalence and AMR status of bacteria from an animal type, production practice or as a result of clinical use. The aim of this study was to determine the prevalence and phenotypic AMR status of *Salmonella*, *E. coli* and *Enterococcus* isolates from Australian cattle populations.

Materials and methods

Sample collection

Australian beef cattle destined for export can be classified into three animal groups: beef cattle, dairy cattle, and veal calves. A total of 31 abattoirs representing >85% of total beef exports agreed to participate in the survey. The number of cattle to be sampled at each abattoir was stratified based on animal group and slaughter volumes. Sample collection targets of 900, 300 and 300 were established for beef cattle, dairy cattle and veal calves, respectively. Samples were collected across two sampling windows with sample numbers collected from each participating abattoir ranging from 8-80 (mean 24) per sampling window. Systematic random sampling was used to collect the samples across a consecutive two day period in each of the sampling windows. A sampling day consisted of eight hours of production with each abattoir expected to sample evenly across the day. Abattoirs were expected to collect up to a maximum of 40 samples per sampling day therefore all samples were expected to be collected a minimum of 12 minutes apart. Each sampling window occurred over an eight week period with the first window occurring in February and March, 2013 and the second sampling window occurring in August and September, 2013. Faecal samples were collected post-evisceration by cutting the intestine 15-30 cm from the rectal end and squeezing at least 40 g of material into a sterile jar. Samples were kept chilled and returned to the laboratory by overnight courier for processing.

Isolation of target organisms

Salmonella

Faecal slurries prepared by diluting 10 g of faeces 1 in 10 with buffered peptone water (BPW; Oxoid, UK) and homogenising for 1 min were then incubated at 42±1°C for 6 h and subsequently tested for the presence of *Salmonella* using automated immunomagnetic separation (AIMS) with Dynabeads anti-*Salmonella* (Invitrogen, Norway) following the manufacturer's instructions. Following AIMS, Dynabeads were inoculated into 10 ml of Rappaport-Vassiliadis soy broths (RVS; BioMerieux, France) and incubated for 20 h at 42±1°C. A loopful of RVS broth was plated onto brilliant green agar (BGA; Oxoid) and xylose lysine desoxycholate (XLD; BioMerieux) agar and incubated at 37±1°C for 24 h. Following incubation, plates were examined for the presence of *Salmonella* using the *Salmonella* latex agglutination test kit (Oxoid). Colonies that agglutinated with the latex agglutination test kit were plated onto 5% sheep blood agar (SBA; BioMerieux) and confirmed as *Salmonella* by *invA* PCR (Chiu and Ou 1996) and biochemical tests (Microbact 24E; Oxoid). Up to two confirmed *Salmonella* isolates were stored at -80°C using Microbank (Pro-Lab Diagnostics, USA). A multiplex PCR-based method capable of identifying and discriminating common clinical serovars of *Salmonella* was used to determine the identity of *Salmonella* serovars (Kim, Frye et al. 2006). Conventional serotyping of isolates not identified using the molecular serotyping approach was conducted by Queensland Health (Brisbane, Australia).

E. coli

E. coli were isolated by plating 1 ml of serial dilutions of the unenriched faecal slurries onto Petrifilm *E. coli*/coliform count plates (3M; St. Paul, Minnesota, USA). Presumptive *E. coli* were recovered by plating representative colonies onto eosin methylene blue agar (EMB; Oxoid) and incubating at 37 ± 2°C for 18 h. Colonies displaying the typical metallic green sheen were subsequently plated onto 5% sheep blood agar (SBA; BioMerieux, France) and incubated at 37 ± 2°C for 18 h. The resulting isolates were confirmed as *E. coli* using the Microbact 12E or 24E system (Oxoid) and stored at -80°C using MicroBank (Pro-Lab Diagnostics, USA).

Enterococcus

The presence of *Enterococcus* was determined by enriching 1 g of faeces in 10 ml of BBL Enterococcosel Broth (BD, Maryland, USA) for 18-24 h at 35 ± 2°C. Enriched broths were then plated onto BBL Enterococcosel Agar (BD) and incubated for 18-24 h at 35 ± 2°C. Translucent colonies with

brownish-black to black zones were then streaked onto SBA and incubated for 18-24 h at 35 ± 2°C. Isolates were confirmed as *Enterococcus* spp. by PCR (Ke, Picard et al. 1999). A species specific PCR was then used to identify *E. faecalis* and *E. faecium* strains (Dutkamalen, Evers et al. 1995; Dutkamalen, Evers et al. 1995). Further speciation was not performed and the remaining isolates were labelled *Enterococcus spp*.

Phenotypic detection of antimicrobial resistance

The antimicrobial resistance phenotype of isolates was determined using the broth microdilution method and the Sensititre apparatus. Custom susceptibility panels for *E. coli* & *Salmonella* (AUSVN2) and *Enterococcus* (AUSVP2; TREK Diagnostic Systems, UK) were used to test all isolates. The dilution ranges and breakpoints for each antimicrobial are shown in Table1. Interpretation of the MIC values was based on CLSI interpretive criteria when available; otherwise EUCAST and NARMS values were used. The breakpoint listed for florfenicol is the susceptible breakpoint. Isolates that exceeded the MIC value of the susceptible breakpoint were reported as non-susceptible. *Enterococcus faecalis* ATCC 29212, *Salmonella typhimurium* ATCC 14028 and *Escherichia coli* ATCC 25922 were used as the control strains.

Statistical analysis

Univariate statistical analysis, comparison of simple proportions and data management was performed using Stata v12.1 (StataCorp, USA). Confidence intervals were calculated using the 'exact' confidence interval method of Clopper and Pearson (Clopper and Pearson 1934).

Results

Prevalence and identity

In total, 1500 faecal samples comprising 910 beef cattle faeces, 290 dairy cattle faeces and 300 veal calf faeces were tested for the presence of *Salmonella*, *E. coli* and *Enterococcus*.

Salmonella

Salmonella was isolated from 105 (11.5%) beef cattle, 75 (25.9%) dairy cattle and 36 (12.0%) veal calf faecal samples for an overall prevalence in Australian cattle of 14.4%. Univariate analysis determined that *Salmonella* was significantly ($p < 0.05$) more likely to be isolated from dairy cattle than from

beef cattle or veal calves. Of the 31 abattoirs participating in the survey, 29 provided samples in sampling window 1 and 30 provided samples in sampling window 2. S*almonella* was isolated from 25 of 29 (86.2%) abattoirs in sampling window 1 and 19 of 30 (63.3%) in sampling window 2. No significant differences were observed for *Salmonella* prevalence in beef or dairy cattle across the sampling windows (Table 2). However, veal calves were significantly ($p < 0.05$) more likely to be positive for *Salmonella* in sampling window 2 than sampling window 1. The overall prevalence of *Salmonella* in grain-fed beef cattle samples (9.6%) was lower than grass-fed beef cattle samples (13.0%), however this difference was not considered significant. Interestingly, grain-fed beef cattle samples from sampling window 1 were three times more likely to yield *Salmonella* than grain-fed beef cattle samples from sampling window 2 and this difference was shown to be significant ($p < 0.05$). A similar relationship was observed between grain-fed beef cattle samples and grass-fed beef cattle samples in sampling window 2 (Table 3).

Attempts were made to determine the serovar of all *Salmonella* isolates using a multiplex PCR approach. Where possible, two isolates (one from BGA and one from XLD) were subjected to analysis. Serotyping using the multiplex PCR approach determined the identity of *Salmonella* in 161 of 216 (74.5%) samples. With the exception of one beef cattle sample harbouring a Saintpaul and Chester serovar and one dairy cattle sample harbouring an Anatum and Newport serovar, all samples contained a single *Salmonella* serovar. A total of 19 different serovars were identified across the three animal groups. The distribution of *Salmonella* serovars for each animal group is shown in Figure 1. The most frequently detected serovar for each animal group was Typhimurium comprising between 28% and 45% of all isolates. The next most prevalent serovars were Anatum (11%) in beef cattle, Bovismorbificans (9%) in dairy cattle and Saintpaul (11%) in veal calves. The serovar Newport was found in 3.8% of beef cattle isolates and 3.9% of dairy cattle isolates whilst Heidelberg was found in just one (2.8%) of veal calf isolates. *S.* Hadar was not recovered from any animal group. Unknown serovars represent strains that are seldom implicated in human clinical disease.

E. coli

Attempts were made to isolate *E. coli* from samples that had concentrations of *E. coli* >1.00 $\log_{10}$ CFU/g with *E. coli* recovered from 1385 (92.3%) of all samples. Veal samples were most likely to yield *E. coli* with isolates recovered from 294 (98.0%) of 300 samples. *E. coli* was recovered from 93.0% of dairy cattle samples and 90.2% of beef cattle samples.

Enterococcus

Enterococcus were isolated from 805 (88.5%) beef cattle faeces, 244 (84.1%) dairy cattle faeces and 247 (82.3%) veal calf faeces. Species specific PCR determined that 6.4% of all isolates were *E. faecalis* and 8.0% were *E. faecium*. Veal samples (14.3%) were significantly ($p < 0.05$) more likely to contain *E. faecalis* than dairy (3.1%) or beef (4.8%) samples. No significant differences in prevalence were observed between the three animal groups for *E. faecium*.

Antimicrobial susceptibility testing

Salmonella

All 218 distinct *Salmonella* isolates were submitted for AMR analysis. The distribution of minimum inhibitory concentrations (MICs) for each antimicrobial and animal group is shown in Table 4. When all isolates are considered the rates of resistance were low with resistance to any one antimicrobial not exceeding 7.4%. The majority (87.2%) of isolates, including all veal isolates, remained susceptible to all antimicrobials except florfenicol. Non-susceptibility to florfenicol was observed in 28.3%, 23.7% and 38.9% of isolates from beef cattle, dairy cattle and veal calves, respectively. Differences in the rates of resistance to specific antimicrobials were observed between animal groups (Figure 2). Resistance to streptomycin (8.5%), ampicillin (7.5%), trimethoprim / sulfamethoxazole (7.5%) and tetracycline (6.6%) were the most common resistances identified in beef cattle isolates. Resistance to cephalosporins and fluorquinolones was low amongst beef cattle isolates with resistance to ceftiofur, cefotaxime, cefazolin and nalidixic acid found in no more than two isolates. In contrast, resistance to cephalosporins and fluorquinolones was more often observed in dairy cattle isolates. Resistance was most frequently detected to cefazolin (18.4%), ceftiofur (17.1%), cefotaxime (15.8%), nalidixic acid (15.8%) and Ceftriaxone (14.5%). A full summary of the *Salmonella* AMR testing, including a further breakdown of beef cattle isolates into grass- or grain-fed categories is presented in Table 5.

Multiple resistance (MDR) to 3 or more antimicrobials was observed in a total of 21 (9.6%) of 218 isolates. MDR was observed in 13 (17.1%) of 76 dairy cattle isolates and eight (7.5%) of beef cattle isolates. MDR antibiograms specific to animal groups could be identified and are outlined in Table 6. The antibiogram AMP-STR-TET-SXT was only found in *Salmonella* isolated from beef cattle which were grain-fed, three of which were the serovar Typhimurium and one was Newport. In dairy cattle

isolates, a base antibiogram of FAZ-FOT-XNL-NAL was present in 11 (73%) of the 15 *Salmonella* isolates that were resistant to at least one antimicrobial. Of these 11 isolates, eight were Typhimurium and one was Newport. Importantly, all 15 resistant dairy cattle *Salmonella* isolates were recovered from samples collected at a single abattoir across a two hour period. The sampling frequency employed at this abattoir was 50% greater than requested thereby resulting in the oversampling of related animals.

E. coli

A total of 800 *E. coli* isolates were randomly selected from a pool of 1385 isolates and submitted for AMR analysis. The group comprised *E. coli* from 469 beef cattle, 155 dairy cattle and 176 veal calves. The distribution of MICs for each antimicrobial and animal group is shown in Table 7. AMR was generally low across the three animal groups with 92.1%, 96.8% and 93.2% of *E. coli* from beef cattle, dairy cattle and veal calves susceptible to all antimicrobials tested. Non-susceptibility to florfenicol was observed in 55.4%, 58.7% and 59.7% of beef cattle, dairy cattle and veal calf isolates, respectively. With the exception of tetracycline in beef cattle *E. coli*, resistance to any one antimicrobial did not exceed 5.0% (Figure 3). Tetracycline resistance was present in 48 (6.0%) of all *E. coli* tested but was significantly ($p < 0.05$) more likely to be present in *E. coli* from grain-fed cattle than any other animal group (Table 8). Resistance of *E. coli* to fluoroquinolones was not observed in any animal group and resistance to 3rd and 4th generation cephalosporins was not present in isolates from grass- or grain-fed beef cattle and dairy cattle. Resistance to amoxicillin / clavulanic acid (1.1%), kanamycin (1.1%), gentamicin (0.6%) and ceftiofur (0.6%) although infrequent, were only observed in *E. coli* from veal calves. Resistance to three or more antimicrobials was not observed in any beef cattle *E. coli* but was present in two (1.3%) and seven (4.0%) dairy cattle and veal calf *E. coli*, respectively. TET alone was the most common antibiogram identified with STR-TET the only other antibiogram present in more than two isolates (Table 9).

Enterococcus

A total of 800 *Enterococcus* isolates comprising 96 *E. faecalis*, 120 *E. faecium*, and 584 *Enterococcus spp.* were submitted for AMR analysis. The distribution of MICs for each antimicrobial and species group is shown in Table 10. Streptogramin MIC values for *E. faecalis* are not presented as this species is intrinsically resistant. The resistances observed for each species and animal group are shown in Figure 4. Irrespective of animal group and species, resistance to flavomycin (77.3 – 92.5%) and lincomycin (77.8 – 100.0%) was common. Resistance to quinupristin / dalfoprisitin was observed

in 38 – 48% of all non-*faecalis* isolates but was not correlated with similar resistances to virginiamycin. There was a strong association between daptomcyin resistant *E. faecalis* and veal calves, however this was not considered to be statistically significant ($p < 0.05$). Resistance to tetracycline (7.3 – 11.7%) and erythromycin (6.0 – 10.4%) were observed in all three species groups but was not identified in *E. faecium* from veal calves. Analysis of isolates from grass- and grain-fed cattle (Table 11) showed that daptomycin resistance in *E. faecalis* and erythromycin resistance in *E. faecium* were more likely in grass-fed animals than grain-fed. Furthermore, tigecycline resistance was only observed in *E. faecium* and *E. faecalis* from grass-fed animals, and whilst tetracycline resistance in *E. faecalis* was more common in grain-fed isolates, the opposite relationship existed in *E. faecium* with tetracycline resistance only detected in isolates from grass-fed animals. None of the differences observed between isolates from grass- and grain-fed animals were considered significantly different ($p < 0.05$). Resistance to ampicillin, chloramphenicol, gentamicin, linezolid, penicillin, teicoplanin and vancomcyin was not observed in any *E. faecalis* or *E. faecium* isolate, regardless of source.

The high levels of resistance to flavomycin and lincomycin meant that MDR isolates were common among *Enterococcus* isolates. Table 12 shows the antibiograms for each *Enterococcus* grouping. MDR was observed in 51 (42.5%) *E. faecium*, 21 (21.9%) *E. faecalis* and 260 (44.5%) *Enterococcus spp.* isolates. Resistance to four or more antimicrobials was less commonly observed with 10 (8.3%) *E. faecium* and four (4.2%) *E. faecalis* isolates falling into this category. Antibiogram profiles across the species groups were dominated by the FLV-LIN profile with 262 (32.8%) of all isolates harbouring this combination. The main MDR profiles for *E. faecium* included FLV-LIN-QDA (24.2%), FLV-LIN-TET (4.2%) and ERY-FLV-LIN-QDA-TET (4.2%). Similarly for *E. faecalis*, DAP-FLV-LIN (8.3%) and ERY-FLV-LIN (5.2%) were the main MDR antibiograms identified.

Discussion

Zoonotic bacteria that are resistant to antimicrobials are of increased concern to public health officials throughout the world as they may compromise the ability of various treatment regimes to address disease and infection in human medical settings. Knowledge and understanding of the types of AMR present in food production animals is key to determining the ongoing risk that AMR bacteria pose to human health. Australia currently does not have a nationally coordinated program for the ongoing surveillance and analysis of AMR bacteria in animals, bacteria in food derived from animals, or bacteria from humans. Consequently it relies heavily on routine testing of human and animal

clinical isolates as well as infrequent surveys of isolates from animals or from food of animal origin to understand AMR development and trends. Australia most recently conducted pilot surveys for AMR in bacteria of animal origin and in retail foods in 2003/4 and 2007/8. Both studies concluded that resistance to clinically significant antimicrobials such as 3rd and 4th generation cephalosporins as well as fluoroquinolones was non-existent or very low regardless of animal, food or bacterial type (DAFF 2007; Barlow and Gobius 2008). The study detailed here was conducted as an adjunct to a survey of pathogenic STEC (pSTEC) in Australian cattle populations (refer pSTEC manuscript) and is consequently narrower in focus than previous studies as it solely focuses on isolates from cattle at slaughter. Nevertheless, the large volume of isolates being analysed ensures it provides a comprehensive snapshot assessment of AMR in Australian cattle.

Despite the potential limitations that point prevalence surveys have in comparison to ongoing surveillance programs, the methodology used in this study does allow for the results to be placed in a global context and contrasted with overseas data. Of prime importance to this study is the prevalence of AMR in *Salmonella* from Australian cattle populations. This importance is in response to the petition submitted to USDA that requests that specific serovars of MDR *Salmonella* be classified as adulterants of raw, non-intact beef products (Center for Science in the Public Interest 2011). *Salmonella* were isolated from 14.4% of samples which represents a substantial increase from a previous Australian cattle survey that detected *Salmonella* in 6.8% of samples (Fegan, Vanderlinde et al. 2004). Importantly, this survey included dairy cattle faecal samples and in univariate analysis these were shown to be significantly more likely to harbor *Salmonella* than samples from beef cattle or veal calves. However, even after taking this difference between the surveys into account, the prevalence of *Salmonella* in beef cattle samples in this survey was 11.5% and remains higher than previously estimated.

In general, the prevalence of resistance in *Salmonella* to any of the antimicrobials tested in this study is low with 87.2% of all *Salmonella* susceptible to all antimicrobials tested. Resistance to streptomycin, ampicillin, chloramphenicol and tetracycline were well below the rates observed in the European Union (EU) and the USA (NARMS). Resistance to the abovementioned antimicrobials did not exceed 10% in this study compared with >25% resistance in the 2010 NARMS study and 29.1% and 31.1% for ampicillin and tetracycline, respectively in the EU study. (USDA 2012; European Food Safety Authority 2013). Resistance to the cephems was generally absent from beef cattle and

veal calves but was elevated in *Salmonella* from dairy cattle with ceftiofur, ceftriaxone and cefotaxime resistance ranging from 14.5% to 17.1%. Globally, cepehem resistance varies substantially between the EU where resistance levels are very low and the USA where resistance levels have steadily increased during the last decade to exceed 20% across all *Salmonella* in 2010 (USDA 2012; European Food Safety Authority 2013). The increased cephem resistance observed in dairy cattle isolates is thought to be linked to the use of ceftiofur. Ceftiofur is a 3rd generation cephalosporin licensed for use in Australia for the treatment of bovine respiratory diseases. Whilst resistance has typically been low, it is often highly correlated with resistance to ceftriaxone, cefotaxime and a number of unrelated antimicrobial classes via the presence of multi-drug resistant plasmids carrying the AmpC-like β-lactamase CMY-2 (Alcaine, Sukhnanand et al. 2005; Donaldson, Straley et al. 2006). However, the rates of resistance to cephems observed in this study must be interpreted with caution as they are concentrated by time and space. All cephem resistant *Salmonella* were from animals from an identical source slaughtered within a two hour period and is indicative of a horizontal gene transfer event or the proliferation of a resistant clone of *Salmonella* within that animal population as opposed to a widespread issue. Nonetheless, presentation of a group of cattle for slaughter with increased AMR *Salmonella* levels, particularly in the serovars Typhimurium and Newport is undesirable and exerts additional pressure on existing hygiene controls to maintain a safe food supply.

The monitoring of AMR in *E. coli* is a common component of all surveillance programs as *E. coli* have been shown to routinely act as reservoirs of resistance genes that can then spread horizontally to other bacteria. Previous Australian surveys that have investigated phenotypic and genotypic AMR in *E. coli* from cattle have all indicated that resistance to all antimicrobial classes is low and in particular resistance to antimicrobials of human clinical significance is generally absent (DAFF 2007; Barlow, Fegan et al. 2008; Barlow and Gobius 2008). The pattern of low levels of resistance in *E. coli* has continued in this survey with >92% of isolates remaining susceptible to all antimicrobials tested regardless of animal class. *E. coli* that did exhibit AMR were most likely to do so to older antimicrobials such as tetracycline, streptomycin, ampicillin and trimethoprim / sulfamethoxazole. Additionally, tetracycline resistance was significantly more likely to be associated with grain-fed cattle than grass-fed cattle, dairy cattle or veal calves and may be a result of specific production practices employed during feed-lotting of animals. Similar observations around AMR to older antimicrobials, albeit at increased frequencies, have been made in *E. coli* from cattle in EU member states (European Food Safety Authority 2013; Wasyl, Hoszowski et al. 2013). NARMS does not

perform susceptibility testing on *E.* coli isolates from live cattle; however the levels of AMR present in *E. coli* collected from dairy cattle during this study contrast heavily with a retrospective analysis of 3373 US dairy cattle *E. coli* isolates collected between 2004 and 2011 where 71% of isolates were resistant to two or more antimicrobials (Cummings, Aprea et al. 2014). Resistance to antimicrobials of significance to human medicine such as amoxicillin / clavulanic acid, gentamicin and ceftiofur although infrequent, were only observed in *E. coli* from veal calves. Some member states of the EU have reported increased AMR in isolates from younger animals, mainly fattening calves, compared to older animals (European Food Safety Authority 2013). Whist similar observations have been made in North American studies (Gow, Waldner et al. 2008) it has been suggested that the prevalence of AMR *E. coli* in calves may not be a function of antimicrobial use and instead related to AMR neonate-adapted bacteria (Khachatryan, Hancock et al. 2004). There is no evidence for the persistence of neonate-adapted AMR *E. coli* in veal calf populations in Australia and in general, resistance in *E. coli* does not appear to be linked to the age of the animal or the production system from which the isolate was obtained.

Enterococci are ubiquitous bacteria that demonstrate intrinsic resistance to antimicrobials within a number of classes (aminoglycosides, cephalosporins and lincosamides). They are also frequently associated with mobile genetic elements harbouring AMR genes and have the potential for resistance to virtually all antimicrobials of importance to human medicine (Ramos, Igrejas et al. 2012). The importance of Enterococci as the third most commonly isolated nosocomial pathogen (Hidron, Edwards et al. 2008) and the clear relationship between exposure to parental antimicrobials and the development of resistance (Hollenbeck and Rice 2012) warrants their ongoing inclusion in any human, animal or food AMR surveillance program. *Enterococcus faecalis* and *E. faecium* were recovered from 6.4% and 8.0% of samples in this survey and although they are the two Enterococcal species most associated with human infections, monitoring of environmental enterococci is useful as it may provide insights into the development of resistance which may subsequently transfer to the more clinically relevant species. From a human clinical perspective, resistance in *E. faecalis* and *E. faecium* to ampicillin, vancomycin, linezolid, daptomycin, quinupristin / dalfopristin (*E. faecium* only) and tigecycline are the key issues. Resistance to other older antimicrobials such as lincomycin, flavomycin, tetracycline and erythromycin are seldom considered as either resistance is common or the antimicrobials are seldom used in human medicine (Hollenbeck and Rice 2012). The findings of this study reinforce this segregation of concern with high levels of resistance to lincomycin, flavomycin, tetracycline and erythromycin observed across all enterococcal species and animal

groups. Conversely, resistance to ampicillin, linezolid and vancomycin was not observed. This is a pleasing result as ampicillin remains the preferred therapy for uncomplicated enterococcal infections. Similarly, the abscence of vancomycin resistant enterococcus assists in maintaining optimal treatment options. Linezolid and quniupristin / dalfopristin are suggested therapies for vancomycin resistant enterococcus infections. In this study 41.7% of *E. faecium* isolates were shown to be resistant to quniupristin / dalfopristin. Resistance to quniupristin / dalfopristin has been linked to the use of virginiamycin and consequently resistance is most common in enterococci isolated from farm animals and agricultural sewage (Hollenbeck and Rice 2012). EU member states also report a high rate of quinupristin / dalfopristin resistance in cattle isolates regardless of age or production system (European Food Safety Authority 2013). At the human clinical level, several studies report an increase in resistance to quinupristin / dalfopristin among *E. faecium* strains and have suggested that linezolid is a preferred therapy for vancomycin resistant enterococcus infections (Berenger, Bourdon et al. 2011). Similarly there are reports of increased resistance to tigecycline and daptomycin in human medicine (Tsai, Liao et al. 2012). Resistance to these antimicrobials was demonstrated in this study and close monitoring of MICs for daptomycin and tigecycline may be required (Cai, Wang et al. 2011; Kelesidis, Humphries et al. 2011).

Statistical methodology used in this study was kept to simple comparisons for ease of interpretation. A more complex multivariate evaluation of how various factors ("class of animal", "sampling window" and "type of feed") impact on the prevalence and AMR of Salmonella, *E. coli* and *Enterococcus* is to be undertaken. This analysis will account for potential confounding and interaction in the data as well as the implied dependence in the data induced by the cluster-based approach to sampling. This study has determined the AMR status of *Salmonella*, *E. coli* and *enterococcus* isolates from Australian cattle populations. Overall, the results corroborate previous Australian based animal and retail food surveys that have shown a low level of AMR, relatively small proportions of MDR and most importantly the maintenance of susceptibility to most antimicrobials of human clinical significance. Nevertheless the presence of ceftiofur resistant *Salmonella* and enterococci with resistance to quinupristin / dalfopristin, daptomycin and tigecycline is of concern and may warrant an increased level of surveillance. Importantly, it would appear that the production practices at work in Australian cattle populations are not generating pools of resistance that are likely to result in the inability to treat human infections caused by *Salmonella*, *E. coli* and enterococci. Similarly, although some differences in AMR levels were noted between production systems, there is minimal evidence that specific production practices are responsible for

disproportionate contributions to AMR development. Furthermore, comparisons with AMR data from the EU and USA shed a favourable light on Australia's ability to meet any proposed regulations relating to the presence of MDR bacteria in exported beef products. Nevertheless, it is necessary to maintain strict guidelines and controls around the use of antimicrobials in food-production animals in Australia and to continually monitor the effects of all antimicrobial use if Australia's reputation as a supplier of safe and healthy food is to be maintained.

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Table 1. Dilution ranges and breakpoints for antimicrobial susceptibility testing

Antimicrobial	*E. coli* & *Salmonella*		*Enterococcus*	
	Range	**Breakpoint**	**Range**	**Breakpoint**
Amoxicillin / clavulanic acid	1/0.5 - 32/16	≥32/16		
Ampicillin	2-64	≥32	0.5-16	≥16
Cefazolin	2-16	≥8		
Cefotaxime	0.032-8	≥4		
Cefoxitin	0.5–32	≥32		
Ceftiofur	0.5–16	≥8		
Ceftriaxone	0.125-4	≥4		
Chloramphenicol	2-32	≥32	2-32	≥32
Ciprofloxacin	0.0625-4	≥1		
Daptomycin			0.125-4	≥8
Erythromycin			0.25-8	≥8
Flavomycin			1-32	≥32
Florfenicol	2-64			
Gentamicin	0.5-16	≥16	32-1024	≥512
Kanamycin	8-64	≥64	128-1024	≥1024
Lincomycin			1-32	≥8
Linezolid			0.5-8	≥8
Meropenem	0.0625-0.5	8		
Nalidixic Acid	1-32	≥32		
Penicillin			0.5-16	≥16
Quinupristin / dalfopristin			256-1024	≥1024
Streptomycin	16-64	≥64	0.125-4	2
Teicoplanin			2-16	≥16
Tetracycline	2-16	≥16	0.016-0.5	≥0.5
Tigecycline				
Trimethoprim / sulfamethoxazole	0.12/2.38 - 4/76	≥4/76		
Vancomycin			0.25-32	≥32
Virginiamycin			1-32	>8

Shaded boxes indicate that the bacterial / antimicrobial combination was not tested.

Table 2. *Salmonella* prevalence in Australian cattle groups across sampling windows

Group	Window 1	*Salmonella* +ve	Window 2	*Salmonella* +ve	Total
Beef cattle	469	59 (12.6)[*]	441	46 (10.4)	105 (11.5)
Dairy cattle	146	42 (28.8)	144	33 (22.9)	75 (25.9)
Veal calves	138	7 (5.1)	162	29 (17.9)	36 (12.0)
Total	753	108 (14.3)	747	108 (14.5)	216 (14.4)

[*] figures in parentheses are percent

Table 3. *Salmonella* prevalence in grain- and grass-fed beef cattle across sampling windows

Feed type	Window 1	*Salmonella* +ve	Window 2	*Salmonella* +ve	Total
Grain-fed	167	23 (13.8)[*]	135	6 (4.4)	302 (9.6)
Grass-fed	293	36 (12.3)	282	38 (13.5)	575 (12.9)
Total	460	59 (12.8)	417	44 (10.6)[#]	877 (11.7)

[*] figures in parentheses are percent;

[#] feed type information was not available for two *Salmonella* positive samples in sampling window 2

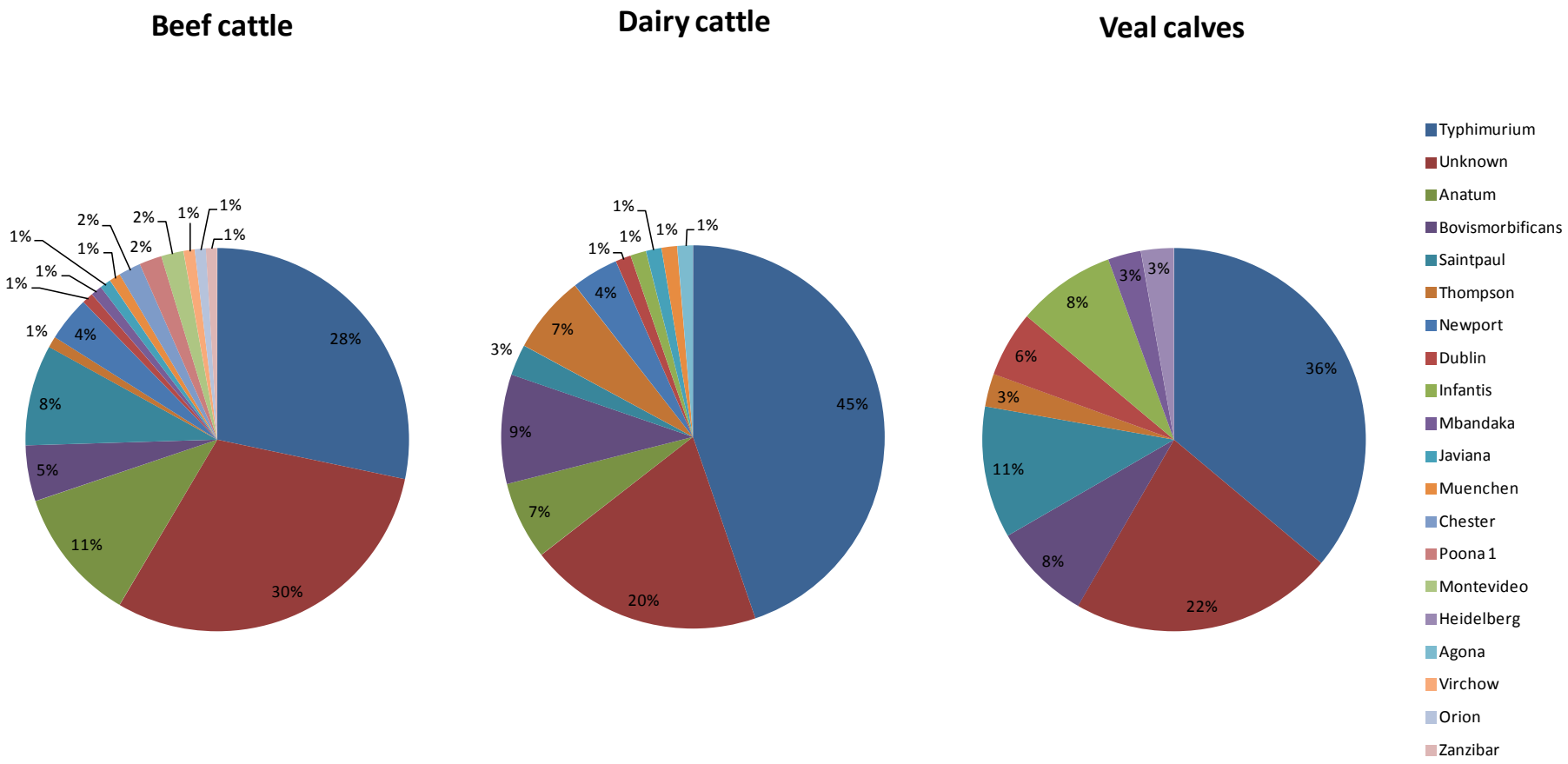

Figure 1. Distribution of *Salmonella* serovars in beef cattle, dairy cattle and veal calf faecal samples

Table 4. Distribution of MICs and occurrence of resistance among *Salmonella* isolates from beef cattle, dairy cattle and veal calf faecal samples

Class	Antimicrobial	Group	N =	% Resistant	95% CI	Antimicrobial concentration (µg/ml)												
						0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	>64
Aminoglycosides	Gentamicin	Beef	106	0.0	0.00 - 3.42					50.9	44.3	4.7						
		Dairy	76	1.3	0.03 - 7.11					56.6	27.6	2.6	3.9	7.9	1.3			
		Veal	36	0.0	0.00 - 9.74					72.2	25.0	2.8						
	Kanamycin	Beef	106	0.0	0.00 - 3.42									95.3	3.8	0.9		
		Dairy	76	3.9	0.82 - 11.11									82.9	5.3	7.9	3.9	
		Veal	36	0.0	0.00 - 9.74									100.0				
	Streptomycin	Beef	106	8.5	3.96 - 15.51										84.0	7.5	2.8	5.7
		Dairy	76	9.2	3.78 - 18.06										80.3	10.5	9.2	
		Veal	36	0.0	0.00 - 9.74										91.7	8.3		
b-lactam/b-lactamase inhibitor combinations	Amoxicillin /Clavulanic acid	Beef	106	0.0	0.00 - 3.42						85.8	6.6	3.8	3.8				
		Dairy	76	0.0	0.00 - 4.74						97.4	2.6						
		Veal	36	0.0	0.00 - 9.74						94.4	5.6						
Carbapenem	Meropenem	Beef	106	0.0	0.00 - 3.42		93.4	2.8	0.9	0.9	1.9							
		Dairy	76	0.0	0.00 - 4.74		77.6	2.6		5.3	14.5							
		Veal	36	0.0	0.00 - 9.74		100.0											
Cephems	Cefazolin	Beef	106	1.9	0.23 - 6.65							89.6	8.5		0.9	0.9		
		Dairy	76	18.4	10.45 - 28.97							78.9	2.6		2.6	15.8		
		Veal	36	0.0	0.00 - 9.74							91.7	8.3					
	Cefotaxime	Beef	106	0.9	0.02 - 5.14		61.3	32.1	4.7	0.9					0.9			
		Dairy	76	15.8	8.43 - 25.96		51.3	27.6	2.6	1.3		1.3		1.3	14.5			
		Veal	36	0.0	0.00 - 9.74		77.8	16.7	2.8	2.8								
	Cefoxitin	Beef	106	0.0	0.00 - 3.42						3.8	61.3	29.2	4.7	0.9			
		Dairy	76	5.3	1.45 - 12.93							46.1	34.2	7.9	6.6	5.3		
		Veal	36	0.0	0.00 - 9.74						5.6	61.1	27.8	5.6				
	Ceftiofur	Beef	106	1.9	0.23 - 6.65					45.3	48.1	4.7				1.9		
		Dairy	76	17.1	9.43 - 27.47					55.3	27.6			1.3	2.6	13.2		
		Veal	36	0.0	0.00 - 9.74					58.3	41.7							

Class	Antimicrobial	Group	N =	% Resistant	95% CI	Antimicrobial concentration (µg/ml)												
						0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	>64
Cephems	Ceftriaxone	Beef	106	0.0	0.00 - 3.42			87.7	6.6	0.9	2.8	1.9						
		Dairy	76	14.5	7.45 - 24.42			76.3	3.9	1.3	3.9		3.9	10.5				
		Veal	36	0.0	0.00 - 9.74			97.2	2.8									
Folate pathway inhibitors	Trimethoprim /Sulfamethoxazole	Beef	106	7.5	3.31 - 14.33			84.9	3.8	1.9	0.9	0.9	7.5					
		Dairy	76	0.0	0.00 - 4.74			88.2	11.8									
		Veal	36	0.0	0.00 - 9.74			100.0										
Penicillins	Ampicillin	Beef	106	7.5	3.31 - 14.33							89.6	0.9	0.9	0.9		0.9	6.6
		Dairy	76	0.0	0.00 - 4.74							100.0						
		Veal	36	0.0	0.00 - 9.74							100.0						
Phenicols	Chloramphenicol	Beef	106	0.0	0.00 - 3.42								13.2	84.9	1.9			
		Dairy	76	0.0	0.00 - 4.74								11.8	86.8	1.3			
		Veal	36	0.0	0.00 - 9.74							2.8	8.3	88.9				
	Florfenicol*	Beef	106	NA	NA								71.7	28.3				
		Dairy	76	NA	NA								76.3	23.7				
		Veal	36	NA	NA							2.8	58.3	38.9				
Quinolones	Ciprofloxacin	Beef	106	0.0	0.00 - 3.42		94.3	1.9	3.8									
		Dairy	76	2.6	0.32 - 9.18		78.9	5.3	10.5	2.6	2.6							
		Veal	36	0.0	0.00 - 9.74		100.0											
	Nalidixic Acid	Beef	106	0.9	0.02 - 5.14							6.6	84.9	7.5		0.9		
		Dairy	76	15.8	8.43 - 25.96							5.3	68.4	7.9	2.6	2.6	13.2	
		Veal	36	0.0	0.00 - 9.74							2.8	91.7	5.6				
Tetracyclines	Tetracycline	Beef	106	6.6	2.70 - 13.13							87.7	3.8	1.9	0.9	5.7		
		Dairy	76	0.0	0.00 - 4.74							98.7	1.3					
		Veal	36	0.0	0.00 - 9.74							100.0						

* Only a susceptible breakpoint (≤4µg/ml) has been established. Isolates with an MIC ≥8µg/ml are reported as non-susceptible

Solid vertical lines indicate breakpoints for resistance. The white fields indicate the dilution range tested for each antimicrobial. Values in the shaded area indicate MIC values greater than the highest concentration tested.

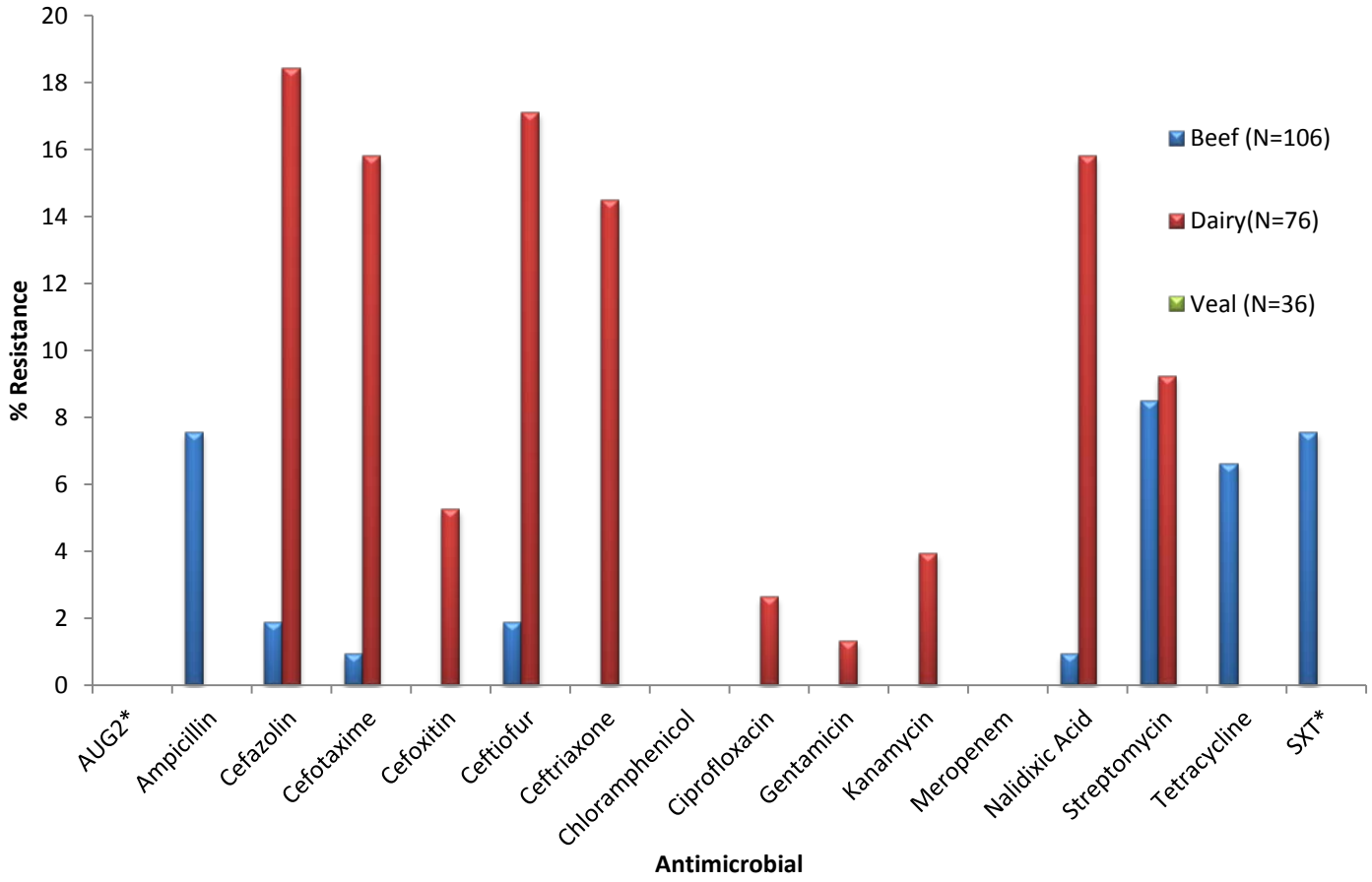

Figure 2. Prevalence of AMR in *Salmonella* isolates from beef cattle, dairy cattle and veal calf faecal samples

* AUG2 – amoxicillin / clavulanic acid; SXT – trimethorpim / sulfamethoxazole

Table 5. Prevalence of AMR in *Salmonella* isolates from grass-fed beef cattle, grain-fed beef cattle, dairy cattle and veal calf faecal samples

Antimicrobial	Grass N=74	Grain N=29	Dairy* N=76	Veal N=36
Amoxicillin / clavulanic acid	0.0	0.0	0.0	0.0
Ampicillin	1.4	24.1	0.0	0.0
Cefazolin	2.7	0.0	18.4	0.0
Cefotaxime	1.4	0.0	15.8	0.0
Cefoxitin	0.0	0.0	5.3	0.0
Ceftiofur	2.7	0.0	17.1	0.0
Ceftriaxone	0.0	0.0	14.5	0.0
Chloramphenicol	0.0	0.0	0.0	0.0
Ciprofloxacin	0.0	0.0	2.6	0.0
Gentamicin	0.0	0.0	1.3	0.0
Kanamycin	0.0	0.0	3.9	0.0
Meropenem	0.0	0.0	0.0	0.0
Nalidixic Acid	1.4	0.0	15.8	0.0
Streptomycin	4.1	20.7	9.2	0.0
Tetracycline	0.0	24.1	0.0	0.0
Trimethoprim / sulfamethoxazole	1.4	24.1	0.0	0.0

**Salmonella* isolates from dairy cattle with resistance to cefazolin, ceftiofur, ceftriaxone and nalidixic acid were isolated from a single abattoir during one sampling day.

Table 6. Antibiograms of *Salmonella* from beef cattle, dairy cattle and veal calf faecal samples

Antibiograms	Beef (N=106)	Dairy (N=76)	Veal (N=36)	Major serovars present
All Susceptible	93	61	36	
FAZ		1		Typhimurium
NAL	1			Anatum
STR	1			Unknown
FAZ FOT	1			Anatum
FAZ XNL	1			Typhimurium
NAL STR		1		Anatum
XNL STR	1			Anatum
AMP STR SXT	1			Unknown
AMP TET SXT	1			Dublin
FAZ FOT XNL		1		Javiana
FAZ XNL AXO		1		Typhimurium
AMP STR TET SXT	6			Typhimurium (3), Newport (1), Unknown (2)
FAZ FOT XNL NAL		1		Newport
FAZ FOT XNL AXO NAL		4		Typhimurium (3), Thompson (1)
FAZ FOT XNL AXO NAL STR		1		Typhimurium
FAZ FOT FOX XNL AXO NAL STR		1		Typhimurium
FAZ FOT XNL AXO KAN NAL STR		1		Bovismorbificans
FAZ FOT FOX XNL AXO CIP NAL STR		1		Typhimurium
FAZ FOT FOX XNL AXO KAN NAL STR		1		Typhimurium
FAZ FOT FOX XNL AXO CIP GEN KAN NAL STR		1		Typhimurium

* FAZ – cefazolin, NAL – nalidixic acid, STR – streptomycin, FOT – cefotaxime, XNL – ceftiofur, AMP – ampicillin, SXT – trimethoprim / sulfamethoxazole, TET – tetracycline, AXO – ceftriaxone, FOX – cefoxitin, KAN – kanamycin, CIP – ciprofloxacin, GEN – gentamicin.

Table 7. Distribution of MICs and occurrence of resistance among *E. coli* isolates from beef cattle, dairy cattle and veal calf faecal samples

Class	Antimicrobial	Group	N =	% Resistant	95% CI	Antimicrobial concentration (µg/ml)												
						0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	>64
Aminoglycosides	Gentamicin	Beef	469	0.0	0.00 - 0.78					21.1	72.1	6.2	0.6					
		Dairy	155	0.0	0.00 - 2.35					29.0	60.0	10.3	0.6					
		Veal	176	0.6	0.01 - 3.12					23.9	68.8	5.1	1.1	0.6		0.6		
	Kanamycin	Beef	469	0.0	0.00 - 0.78									99.4	0.4	0.2		
		Dairy	155	0.0	0.00 - 2.35									98.1	1.9			
		Veal	176	1.1	0.14 - 4.04									97.2	1.1	0.6		1.1
	Streptomycin	Beef	469	1.1	0.35 - 2.47										97.9	1.1	0.2	0.9
		Dairy	155	1.9	0.40 - 5.55										98.1			1.9
		Veal	176	4.0	1.61 - 8.02										96.0		1.1	2.8
b-lactam/b-lactamase inhibitor combinations	Amoxicillin /Clavulanic acid	Beef	469	0.0	0.00 - 0.78						5.3	23.7	59.5	11.5				
		Dairy	155	0.0	0.00 - 2.35						7.7	17.4	56.8	16.8	1.3			
		Veal	176	1.1	0.14 - 4.04						2.3	16.5	69.3	9.7	1.1	0.6	0.6	
Carbapenem	Meropenem	Beef	469	0.0	0.00 - 0.78		99.6	0.2	0.2									
		Dairy	155	0.0	0.00 - 2.35		100.0											
		Veal	176	0.0	0.00 - 2.07		99.4	0.6										
Cephems	Cefazolin	Beef	469	0.2	.01 - 1.18							96.4	3.4		0.2			
		Dairy	155	0.0	0.00 - 2.35							96.8	3.2					
		Veal	176	1.7	0.35 - 4.90							93.8	4.5	0.6		1.1		
	Cefotaxime	Beef	469	0.0	0.00 - 0.78	26.2	63.8	9.4	0.4	0.2								
		Dairy	155	0.0	0.00 - 2.35	27.1	62.6	10.3										
		Veal	176	0.0	0.00 - 2.07	22.2	68.2	6.3	2.3	0.6		0.6						
	Cefoxitin	Beef	469	0.0	0.00 - 0.78						6.4	36.9	46.5	9.6	0.6			
		Dairy	155	0.0	0.00 - 2.35						5.8	43.2	44.5	6.5				
		Veal	176	0.0	0.00 - 2.07						1.7	41.5	47.7	8.5	0.6			
	Ceftiofur	Beef	469	0.0	0.00 - 0.78					99.1	0.9							
		Dairy	155	0.0	0.00 - 2.35					99.4	0.6							
		Veal	176	0.6	0.01 - 3.12					97.2	2.3			0.6				

Class	Antimicrobial	Group	N =	% Resistant	95% CI	Antimicrobial concentration (µg/ml)												
						0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	>64
Cephems	Ceftriaxone	Beef	469	0.0	0.00 - 0.78			98.3	1.5		0.2							
		Dairy	155	0.0	0.00 - 2.35			98.7	1.3									
		Veal	176	0.0	0.00 - 2.07			96.6	2.3	0.6	0.6							
Folate pathway inhibitors	Trimethoprim /Sulfamethoxazole	Beef	469	0.2	0.01 - 1.18			97.7	1.1	0.9		0.2		0.2				
		Dairy	155	1.3	0.16 - 4.58			98.1	0.0	0.6				1.3				
		Veal	176	2.3	0.62 - 5.72			95.5	1.1	0.6	0.6			2.3				
Penicillins	Ampicillin	Beef	469	0.0	0.00 - 0.78							37.7	56.1	5.8	0.4			
		Dairy	155	2.6	0.71 - 6.48							32.3	54.8	10.3		0.6		1.9
		Veal	176	4.5	1.98 - 8.76							34.7	57.4	2.3	1.1		0.6	4.0
Phenicols	Chloramphenicol	Beef	469	0.0	0.00 - 0.78							2.6	26.2	65.0	6.2			
		Dairy	155	0.0	0.00 - 2.35							0.6	20.6	70.3	8.4			
		Veal	176	0.0	0.00 - 2.07							2.8	22.2	72.2	2.8			
	Florfenicol*	Beef	469	NA	NA							5.5	39.0	51.6	3.8			
		Dairy	155	NA	NA								41.3	55.5	3.2			
		Veal	176	NA	NA							5.1	35.2	59.1	0.6			
Quinolones	Ciprofloxacin	Beef	469	0.0	0.00 - 0.78		99.8		0.2									
		Dairy	155	0.0	0.00 - 2.35		100.0											
		Veal	176	0.0	0.00 - 2.07		100.0											
	Nalidixic Acid	Beef	469	0.0	0.00 - 0.78						7.5	63.3	27.9	1.1	0.2			
		Dairy	155	0.0	0.00 - 2.35						6.5	66.5	25.8	1.3				
		Veal	176	0.0	0.00 - 2.07						8.0	69.3	22.2	0.6				
Tetracyclines	Tetracycline	Beef	469	7.7	5.43 - 10.47							83.8	8.1	0.4	0.9	6.8		
		Dairy	155	2.6	0.71 - 6.48							83.2	14.2			2.6		
		Veal	176	4.5	1.98 - 8.76							91.5	4.0			4.5		

*Only a susceptible breakpoint (≤4µg/ml) has been established. Isolates with an MIC ≥8µg/ml are reported as non-susceptible

Solid vertical lines indicate breakpoints for resistance. The white fields indicate the dilution range tested for each antimicrobial. Values in the shaded area indicate MIC values greater than the highest concentration tested.

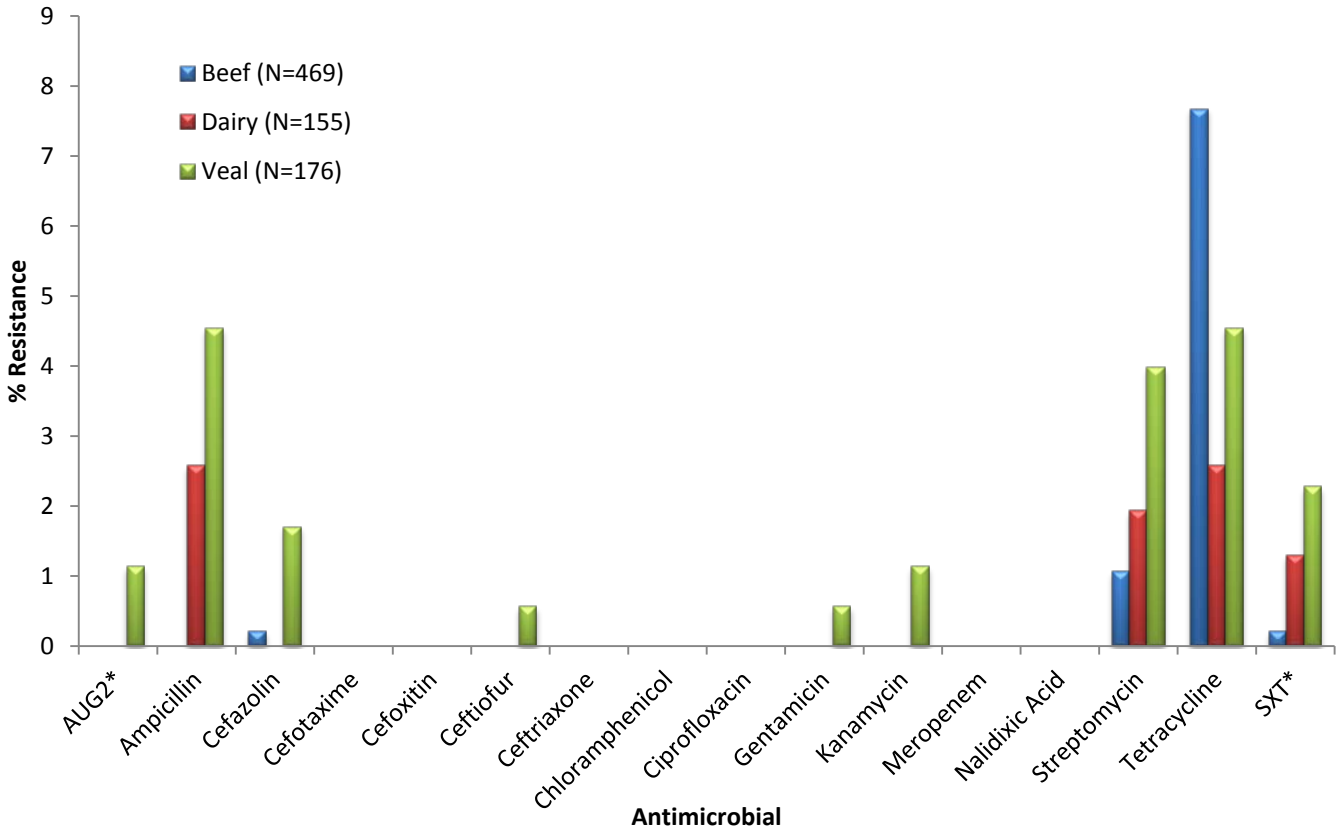

Figure 3. Prevalence of AMR in *E. coli* isolates from beef cattle, dairy cattle and veal calf faecal samples

* AUG2 – amoxicillin / clavulanic acid; SXT – trimethorpim / sulfamethoxazole

Table 8. Prevalence of AMR in *E. coli* isolates from grass-fed beef cattle, grain-fed beef cattle, dairy cattle and veal calf faecal samples

Antimicrobial	Grass N=280	Grain N=173	Dairy N=155	Veal N=176
Amoxicillin / clavulanic acid	0.0	0.0	0.0	1.1
Ampicillin	0.0	0.0	2.6	4.6
Cefazolin	0.0	0.6	0.0	1.7
Cefotaxime	0.0	0.0	0.0	0.0
Cefoxitin	0.0	0.0	0.0	0.0
Ceftiofur	0.0	0.0	0.0	0.6
Ceftriaxone	0.0	0.0	0.0	0.0
Chloramphenicol	0.0	0.0	0.0	0.0
Ciprofloxacin	0.0	0.0	0.0	0.0
Gentamicin	0.0	0.0	0.0	0.6
Kanamycin	0.0	0.0	0.0	1.1
Meropenem	0.0	0.0	0.0	0.0
Nalidixic Acid	0.0	0.0	0.0	0.0
Streptomycin	0.0	2.9	1.9	4.0
Tetracycline	3.6	15.0	2.6	4.6
Trimethoprim / sulfamethoxazole	0.4	0.0	1.3	2.3

Table 9. Antibiograms of *E. coli* from beef cattle, dairy cattle and veal calf faecal samples

Antibiograms*	**Beef (N=469)**	**Dairy (N=155)**	**Veal (N=176)**
ALL SENSITIVE	432	150	164
AMP		1	1
STR	1		1
TET	30		1
AMP FAZ			1
AMP TET		1	
FAZ TET	1		
STR TET	4	1	1
TET SXT	1		
AMP STR TET			1
AUG2 AMP FAZ			1
AMP STR TET SXT		2	1
GEN STR TET SXT			1
AMP KAN STR TET SXT			2
AUG2 AMP FAZ XNL TET			1

* AMP – ampicillin , STR – streptomycin, TET – tetracycline, FAZ – cefazolin, SXT – trimethoprim / sulfamethoxazole, AUG2 – amoxicillin / clavulanic acid, GEN – gentamicin, KAN – kanamycin, XNL – ceftiofur.

Table 10. Distribution of MICs and occurrence of resistance among *Enterococcus* isolates from beef cattle, dairy cattle and veal calf faecal samples

Class	Antimicrobial	Species	N =	% Resistant	95% CI	Antimicrobial concentration (µg/ml)														
						0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Aminoglycoside	Gentamicin	*Enterococcus faecalis*	96	0.0	0.00 - 3.77												100.0				
		Enterococcus faecium	120	0.0	0.00 - 3.03												99.2	0.8			
		Enterococcus spp	584	0.0	0.00 - 0.63												99.7	0.3			
	Kanamycin	*Enterococcus faecalis*	96	1.0	0.03 - 5.67														92.7	5.2	1.0
		Enterococcus faecium	120	0.8	0.02 - 4.56														99.2		
		Enterococcus spp	584	0.9	0.28 - 1.99														95.0	3.8	0.3
	Streptomycin	*Enterococcus faecalis*	96	1.0	0.03 - 5.67															99.0	
		Enterococcus faecium	120	0.0	0.00 - 3.03															100.0	
		Enterococcus spp	584	0.9	0.28 - 1.99															99.1	
Glycopeptides	Teicoplanin	*Enterococcus faecalis*	96	0.0	0.00 - 3.77				30.2	46.9	17.7	5.2									
		Enterococcus faecium	120	0.0	0.00 - 3.03				42.5	40.0	14.2	3.3									
		Enterococcus spp	584	0.0	0.00 - 0.63				33.2	41.1	19.7	6.0									
	Vancomycin	*Enterococcus faecalis*	96	0.0	0.00 - 3.77					1.0	40.6	34.4	11.5	10.4	2.1						
		Enterococcus faecium	120	0.0	0.00 - 3.03						46.7	22.5	15.0	15.0	0.8						
		Enterococcus spp	584	0.0	0.00 - 0.63					0.5	41.6	28.3	13.4	12.3	3.9						
Glycylcycline	Tigecycline	*Enterococcus faecalis*	96	2.1	0.25 - 7.32	2.1	45.8	41.7	3.1	5.2	2.1										
		Enterococcus faecium	120	2.5	0.52 - 7.13		43.3	44.2	5.8	4.2	2.5										
		Enterococcus spp	584	2.9	1.70 - 4.62	2.1	45.0	37.7	8.0	4.3	2.7	0.2									
Lincosamide	Lincomycin	*Enterococcus faecalis*	96	85.4	76.74 - 91.79							10.4	1.0	3.1	8.3	25.0	34.4	17.7			
		Enterococcus faecium	120	94.2	88.35 - 97.62							4.2	0.8	0.8	2.5	27.5	50.0	14.2			
		Enterococcus spp	584	83.7	80.48 - 86.63							12.3	1.9	2.1	6.2	30.7	37.5	9.4			
Lipopeptide	Daptomycin	*Enterococcus faecalis*	96	9.4	4.38 - 17.05								75.0	15.6	9.4						
		Enterococcus faecium	120	2.5	0.52 - 7.13							5.8	45.0	46.7	2.5						
		Enterococcus spp	584	5.1	3.49 - 7.25				0.2		3.3	24.8	40.6	24.5	6.7						
Macrolide	Erythromycin	*Enterococcus faecalis*	96	10.4	5.11 - 18.32					33.3	15.6	14.6	20.8	5.2	1.0	9.4					
		Enterococcus faecium	120	8.3	4.07 - 14.79					53.3	13.3	12.5	9.2	3.3	4.2	4.2					
		Enterococcus spp	584	6.0	4.21 - 8.24					45.9	13.2	11.0	17.6	6.3	1.2	4.8					

Class	Antimicrobial	Species	N =	% Resistant	95% CI	Antimicrobial concentration (µg/ml)															
						0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Oxazolidinones	Linezolid	*Enterococcus faecalis*	96	0.0	0.00 - 3.77							6.3	87.5	6.3							
		Enterococcus faecium	120	0.0	0.00 - 3.03							5.8	87.5	6.7							
		Enterococcus spp	584	0.0	0.00 - 0.63						0.3	5.0	88.2	6.5							
Penicillins	Ampicillin	*Enterococcus faecalis*	96	0.0	0.00 - 3.77						44.8	52.1	3.1								
		Enterococcus faecium	120	0.0	0.00 - 3.03						30.0	60.0	10.0								
		Enterococcus spp	584	0.0	0.00 - 0.63						45.9	29.5	22.3	2.4							
	Penicillin	*Enterococcus faecalis*	96	0.0	0.00 - 3.77						13.5	28.1	26.0	31.3	1.0						
		Enterococcus faecium	120	0.0	0.00 - 3.03						12.5	34.2	30.8	20.8	1.7						
		Enterococcus spp	584	0.3	0.04 - 1.23						19.3	27.1	22.8	21.2	9.2	0.2	0.2				
Phenicol	Chloramphenicol	*Enterococcus faecalis*	96	0.0	0.00 - 3.77									17.7	82.3						
		Enterococcus faecium	120	0.0	0.00 - 3.03								1.7	51.7	40.8	5.8					
		Enterococcus spp	584	0.0	0.00 - 0.63								0.3	38.4	59.4	1.9					
Phosphoglycolipid	Flavomycin	*Enterococcus faecalis*	96	80.2	70.83 - 87.64							15.6	1.0		2.1	1.0	2.1	78.1			
		Enterococcus faecium	120	88.3	81.20 - 93.47							11.7					1.7	86.7			
		Enterococcus spp	584	84.8	81.58 - 87.58							11.8	1.0	0.7	1.0	0.7	2.1	82.7			
Streptogramins	Quinupristin / dalfopristin*	*Enterococcus faecalis*	NA	NA	NA																
		Enterococcus faecium	120	41.7	32.74 - 51.02						5.0	1.7	51.7	30.0	6.7	5.0					
		Enterococcus spp	584	45.2	41.12 - 49.34						11.8	6.2	36.8	35.1	7.2	2.9					
	Virginiamycin*	*Enterococcus faecalis*	NA	NA	NA																
		Enterococcus faecium	120	0.0	0.00 - 3.03							85.0	5.8	9.2							
		Enterococcus spp	584	0.5	0.11 - 1.49							86.6	6.8	6.0	0.2	0.3					
Tetracycline	Tetracycline	*Enterococcus faecalis*	96	7.3	2.98 - 14.45								86.5	2.1	4.2	3.1	4.2				
		Enterococcus faecium	120	11.7	6.53 - 18.80								78.3	8.3	1.7	1.7	10.0				
		Enterococcus spp	584	10.8	8.39 - 13.59								83.2	3.9	2.1	1.9	8.9				

* *Enterococcus faecalis* isolates are intrinsically resistant to streptogramins

Solid vertical lines indicate breakpoints for resistance. The white fields indicate the dilution range tested for each antimicrobial. Values in the shaded area indicate MIC values greater than the highest concentration tested.

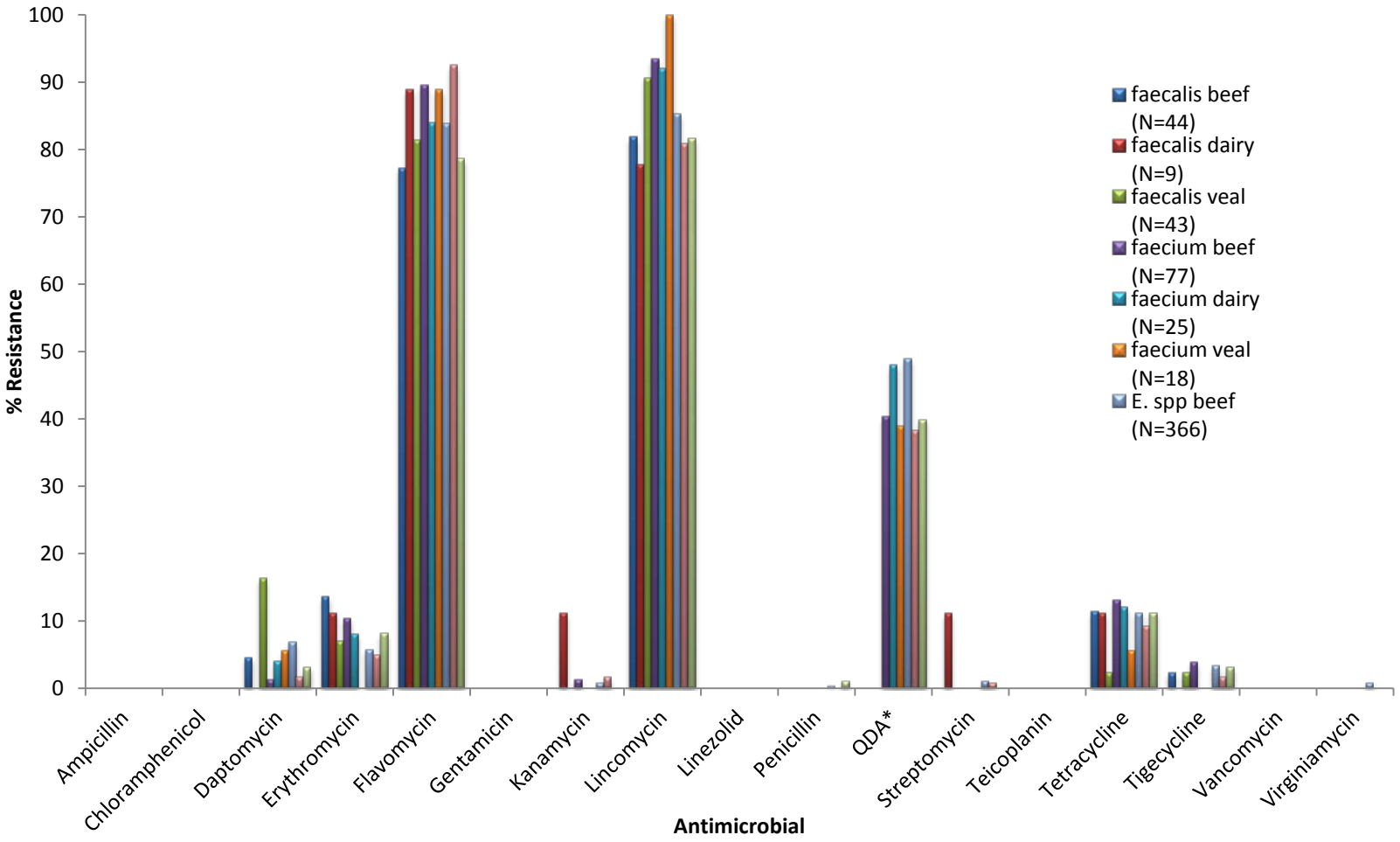

Figure 4. Prevalence of AMR in *Enterococcus* isolates from beef cattle, dairy cattle and veal calf faecal samples

* QDA – quinupristin/dalfopristin

Table 11. Prevalence of AMR in *Enterococcus* isolates from grass-fed and grain-fed beef cattle

Antimicrobial	*Enterococcus faecalis*		*Enterococcus faecium*		*Enterococcus spp*	
	Grass N=27	Grain N=14	Grass N=62	Grain N=12	Grass N=212	Grain N=139
Ampicillin	0.00	0.00	0.00	0.00	0.00	0.00
Chloramphenicol	0.00	0.00	0.00	0.00	0.00	0.00
Daptomycin	7.41	0.00	0.00	0.00	9.91	28.78
Erythromycin	11.11	21.43	12.90	0.00	6.60	5.04
Flavomycin	77.78	85.71	91.94	83.33	83.49	83.45
Gentamicin	0.00	0.00	0.00	0.00	0.00	0.00
Kanamycin	0.00	0.00	1.61	0.00	0.94	0.72
Lincomycin	74.07	92.86	93.55	91.67	88.21	84.17
Linezolid	0.00	0.00	0.00	0.00	0.00	0.00
Penicillin	0.00	0.00	0.00	0.00	0.00	0.00
Quinupristin / dalfopristin	NA	NA	40.32	41.67	52.36	44.60
Streptomycin	0.00	0.00	0.00	0.00	0.94	1.44
Teicoplanin	0.00	0.00	0.00	0.00	0.00	0.00
Tetracycline	7.41	21.43	14.52	0.00	12.74	10.07
Tigecycline	3.70	0.00	4.84	0.00	3.77	2.88
Vancomycin	0.00	0.00	0.00	0.00	0.00	0.00
Virginiamycin	NA	NA	0.00	0.00	0.94	0.72

Table 12. Antibiograms of *Enterococcus* isolates from beef cattle, dairy cattle and veal calf faecal samples

Antibiograms*	***E. faecium* (N=120)**	***E. faecalis* (N=96)**	***Enterococcus spp.* (N=584)**
ALL SENSITIVE	1	1	12
FLV	5	13	69
LIN		16	14
TET			1
TGC			1
DAP FLV			4
ERY LIN			1
FLV LIN	53	44	165
FLV PEN			1
FLV QDA			1
FLV TET	1		3
FLV TGC			2
LIN QDA	8		46
LIN TET	1		4
LIN TGC		1	
DAP FLV KAN			1
DAP FLV LIN	2	8	10
DAP LIN QDA	1		
ERY FLV LIN	1	5	6
ERY LIN TET		1	2
FLV KAN LIN			1
FLV LIN QDA	29		159
FLV LIN TET	5	2	12
FLV LIN TGC		1	4
LIN QDA TET	1		3
LIN QDA TGC	2		
DAP FLV LIN QDA			8
DAP FLV LIN TET			2
ERY FLV LIN QDA	2		5
ERY FLV LIN TET		2	4
ERY FLV LIN TGC	1		
FLV LIN QDA TET	1		8
FLV LIN QDA TGC			9
FLV LIN QDA VIR			2
KAN LIN STR TET			1
LIN STR QDA TET			2
LIN QDA TET TGC			1
DAP ERY FLV LIN TET		1	
DAP FLV LIN QDA TET			3
ERY FLV KAN LIN QDA	1		
ERY FLV LIN QDA TET	5		12
DAP ERY FLV LIN QDA TET			2

ERY FLV KAN LIN STR TET	1	
ERY FLV LIN QDA TET VIR		1
ERY KAN LIN STR QDA TET		1
ERY FLV KAN LIN PEN STR QDA TET		1

* FLV – flavomycin, LIN – lincomycin, TET – tetracycline, TGC – tigecycline, DAP – daptomycin, ERY – erythromycin, PEN – penicillin, QDA – quinupristin/dalfopristin, KAN – kanamycin, VIR – virginiamycin, STR - streptomycin

Comparison of two methods for the isolation of Shiga toxin producing *Escherichia coli* O157 from cattle faeces at slaughter.

Running head:

Comparison of *E. coli* O157 isolation methodologies

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Keywords: *Escherichia coli*, O157, isolation, method, comparison

Abstract

Accurate determination of food-borne pathogen prevalence estimates in animals is largely dependent on the performance of detection and isolation procedures. In this study, we compare two methods for isolating *E. coli* O157 from 1500 enriched cattle faecal samples. In the first method (Method A), detection and isolation of *E. coli* O157 was assessed by broth enrichment in MP media for 18-20 h followed by PCR screening and immunomagentic separation (IMS). Results were compared to broth enrichment in BPW for 6 h followed by IMS (Method B). For each method, cefixime-tellurite sorbitol MacConkey agar was used as the primary culture media, though IMS beads from MP enrichments were plated onto three additional media, including modified Rainbow agar. A total of 96 isolates were obtained from 1500 samples, of which 37 (2.5%) were recovered using Method A and 90 (6.0%) were recovered using Method B. Overall, 6 (6.3%) were exclusively isolated using Method A, 31 (32.3%) were isolated by both methods and 59 (61.5%) were solely isolated by Method B. Further analysis of positive broths revealed that Method B outperformed Method A when samples had low *E.* coli O157 counts (< 0.3 log MPN/g faeces). Many of the samples that yielded an isolate using Method B did not proceed to confirmation in Method A, as the genetic marker for O157 was not detected in the screening PCR. The results of this work would suggest that Method B should be used in preference to Method A when estimating the prevalence of *E. coli* O157 in cattle faeces.

Introduction

E. coli O157 is a much publicised foodborne pathogen that has garnered significant interest from industry, regulators and public health over the past two decades. Considerable efforts have been made to understand the epidemiology of these organisms, particularly with respect to beef cattle which are widely accepted as the primary reservoir for *E. coli* O157. As a result of these efforts, numerous technologies have been developed to detect and isolate *E. coli* O157 from various matrices including cattle faeces, soil, water and food products destined for human consumption such as ground beef, fresh produce and dairy products (Chapman 2000; Vimont, Vernozy-Rozand et al. 2006).

Traditionally, direct plating methods of isolation have been used to obtain prevalence estimates of bacteria in samples. In the early 1990's, the advent of automated immunomagentic separation (IMS) technologies for *E. coli* O157 isolation enabled targeted concentration of isolates prior to plating (Fratamico, Schultz et al. 1992) and is now a standard component of most isolation procedures. While IMS has repeatedly been shown to outperform direct plating methods of isolation, particularly in samples with low bacterial counts (Chapman, Wright et al. 1994; Chapman and Siddons 1996), IMS is an expensive, laborious process to perform in large surveys or for routine testing by industry or diagnostic laboratories. Attempts to streamline detection and isolation methods have led to the development of reliable real-time PCR methodologies to pre-screen samples for *E. coli* O157 prior to isolation via IMS (Perelle, Dilasser et al. 2007). This two stage approach has enormous appeal to industry and the broader scientific community as it greatly reduces the number of samples that require confirmation. However, the effectiveness of both PCR and IMS can vary between matrices and target organisms and isolation of bacterial targets using these methods is not always guaranteed.

While real-time PCR detection followed by IMS is an industry accepted method for detecting and isolating *E. coli* O157 from ground beef samples little is known about its effectiveness in more complex matrices such as cattle faeces. In this study we investigate two methods for isolating *E. coli* O157 from enriched cattle faecal samples. The first method (Method A) involved enriching samples for 18 – 20 h and screening for *E. coli* O157 targets (*stx*, *eae* and O serotype) using real-time PCR prior to isolation via IMS. For Method A, IMS beads were plated onto three additional media. In contrast, the second method (Method B) was based on a short enrichment (6 h) followed by IMS without an initial screening test (Fegan, Vanderlinde et al. 2004).

Materials and methods

Sample collection

Samples were collected from Australian beef cattle, dairy cattle, and veal calves with a collection total of 910, 290 and 300 samples respectively for the three animal groups. Faecal samples were collected at slaughter, post-evisceration, across two sampling windows following the method detailed in the pSTEC report.

Method A

Sample enrichments

Faecal slurries were prepared according to the method described in the pSTEC report. Briefly, 20 g of faeces was diluted 10^{-1} in MP Media (DuPont Qualicon, UK) and enriched at 41 ± 1°C for 18 - 20 h without agitation.

PCR detection of *E. coli* O157

Faecal samples that were enriched in MP media were tested for the presence of *stx*, *eae* and *E. coli* O157 serogroup markers using the BAX System PCR Assay for *E. coli* O157:H7 MP (DuPont Qualicon, UK). Boiled cell lysates were prepared for use as templates in BAX PCR Assays according to the method described previously (pSTEC report). Following this, assays were performed as per the manufacturer's recommendations.

Isolation and confirmation of PCR positive *E. coli* O157 samples

This study formed part of a previous study in which Poly-GDS Top 7 IMS beads were used to target seven serogroups, one of which was *E. coli* O157. Samples that tested positive for *stx*, *eae* and *E. coli* O157 were deemed to be potentially positive and subjected to the following isolation procedure. IMS was performed using Assurance GDS Poly IMS – Top 7 STEC (BioControl, USA) and an automated bead retriever (Life Technologies, Australia). The resulting bead-bacteria complexes were plated onto Rainbow Agar O157 (Biolog, USA) supplemented with 5.0 mg/L sodium novobiocin, 0.05 mg/L cefixime trihydrate and 0.15 mg/L potassium tellurite, cefixime-tellurite sorbitol MacConkey agar (CT-SMAC; Oxoid, UK), USMARC chromogenic agar medium (Kalchayanand, Arthur et al. 2013) and washed sheep blood supplemented with 0·5 µg ml^{-1} mitomycin C (WBAM) (Sugiyama, Inoue et al. 2001) . All agar plates were incubated at 37 ± 2°C for 20-24 h. Resultant colonies were picked and confirmed according to the method described in the pSTEC report.

Method B

Sample enrichments

Faecal enrichments were prepared according to Fegan et al. (Fegan, Vanderlinde et al. 2004). Briefly, 10 g of faeces was diluted 10^{-1} in buffered peptone water (BPW; Oxoid, UK) and subsequently incubated at 42 ± 1°C for 6 h.

Isolation of *E. coli* O157 from BPW

Attempts were made to isolate *E. coli* O157 from all samples using the method described by Fegan et al. (Fegan, Vanderlinde et al. 2004). IMS was performed using anti-*E. coli* O157 Dynabeads (Life Technologies, Australia) and an automated bead retriever (Life Technologies, Australia) and the resulting O157 bead-bacteria complexes were plated onto CT-SMAC only. CT-SMAC plates were incubated at 37 ± 2°C for 20-24 h and non sorbitol-fermenting colonies were serotyped using latex agglutination kits (Oxoid, UK). Resultant colonies were picked and confirmed according to the method described in the pSTEC report.

Enumeration of E. coli *O157 and generic* E. coli *isolates*

Attempts were made to enumerate *E. coli* O157 and generic *E. coli* from positive samples using the method described in the pSTEC report.

Statistical analysis

Statistical analyses were performed in Stata version 12.1 using a 2x2 contingency table and Fisher's exact test (StataCorp U.S.). *P* values were two-tailed and groups were considered significantly different if *P* values were <0.05. Group comparisons were performed using a one-way ANOVA with Bonferroni correction to *P* values to account for multiple comparisons.

Results and discussion

In total, 96 *E. coli* O157 isolates were obtained from 1500 samples. *E. coli* O157 were isolated from 37 (2.5%) samples using Method A and 90 (6.0%) samples using Method B. The number of samples yielding *E. coli* O157 was significantly higher for Method B than Method A ($P < 0.05$). Of the 96 positive samples, 6 (6.3%) yielded isolates by Method A alone, 31 (32.3%) yielded isolates by both Methods A and B and 59 (61.5%) isolates were recovered from Method B alone (Fig. 1).

For Method A, 157 MP enrichments (10.5%) tested PCR screen positive for *stx*, *eae* and O157 markers (potentially positive for *E. coli* O157) and proceeded to confirmation, with an *E. coli* O157

isolate obtained from 37 (23.6%) of these samples. Method B also recovered *E. coli* O157 from 31 (19.7%) of the same 37 samples that yielded an isolate by Method A. This difference in isolation (23.6% for Method A vs 19.7% for Method B) is not significant ($P > 0.05$) and suggests that the use of different IMS beads (GDS in Method A vs Dynal in Method B) in each of the methods did not significantly influence the recovery of isolates from potentially positive samples. The isolation rate of *E. coli* O157 from MP enrichments that were PCR screen positive (23.6%) was lower than previous studies that report conversion rates ranging from 40 to 100% (Barlow and Mellor 2010; Hofer, Stephan et al. 2012; Bibbal, Loukiadis et al. 2014). While those studies also employed a real-time PCR screening step prior to confirmation by IMS, comparison of isolation rates with our study are confounded by the use of different sample preparation and screening methodologies.

Further comparisons between the methods revealed significant differences in the total *E. coli* O157 counts for samples that tested positive by each of the methods. *E. coli* O157 counts ranged from <-0.52 $\log_{10}$ MPN/g faeces to 4.04, 4.38 and 6.89 $\log_{10}$ MPN/g faeces for samples that yielded isolates using Methods A, both A and B, and Method B, respectively. The mean counts in samples that tested positive by Method A either alone (3.53 $\log_{10}$ MPN/g faeces) or in combination with Method B (3.36 $\log_{10}$ MPN/g faeces) were significantly higher ($P < 0.05$) than counts in samples that tested positive for Method B alone (0.57 $\log_{10}$ MPN/g faeces) (Fig. 2). Despite this, Methods A and B were individually shown to harbor generic *E. coli* at a mean of 5.8 $\log_{10}$ MPN/g faeces, while samples testing positive for both methods had a mean count of 6.0 $\log_{10}$ MPN/g faeces. Since no significant difference was observed in the mean generic *E. coli* counts in samples that tested positive by each method ($P > 0.05$) it is unlikely that background flora has impeded isolation of *E. coli* O157 in either method. Low *E. coli* O157 counts (<-0.52 $\log_{10}$ MPN/g faeces) were detected in a total of 39 positive samples, of which 37 yielded an isolate using Method B compared with just 2 samples using Method A. These findings suggest that Method B was a superior method for isolating *E. coli* O157 from samples with counts below the MPN limit of detection.

Further investigation of PCR screening results revealed significant differences in the detection of virulence genes and serogroup markers. Of the 59 samples that were positive by Method B alone, 65% tested positive for *stx* and *eae* while only 1.7% were positive for the O157 marker using the PCR screen employed in Method A. Virulence markers *stx* and *eae* may have been present in non-O157 *E. coli* or other bacterial species that were present in MP enrichments (Schmidt, Montag et al. 1993; Paton and Paton 1996; Gyles 2007). In addition, the low prevalence of the O157 serogroup marker may indicate that growth of *E. coli* O157 was not supported appropriately in MP enrichments, *E. coli*

O157 failed to exceed the limit of detection for the PCR screen or inhibition of the PCR occurred. Differences in enrichment media (BPW vs MP) and incubation times (6 h vs 18-20 h) between Method A and B are possible factors contributing to the variable isolation rates of *E. coli* O157. While there is some evidence that higher temperatures may increase the ratio of *E. coli* O157 to background flora (LeJeune, Besser et al. 2001) it is less likely that temperature differences impacted on isolate recovery in the current study as incubation temperatures of both enrichment broths (42°C vs 41°C) were consistently high.

Alternative sample types may pose unique challenges to detection methods that employ RT-PCR methods and the disparity in isolation rates between the methods examined here may be related to the performance of RT-PCR screening in the different sample types tested (beef, dairy or veal). To investigate this, we examined the distribution of animal types in positive samples from each of the two methods. Of the 37 *E. coli* O157 isolated using Method A, 20 (54.1%) were associated with beef cattle, 4 (10.8%) were associated with dairy cattle and 13 (35.1%) were associated with veal calves. Similarly, of the 90 samples yielding an isolate using Method B, 50 (55.6%) were isolated from beef cattle, 14 (15.6%) were isolated from dairy cattle and 26 (28.9%) were isolated from veal calves. Isolation of *E. coli* O157 from each of the different animal types was not significantly different between methods ($P > 0.05$) suggesting that sample type was not responsible for the lower detection and isolation observed in Method A.

Accurate assessment of the microbiological composition of a sample is largely dependent on the performance of methods employed for detection and isolation. Here we demonstrate that Method B (enrichment of cattle faeces in BPW for 6 h followed by IMS) yielded significantly more *E. coli* O157 than Method A (enrichment in MP for 18 – 20 h followed by PCR screening and IMS isolation of PCR screen positive samples). While it is possible that PCR inhibition in faecal enrichments may have been responsible for the lower prevalence estimates in MP enrichments it is equally possible that enrichment conditions (media and incubation times) favoured growth of *E. coli* O157 in BPW over MP broth. Further investigation into the growth properties of MP and BPW enrichments and possible inhibitory effects associated with using PCR screening tests in faeces, particularly with respect to O serogroup markers, should be conducted. The results of this work would suggest that when estimating the prevalence of *E. coli* O157 in cattle faeces, Method B should be used in preference to Method A.

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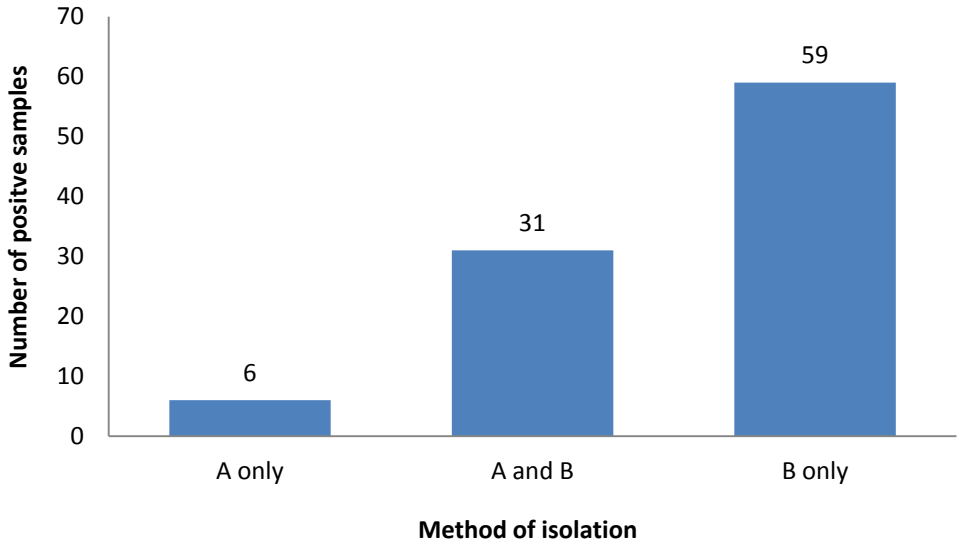

Figure 1. Number of samples that yielded *E. coli* O157 from Methods A and B alone or in combination.

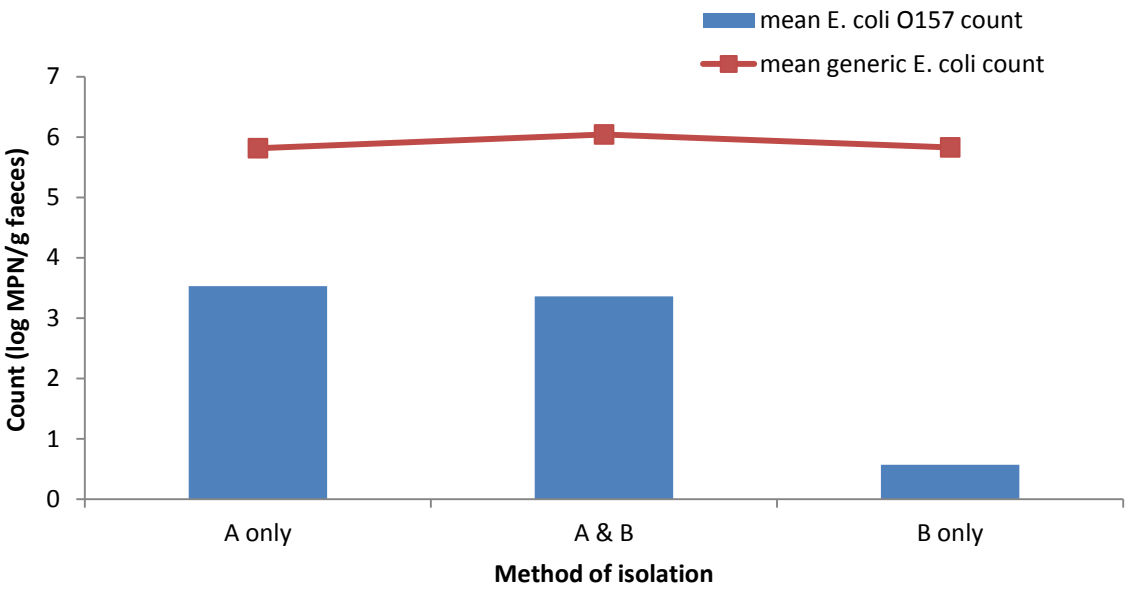

Figure 2. Mean count of *E. coli* O157 and generic *E. coli* in samples that were recovered from Methods A and/or B.

Appendix 2: Comparison of Australian animal isolates AMR surveys

Background

The present study into the AMR status of *E. coli, Salmonella* and *Enterococcus* isolates from Australian cattle populations was designed so that comparisons with recently completed surveys of AMR in Australian cattle populations were possible. The results of this study are to be compared with the *E. coli* and *Enterococcus* AMR results from the pilot surveillance program for antimicrobial resistance in bacteria of animal origin[2] and the *Salmonella* AMR results from a retrospective analysis of sequential *S. enterica* isolates from confirmed cases of salmonellosis in livestock[1]. Comparing and contrasting AMR data from different studies can be problematic if key parameters are not well understood. The key parameters for the surveys in question are described below.

Survey Design

Pilot surveillance program: Antimicrobial resistance in bacteria of animal origin (DAFF)

- Surveillance duration: November 2003 to July 2004
- Sample number: 204 cattle faecal specimens collected from 11 export abattoirs
- Sample independence: No two specimens were obtained from animals belonging to the same processing lot
- Animal classes: Dairy cattle (n=65), grass-fed cattle (n=69) and feedlot cattle (n=70)
- Bacterial targets: *E. coli* (n=194) and *Enterococcus* (n=140)
- AMR method: *E. coli* – broth microdilution technique; *Enterococcus* – agar dilution procedure
- Antimicrobials tested:
    - *E. coli* – ampicillin, chloramphenicol, florfenicol, ceftiofur, cefotaxime, ciprofloxacin, gentamicin, nalidixic acid, trimethoprim/sulfamethoxazole and tetracycline
    - *Enterococcus* – ampicillin, erythromycin, gentamicin, teicoplanin, vancomycin and virginiamycin

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1. Abraham, S. et al. (2014). International Journal of Antimicrobial Agents **43**(2): 126-130.
2. DAFF (2007). Australian Government Department of Agriculture, Fisheries and Forestry. Retrospective analysis of sequential *S. enterica* isolates from confirmed cases of salmonellosis in livestock (NSW DPI).

- Surveillance duration: 2007 to 2011
- Sample number: 106 *Salmonella*
- Sample independence: isolates were recovered from sequential unrelated salmonellosis cases in livestock
- Animal classes: Dairy cattle (n=85) and beef cattle (n=21)
- Bacterial targets: *Salmonella*
- AMR method: Disc diffusion assay
- Antimicrobials tested: ampicillin, amoxicillin/clavulanic acid, ticarcillin/clavulanic acid, cefalexin, cefoxitin, cefotaxime, cefepime, nalidixic acid, ciprofloxacin, imipenem, sulfafurazole, trimethoprim, tetracycline, apramycin, neomycin, gentamicin, azithromycin and chloramphenicol.

AMR status of Australian cattle (CSIRO/MLA)

- Surveillance duration: Two sampling windows – February to March 2013 and August to September 2013
- Sample number: 800 *E. coli*, 800 *Enterococcus* and 218 *Salmonella* collected from faeces from 31 export abattoirs
- Sample independence: Multiple isolates collected from individual lots. A maximum of 47 isolates per abattoir and sampling window may have been included in the analysis.
- Animal classes:
    - *E. coli* – beef cattle (n=469), dairy cattle (n=155) and veal calves (n=176)
    - *Enterococcus* – beef cattle (n=487), dairy cattle (n=154) and veal calves (n=159)
    - *Salmonella* - cattle (n=106), dairy cattle (n=76) and veal calves (n=36)
- Bacterial targets: *E. coli, Enterococcus* and *Salmonella*
- AMR method: broth microdilution (Sensititre)
- Antimicrobials tested:
    - *E. coli* and *Salmonella* – amoxicillin/clavulanic acid, ampicillin, cefazolin, cefotaxime, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, florfenicol, gentamicin, kanamycin, meropenem, nalidixic acid, streptomycin, tetracycline and trimethoprim/sulfamethoxazole
    - *Enterococcus* – ampicillin, chloramphenicol, daptomycin, erythromycin, flavomycin, gentamicin, kanamycin, lincomycin, linezolid, penicillin, quinupristin/dalfopristin, streptomycin, teicoplanin, tetracycline, vancomycin, virginiamycin

Results

E. coli

All antimicrobials included in the DAFF survey were used in the CSIRO/MLA study. The CSIRO/MLA study also included cefazolin, cefoxitin, ceftriaxone, kanamycin, meropenem and streptomycin. The prevalence of resistance to all antimicrobials tested is shown in Table 2A. *E. coli* isolates from both surveys were generally susceptible to all antimicrobials tested with >90% of all isolates demonstrating no resistance to the tested antimicrobials regardless of source. Resistance to clinically relevant antimicrobials such as cefotaxime, cefoxitin, ceftriaxone, ciprofloxacin, meropenem and nalidixic acid was not observed.

Resistance to any one antimicrobial occurred in no more than 7.7% of isolates from any group in either survey. The most frequently observed resistance was to tetracycline with feedlot cattle from the DAFF survey (7.6%) and beef cattle from the CSIRO/MLA survey (7.7%) most likely to harbor this resistance. Further analysis of the beef cattle group in the CSIRO/MLA study determined that grain-fed beef cattle were significantly more likely to harbor tetracycline resistance than any other animal group. This trend is consistent with the DAFF survey. Resistance to ampicillin, amoxicillin/clavulanic acid, ceftiofur and trimethoprim/sulfamethoxazole were only present in small numbers of *E. coli* isolated during the CSIRO/MLA survey. However, resistance to any of these four antimicrobials did not exceed 4.5% for any animal group and was not significantly different to the DAFF survey. Low levels of resistance (<4.0%) to streptomycin and cefazolin were observed in isolates from the CSIRO/MLA study but were not included in the DAFF survey therefore comment relating to trends in AMR development are not possible.

Enterococcus

All antimicrobials included in the DAFF survey were used in the CSIRO/MLA survey. The CSIRO/MLA study also included chloramphenicol, daptomycin, flavomycin, kanamycin, lincomycin, linezolid, penicillin, quinupristin/dalfopristin, streptomycin, tetracycline and tigecycline. Resistance to macrolide, lincosamides and streptogramin (MLS) antimicrobials was a prominent feature of *Enterococcus* isolates from the CSIRO/MLA survey. Only one macrolide (erythromycin) and one streptogramin (virginiamycin) was used in both the DAFF and CSIRO/MLA survey. Comparisons of the prevalence to erythromycin and virginiamycin determined that there were no significant differences between the prevalence of resistance in the DAFF survey to that observed in the CSIRO/MLA survey regardless of enterococcal species or source. Resistance to ampicillin, gentamicin, teicoplanin and vancomycin was not observed in either survey and when combined with the absence of linezolid

resistance in isolates from the CSIRO/MLA it suggests that *Enterococcus* isolates from cattle do not harbor resistances that pose a risk to the treatment of uncomplicated or complicated Enterococcal infections.

Salmonella

A total of eight antimicrobials were used in both the NSW DPI and CSIRO/MLA survey. These include amoxicillin/clavulanic acid, ampicillin, cefoxitin, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid and tetracycline. An additional 17 antimicrobials were included across both surveys with the NSW DPI using an additional nine antimicrobials and the CSIRO/MLA eight. The differences relate to the specific selection of antimicrobials within classes as opposed to the absence of an antimicrobial class from either survey. For example, carbepenem resistance was evaluated in both surveys with imipenem used in the NSW DPI study and meropenem used in the CSIRO/MLA study. Similarly, the NSW DPI survey selected cefalexin and cefepime as part of their suite of cephems whereas the CSIRO/MLA study had cefazolin, ceftiofur and ceftriaxone as unique cephem antimicrobials.

Ampicillin resistance in beef cattle isolates from the NSW DPI survey was significantly higher than resistance in any animal group from the CSIRO/MLA survey. Similarly, resistance to ampicillin in dairy cattle in the NSW DPI survey was significantly higher than ampicillin resistance in isolates from dairy cattle and veal calves in the CSIRO/MLA survey. It is known that all NSW DPI isolates were recovered from infected cattle. It is possible to suggest that the elevated ampicillin resistance may be due to the antimicrobial therapy that the animal may have received prior to isolation of *Salmonella.* A similar trend, albeit not statistically significant was observed with tetracycline resistance, with resistance to tetracycline elevated in isolates from the NSW DPI survey in comparison to isolates from the CSIRO/MLA. It should be noted that co-resistance to ampicillin and tetracycline was a prominent feature of isolates from both surveys. Additionally, isolates resistant to ampicillin and tetracycline were routinely resistant to trimethorprim and sulfafurazole (NSW DPI survey) or trimethoprim/sulfamethoxazole (CSIRO/MLA survey). Molecular investigations into the *Salmonella* isolates from the NSW DPI determined that class 1 integron carriage may be a reason for the co-resistance observed[1]. It is plausible to suggest that similar mechanisms are present in the beef cattle isolates from the CSIRO/MLA survey. However, they are not widespread which suggests selective pressure via the indiscriminate use of antimicrobials is low.

Resistance to fluoroquinolones and cephems was very low or absent from all NSW DPI *Salmonella* isolates as well as *Salmonella* from beef cattle or veal calves in the CSIRO MLA survey. In contrast, resistance to the cephems and fluoroquinolones, particularly ceftiofur, ceftriaxone and nalidixic acid was elevated in *Salmonella* collected from dairy cattle as part of the CSIRO/MLA survey. Caution is required when interpreting these results as the majority of isolates contributing to the elevated resistances are related by time of collection, place of collection and source of animal. For example, 13 of the 13 ceftiofur resistant *Salmonella* from dairy cattle were collected from a single abattoir over a 97 minute period. The same isolates were also routinely resistant to cefazolin, cefotaxime, ceftriaxone and nalidixic acid. These data confirm that the AMR prevalences for *Salmonella* collected from dairy cattle as part of the CSIRO/MLA survey are heavily influenced by the clustering of related isolates. Nonetheless, it remains a finding of the CSIRO/MLA survey that ceftiofur resistant *Salmonella* were isolated on three independent occasions during the CSIRO/MLA survey. The association of ceftiofur resistance with resistance to clinically significant antimicrobials in human medicine demands that the prudent use of antimicrobials by Australia producers is required.

Conclusion

The comparison of recent surveys aimed at determining the AMR status of *E. coli*, *Enterococcus* and *Salmonella* from Australian cattle populations indicates that, in general, resistance to clinically relevant antimicrobials remains low or absent regardless of animal production type, source or bacteria of concern. Nevertheless, there is evidence to suggest that the industry may benefit from further surveillance of particular bacteria-antimicrobial combinations that weren't adequately analysed in the comparisons above. These would include, but are not limited to ceftiofur and *Salmonella* as well as quinupristin/dalfopristin, daptomycin and tigecycline in *Enterococcus*.

Table 2A. Prevalence of AMR in *E. coli* from surveys of Australian cattle

Class	Antimicrobial	DAFF Dairy n=60	DAFF Grass-fed n=68	DAFF Feedlot n=66	CSIRO/MLA Dairy cattle n=155	CSIRO/MLA Grass-fed n=280	CSIRO/MLA Grain-fed n=173	CSIRO/MLA Veal calves n =176
	All susceptible (DAFF survey)	60 (100.0%)	66 (97.1%)	60 (90.9%)	NA	NA	NA	NA
	All susceptible (CSIRO/MLA survey)	NA	NA	NA	150 (96.8%)	270 (96.4%)	146 (84.4%)	164 (93.2%)
Aminoglycosides	Gentamicin	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.6%)
	Kanamycin				0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (1.1%)
	Streptomycin				3 (1.9%)	0 (0.0%)	5 (2.9%)	7 (4.0%)
b-lactam/b-lactamase inhibitor combinations	Amoxicillin/clavulanic acid	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (1.1%)
Carbapenem	Meropenem				0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Cephems	Cefazolin				0 (0.0%)	0 (0.0%)	1 (0.6)	3 (1.7%)
	Cefotaxime	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	Cefoxitin				0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	Ceftiofur	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (0.6%)
	Ceftriaxone				0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Folate pathway inhibitor	Trimethoprim / sulfamethoxazole	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (1.3%)	1 (0.4%)	0 (0.0%)	4 (2.3%)
Penicillins	Ampicillin	0 (0.0%)	0 (0.0%)	0 (0.0%)	4 (2.6%)	0 (0.0%)	0 (0.0%)	8 (4.5%)
Phenicols	Chloramphenicol	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	Florfenicol	0 (0.0%)	1 (1.5%)	1 (1.5%)	NA	NA	NA	NA
Quinolones	Ciprofloxacin	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
	Nalidixic acid	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Tetracycline	Tetracycline	0 (0.0%)	1 (1.5%)	5 (7.6%)	4 (2.6%)	10 (3.6%)	26 (15.0%)	8 (4.5%)

DAFF = isolates collected as part of the 2003-2004 DAFF coordinated animal isolates survey
CSIRO = isolates collected as part of the 2013 CSIRO coordinated cattle population survey
Shaded areas indicate antimicrobials that were not included in a particular survey

Table 2B. Prevalence of AMR in *Enterococcus* from surveys of Australian cattle

Class	Antimicrobial	DAFF	DAFF	DAFF	CSIRO/MLA	CSIRO/MLA	CSIRO/MLA
		***E. faecium* n=21**	***E. faecalis* n=17**	***Enterococcus* spp. n=102**	***E. faecium* n=120**	***E. faecalis* n=96**	***Enterococcus* spp. n=584**
	All susceptible	19 (90.5%)	17 (100.0%)	99 (97.1%)	1 (0.8%)	1 (1.0%)	12 (2.1%)
Aminoglycosides	Gentamicin	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Kanamycin				1 (0.8%)	1 (1.0%)	5 (0.9%)
	Streptomycin				0 (0%)	1 (1.0%)	5 (0.9%)
Glycopeptides	Teicoplanin	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Vancomycin	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Glycylcycline	Tigecycline				3 (2.5%)	2 (2.1%)	17 (2.9%)
Lincosamide	Lincomycin				113 (94.2%)	82 (85.4%)	489 (83.7%)
Lipopeptide	Daptomycin				3 (2.5%)	9 (9.4%)	30 (5.1%)
Macrolide	Erythromycin	2 (9.5%)	0 (0%)	2 (2.0%)	10 (8.3%)	10 (10.4%)	35 (6.0%)
Oxazolidinones	Linezolid				0 (0%)	0 (0%)	0 (0%)
Penicillins	Ampicillin	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Penicillin				0 (0%)	0 (0%)	2 (0.3%)
Phenicol	Chloramphenicol				0 (0%)	0 (0%)	0 (0%)
Phosphoglycolipid	Flavomycin				106 (88.3%)	77 (80.2%)	495 (84.8%)
Streptogramins	Quinupristin / dalfopristin				50 (41.7%)	NA	264 (45.2%)
	Virginiamcyin	2 (9.5%)	NA	3 (2.9%)	0 (0%)	NA	3 (0.5%)
Tetracycline	Tetracycline				14 (11.7%)	7 (7.3%)	63 (10.8%)

DAFF = isolates collected as part of the 2003-2004 DAFF coordinated animal isolates survey
CSIRO = isolates collected as part of the 2013 CSIRO coordinated cattle population survey
Shaded areas indicate antimicrobials that were not included in a particular survey

Table 2C. Prevalence of AMR in *Salmonella* from surveys of Australian cattle

Class	Antimicrobial	NSW DPI Dairy cattle n=85	NSW DPI Beef cattle n=21	CSIRO/MLA Dairy cattle n=76	CSIRO/MLA Grass-fed n=74	CSIRO/MLA Grain-fed n=29	CSIRO/MLA Veal calves n =36
	All susceptible (DAFF survey)	59 (69%)	14 (66.7%)	NA	NA	NA	NA
	All susceptible (CSIRO/MLA survey)	NA	NA	61 (80.3%)	67 (90.5%)	23 (79.3%)	36 (100.0%)
Aminoglycosides	Apramycin	1 (1.2%)	1 (4.8%)				
	Gentamicin	0 (0%)	1 (4.8%)	1 (1.3%)	0 (0%)	0 (0%)	0 (0%)
	Kanamycin			3 (3.9%)	0 (0%)	0 (0%)	0 (0%)
	Neomycin	4 (4.7%)	1 (4.8%)				
	Streptomycin			7 (9.2%)	3 (4.1%)	6 (20.7%)	0 (0%)
b-lactam/b-lactamase inhibitor combinations	Amoxicillin/clavulanic acid	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Ticarcillin/clavulanic acid	0 (0%)	1 (4.8%)				
Carbapenems	Imipenem	0 (0%)	0 (0%)				
	Meropenem			0 (0%)	0 (0%)	0 (0%)	0 (0%)
Cephems	Cefalexin	0 (0%)	0 (0%)				
	Cefazolin			14 (18.4%)	2 (2.7%)	0 (0%)	0 (0%)
	Cefepime	0 (0%)	0 (0%)				
	Cefotaxime			12 (15.8%)	1 (1.4%)	0 (0%)	0 (0%)
	Cefoxitin	0 (0%)	0 (0%)	4 (5.3%)	0 (0%)	0 (0%)	0 (0%)
	Ceftiofur			13 (17.1%)	2 (2.7%)	0 (0%)	0 (0%)
	Ceftriaxone			11 (14.5%)	0 (0%)	0 (0%)	0 (0%)
Folate pathway inhibitors	Trimethoprim	6 (7.1%)	2 (9.5%)				
	Trimethoprim / sulfamethoxazole			0 (0%)	1 (1.4%)	7 (24.1%)	0 (0%)
Macrolides	Azithromycin	0 (0%)	0 (0%)				
Penicillins	Ampicillin	10 (11.8%)	6 (28.6%)	0 (0%)	1 (1.4%)	7 (24.1%)	0 (0%)
Phenicols	Chloramphenicol	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Quinolones	Ciprofloxacin	0 (0%)	0 (0%)	2 (2.6%)	0 (0%)	0 (0%)	0 (0%)
	Nalidixic acid	0 (0%)	0 (0%)	12 (15.8%)	1 (1.4%)	0 (0%)	0 (0%)
Sulfonamide	Sulfafurazole	25 (29.4%)	5 (23.8%)				
Tetracycline	Tetracycline	8 (9.4%)	4 (19.0%)	0 (0%)	0 (0%)	7 (24.1%)	0 (0%)

NSW DPI = isolates collected from confirmed cases of salmonellosis in livestock between 2007 and 2011
CSIRO = isolates collected as part of the 2013 CSIRO coordinated cattle population survey
Shaded areas indicate antimicrobials that were not included in a particular survey
Salmonella isolates from dairy cattle with resistance to cefazolin, ceftiofur, ceftriaxone and nalidixic acid were isolated from a single abattoir during one sampling day.

Appendix 3. Assessment of key parameters included in the design of a pSTEC survey of Australian cattle populations.

The studies detailed in this report are based on the collection of 1500 faecal samples from 31 beef export registered abattoirs across two sampling periods scheduled to coincide with warmer months (Window 1) and cooler months (Window 2). Three animal groups; beef cattle, dairy cattle and veal calves were selected as the base animal groupings and a sampling strategy was developed and implemented successfully. Nonetheless there is opportunity to review the key parameters of the survey in order to understand if improvements are available for implementation into future studies. The advantages and disadvantages of the parameters used to design the survey are summarised below.

- Sample type – faecal samples collected post-evisceration were used for the survey. Samples were collected directly from the intestine of each animal to ensure that minimal cross-contamination of samples occurred at the abattoir. Faecal samples were chosen as they harbor a greater microbial load than other commonly collected samples such as hide, carcase or beef trim. It is routinely proposed that testing faecal samples provides a worst case scenario for contamination of beef carcases and subsequently beef products destined for market. However, there is evidence to suggest that hide contamination is much more closely aligned to carcase contamination and may represent a more appropriate sample site. Ideally, future surveys would have an opportunity to test samples from multiple sites.

- Sample collection – samples were collected by local abattoir staff. Clear instructions were provided on how each sample should be collected and all abattoirs were provided with a minimum time that should elapse between the collection of consecutive samples. Operational requirements or misinterpretation of the supplied information did result in the collection of samples that weren't separated by the required time. This has the potential to exacerbate the effect of clustering as increased focus on a specific herd or lot may occur as a result. This issue can be rectified by employing project staff to collect samples are per the sampling plan. Whilst this approach may be optimal it would come at significant cost to the project and was considered an inappropriate use of funding during the design phase of the current study. An alternative solution is to filter out samples that don't meet the sampling requirements of the study. This would limit the addition of further clustering effects but may result in the study not achieving its sample targets within the allotted time frame.

- Sample clustering – the sampling strategy employed during this survey resulted in clustering of some samples as consecutive samples could not be separated by herd, source, or lot. As all plants sampled over a two day period regardless of the number of samples required, larger throughput plants are more likely to have clustering of samples than small throughput plants when herd, source or lot status is considered. Clustering would be minimised if a longitudinal survey had been performed instead of a point prevalence study however there are additional benefits and limitations associated with each study type requiring attention.

- Data collection – each abattoir was provided with a worksheet to record a number of data variables for each sample. The variables for each field were not defined and the worksheet was generally completed manually and then transcribed at the laboratory. There are a number of terms relating to variables such as animal and feed that are used interchangeably throughout the industry. Substantial effort was required to ensure the provided data was categorised correctly. The provision of an electronic or online system with defined variables for each field would greatly reduce the effort required by abattoir and laboratory staff and the ambiguity associated with categories like those mentioned above.

- Sample numbers – the survey collected a total of 1500 cattle faecal samples across three animal groups. Each abattoir was assigned to an animal group based on typical slaughter data and the number of samples required was then determined using weekly slaughter volumes. As fewer abattoirs slaughter veal calves and dairy cattle, the sample numbers required from abattoirs slaughtering these animals were elevated when compared to beef cattle abattoirs with comparable slaughter volumes. As a consequence the ability to allocate sufficient staff resources to the collection of samples was problematic. Future surveys should ensure sample numbers required per day are consistent with slaughter volumes and if required additional sampling occasions should be allocated within each sampling window to offset the staffing demands.