final report

Project code: G.MFS.0287

Title: An investigation of *Clostridium difficile* carriage on Australian bobby calf carcases

Prepared by: Mr Daniel Knight Professor Thomas V Riley

Microbiology & Immunology
The University of Western Australia
and
Division of Microbiology & Infectious Diseases
PathWest Laboratory Medicine Queen Elizabeth II Medical Centre Nedlands 6009
Western Australia

Date submitted: August 2013

Meat & Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government and contributions from the Australian Meat Processor Corporation to support the research and development detailed in this publication.

Executive summary

Clostridium difficile has been isolated from a wide variety of animals, particularly production animals, including cattle and pigs. *C. difficile* has also been found in retail meats of these production animals in North America and Europe. The incidence of *C. difficile* infection (CDI) in humans has increased in the community with some suggestions that food-borne transmission of *C. difficile* is occurring. This raises the possibility of a public health risk.

To assess the situation in Australia, three previous MLA studies (A.MFS.0124, A.MFS.0157 and A.MFS.0254) looked at the prevalence of *C. difficile* in cattle and calves. These initial studies concluded that neonatal calves, unlike juvenile or adult cattle are potentially a source/reservoir of *C. difficile*, including strains known to cause disease in humans and could present a risk to public health through the contamination of retail meat if inadequately managed during slaughter, dressing, transport and storage. In order to further determine any risk to public health, this current study investigated the prevalence, concentration and genetic diversity of *C. difficile* in the faeces (n=30) and on the carcases (n=300) of neonatal Australian bobby veal calves at slaughter. Sampling took place in April 2013 and covered three abattoirs, comprising numerous 'lots' in South Australia (n=1) and Victoria (n=2).

Selective culture (both direct and enrichment) was performed and isolates were characterised by PCR for toxin A, B and binary toxin genes, and PCR ribotyping. *C. difficile* was found in animals from all abattoirs sampled, although prevalence varied between sites. Overall *C. difficile* prevalence was 25.3% (76/300) on carcases and 60.0% (18/30) in faeces. Of those carcase samples with *C. difficile* levels above the limit of detection (n=25/150), the median count was 3 cfu/cm^2, (highest concentration of 33 cfu/cm^2). Of those faecal samples with *C. difficile* levels above the limit of detection (n=10/30), the mean concentration of *C. difficile* in faeces was 2.8 x 10^5 cfu/mL.

The vast majority of isolates (88.2%) were positive for toxin A and toxin B, 41% of which were also positive for binary toxin genes (A+B+CDT+). Multiple PCR ribotypes were identified, including PCR ribotype (RT) 127 which was the most prominent ribotype comprising 33.3% of isolates. RT127 belongs to sequence type (ST) 11 (by MLST) which falls into clade 5, the same as RT078, the most common animal ribotype worldwide (1) and increasingly associated with community CDI in the northern hemisphere (2). Ribotypes QX 150 and UK 137 comprised 28.4% and 14.7% of isolates respectively, and along with RT 127 have been isolated from humans in Australia in the last decade.

Contents

1 Background

C. difficile is a ubiquitous, spore forming, Gram positive anaerobe, the leading cause of antimicrobial and health care-associated diarrhoea in humans (3). *C. difficile* infection (CDI) is a significant economic burden to global health care systems (3, 4) and an increasing problem in the community (5).

C. difficile is also a recognized enteric pathogen in a variety of animals including companion animals (cats, dogs, horses) and food animals (cattle, sheep, goats, pigs) (6, 7). In Australia, *C. difficile* has been isolated from piglets, sheep, lambs, horses, cats, dogs, and cattle, with the highest prevalence in neonatal animals due to a lack of established gut flora at birth (8-10). The predominant genotype isolated from food production animals outside Australia is PCR ribotype (RT) 078 (1). This RT is now the third most common strain causing human infection in Europe (2). These findings have raised concerns that contaminated meat products could be a potential source of CDI in humans (11). Of significance to Meat and Livestock Australia (MLA) are reports from North America and Europe of *C. difficile* (predominantly RT078) being isolated from bovines and their processed meat products destined for human consumption (12, 13).

Contamination during slaughter occurs when faecal bacteria from the gut or hide are deposited on the carcase. Environmental contamination may also play a role as *C. difficile* spores survive in treated piggery effluent, the by-products of which may be applied to agricultural land, used in retail compost manufacture, or recycled within the swine facility (14).

MLA has previously completed 3 studies (A.MFS.0124, A.MFS.0157 and A.MFS.0254) on the prevalence of *C. difficile* in cattle, the results of which were published earlier this year (15). In the most recent study (A.MFS.0254) prevalence of *C. difficile* was 72% (63/88) in faeces from <7-day old dairy calves and 3.8% (1/26) in 2-6 month old calves. The observed decline in prevalence with increasing age supports studies in other animals (16, 17). This current study aims to provide further data on the prevalence and concentration of *C. difficile* on Australian veal calf carcasses, as well as the concentration in the gut of bobby calves.

2 Project objectives

The objectives of this project were fivefold:

1. Undertake a survey of Australian bobby calves (faecal samples and carcase swabs) for *C. difficile* at processing plants at three sites in two States.
2. Determine the prevalence of viable *C. difficile* spores and vegetative cells in faeces and on carcases.
3. Determine the concentration of viable spores and vegetative cells in faeces and on carcases.
4. Type *C. difficile* isolates recovered and investigate possible alignment with human isolates in Australia.
5. Assess any risk of food-borne transmission of *C. difficile* from contamination.

3 Methodology

3.1 Samples

Samples of faeces (n=30) and carcase washings (sponges) (n=300) were collected by Food Safety Services (Lyndoch, SA) from veal calves aged <14 days at slaughter. Sampling took place in April 2013 and covered three abattoirs in South Australia (n=1) and Victoria (n=2). At each site, samples were taken from at least two 'lots' with each lot having originated from a separate veal farm. It was not possible to select carcases on a statistical basis and carcases were sampled as they became available to the sampler. Sampling of carcases followed the meat industry standard carcase swabbing technique for calves as outlined in Australian Quarantine and Inspection Service (AQIS) Meat Notice 2000/09 (18) with the following variations:

(1) Pre-hydrated and sterilised Polywipes^{TM} (MWE, Corsham, UK) were used. Sterilisation of each 25 cm^2 template was achieved by immersion of the template in a 3.5% solution of sodium hypochlorite (household bleach) for approximately 30 seconds. Afterwards the template was rinsed with potable water (spray) and shaken dry.

(2) The three carcase sites (S1-S3) identified in Meat Notice 20000/09 (18) were used, except that the mid-loin area (S3) could not be used due to the extreme unevenness of the carcase in this area with the backbone protruding several centimetres above the meat. A site immediately adjacent to the mid-loin site was used on all carcases to obtain a more

regular and replicable surface for swabbing. In total, 3 x 25 cm^2 (75 cm^2) areas were swabbed per carcase.
(3) Sampling of carcases took place whilst still 'hot', either at the end of the processing line or immediately as they entered the chiller after hosing or washing of the whole carcass was complete.
All samples were transported under ambient conditions to The University of Western Australia, stored at 4ºC and processed within 24 h. Carcase swabs were processed in 6 batches (A-F).

3.2 Culture for *C. difficile*

Isolation of *C. difficile* was based on our previously described methods (19) with some modifications. Faeces were cultured both directly on *C. difficile* ChromID™ agar (BioMérieux, Marcy l'Etoile, France) and in an enrichment broth containing gentamicin (5 mg/L), cycloserine (200 mg/L) and cefoxitin (10 mg/L) (GCC) (20, 21). After 48 h incubation, 1 mL of all enrichment broths was mixed with an equal volume of absolute alcohol, to enhance spore selection and then 0.2 mL plated onto selective agar plates [cycloserine cefoxitin fructose agar (CCFA)] containing sodium cholate (a germinant). All plates were incubated in an anaerobic chamber (Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK) at 37°C, in an atmosphere containing 80% nitrogen, 10% hydrogen and 10% carbon dioxide.

Carcase sponges were transferred aseptically from the collection bag to a Stomacher bag (Colworth, London UK), 25 mL of peptone saline added and the bag placed in a Stomacher 400 (Colworth, London UK) for 30 s. The fluid was then transferred to a sterile container, 100 µL was spread-plated onto a ChromID plate and the remainder centrifuged at 3000 x rcf for 10 min using a 5470 centrifuge (Eppendorf GmbH, Hamburg Germany). The supernatant was decanted leaving approximately 500µL. The pellet was resuspended by flicking and 100 µL plated onto a ChromID plate and 100 µL added to a cooked meat broth containing GCC. Broths were incubated at 37°C for 7-14 days. All plates were incubated in an anaerobic chamber at 37°C and read at 24 and 48 h.

3.3 Plate counts

Serial dilutions of faeces were made in 0.85% saline. A 10µl loop of each dilution was then spread-plated onto a ChromID agar plate, incubated at 37°C for 48 h in an anaerobic chamber before performing total plate counts. The mean and range values were calculated and all results were given in cfu/mL. Carcase washings were processed as described in 3.2

and colony counts were performed on the ChromID agar (for batches A, B and C after 48 h. The median and highest count values were calculated and all results were given counts per cm^2 as described previously described (18).

3.4 Identification of *C. difficile*

Putative *C. difficile* colonies on either ChromID or CCFA were subcultured onto blood agar plates and identified on the basis of their characteristic colony morphology (yellow, ground glass appearance), odour (horse dung smell) and their characteristic chartreuse fluorescence under long-wave UV light (~360nm). The identity of uncertain isolates was confirmed by presence of the L-proline aminopeptidase (Remel Inc., Lenexa, KS, USA) activity and Gram stain.

3.5 Toxin profiling and PCR ribotyping

All isolates were screened by PCR for the presence of toxin A and B genes (*tcdA/tcdB*), binary toxin genes (both *cdtA* and *cdtB*) and for changes in the repetitive region of the toxin A gene using previously described methods (22-24). PCR ribotyping was performed as previously described (25). PCR ribotyping reaction products were concentrated using the Qiagen MinElute PCR Purification kit (Qiagen) and run on the QIAxcel capillary electrophoresis platform (Qiagen). Analysis of PCR ribotyping products was performed using BioNumerics™ software package v.6.5 (Applied Maths, Saint-Martens-Latem, Belgium). Dendrograms were generated for all isolates using an unweighted-pair group method UPGMA and Dice coefficient to assess the clostridial diversity in the populations. PCR ribotypes (RTs) were identified by comparison of banding patterns with our reference library at the time, consisting of a collection of 15 reference strains from the European Centre for Disease Prevention and Control (ECDC) and a collection of the most prevalent RTs currently circulating in Australia (unpublished data). Isolates that could not be identified with the available reference library were designated with internal nomenclature, prefixed with QX.

4 Results and discussion

4.1 Sample collection

In total, 330 samples from three abattoirs were collected and analysed (Table 1).

Table 1. Summary of sampling.

Abattoir	State	Date rec'd	Batch ID	Carcass washes (N)	Faeces (N)
I	SA	03-Apr-13	A	50	5
II	VIC	09-Apr-13	B	50	10
			C	50	0
III	VIC	15-Apr-13	D	50	10
			E	50	0
I	SA	17-Apr-13	F	50	5
			Total	**300**	**30**

4.2 Prevalence of carriage

The prevalence of *C. difficile* on the carcases and in the faeces of veal calves is presented in Tables 2 and 3, respectively. Recovery of *C. difficile* from carcases varied across the abattoirs sampled (range 10.0 - 58.0%, Table 2). In addition, prevalence appeared to vary with herds, as marked differences were observed with the two batches sent from abattoir I [batch A (58.0%) and batch F (10.0%)]. Of those samples positive for *C. diffi*cile, 88.2% (n=67/76) were by direct culture methods. Several samples (n=6) contained more than one strain.

Table 2. Recovery of *C. difficile* from carcase washings.

Abattoir	State	Batch ID	N samples	N positive‡ n (%)	Direct and enrichment	Enrichment only	N Isolates‡
I	SA	A	50	29 (58.0)	29	0	34
II	VIC	B,C	100	32 (32.0)	23	9	33
III	VIC	D,E	100	10 (10.0)	10	0	10
I	SA	F	50	5 (10.0)	5	0	5
	Total		**300**	**76 (25.3)**	**67**	**9**	**82**

‡ some samples contained multiple strains

Recovery of *C. difficile* from faeces varied across the abattoirs sampled (range 30.0 - 90.0%) (Table 3). Of those samples positive for *C. difficile*, 50.0% (n=9/18) were by direct culture methods. Two samples contained more than one strain.

Table 3. Recovery of *C. difficile* from faeces.

Abattoir	State	Batch ID	N samples	N positive‡ n (%)	Direct and enrichment	Enrichment only	N Isolates‡
I	SA	A	5	4 (80.0)	0	4	4
II	VIC	B,C	10	3 (30.0)	1	2	5
III	VIC	D,E	10	9 (90.0)	6	3	9
I	SA	F	5	2 (40.0)	1	1	2
Total			**30**	**18 (60.0)**	**8**	**10**	**20**

‡ some samples contained multiple strains

4.3 Viable counts

Viable counts were performed on 15 faecal samples, 10/15 of which had counts above the limit of detection. Of the samples with detectable counts of *C. difficile* (n=10), the mean concentration of *C. difficile* was 2.8 x 10^5 cfu/mL (range 1.2 x 10^4 to 2.3 x 10^6 cfu/mL) (Figure 1). Viable counts were performed on 150 carcases samples. Only 25 samples (17%) had counts above the limit of detection. Of these, the median count was 3 cfu/cm^2. The highest concentration detected was 33 cfu/cm^2 (Figure 2).

Figure 1. Concentration of viable *C. difficile* in 10 different faecal samples.

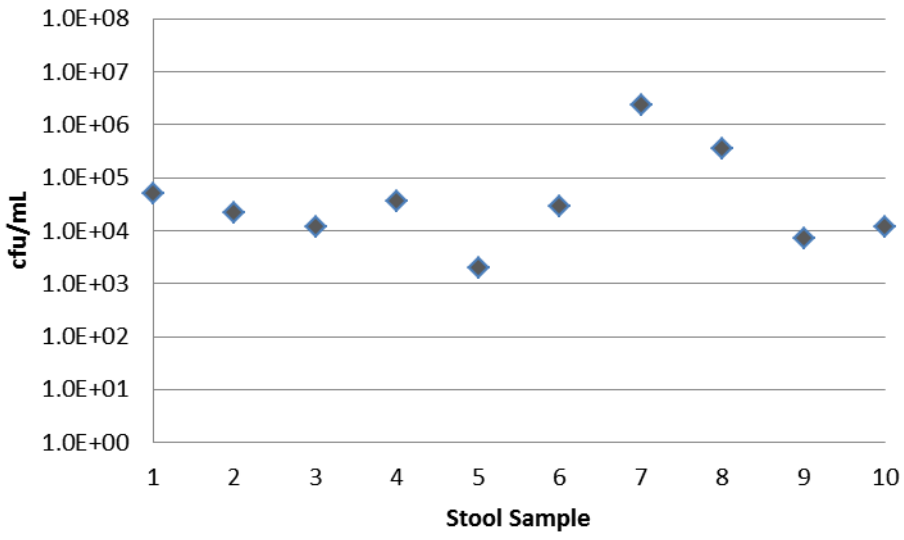
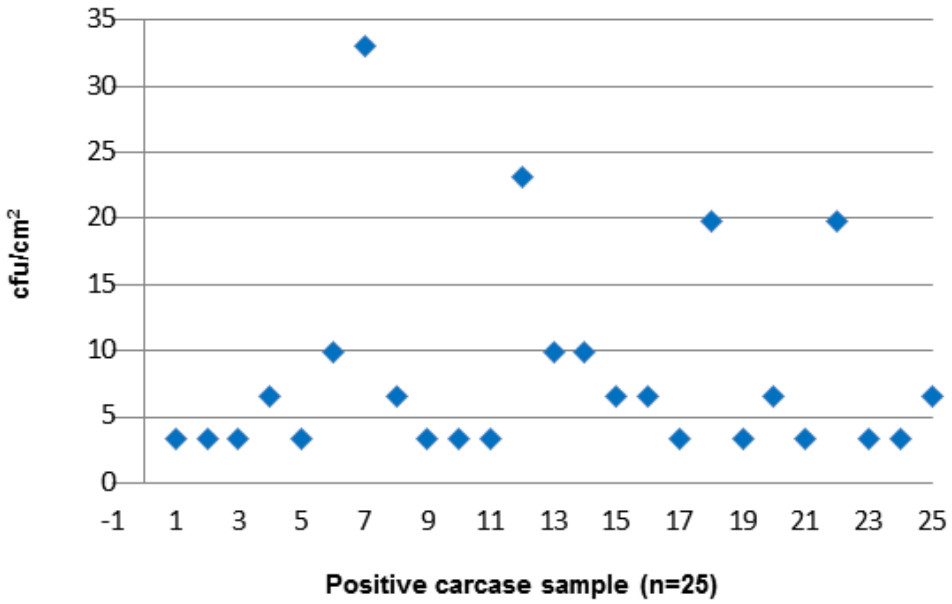


Figure 2. Concentration of viable *C. difficile* in 25 positive carcase samples.



4.4 Toxin gene profiles

Of the 102 isolates of *C. difficile* recovered from calves, 90 (88.2%) were positive for *tcdA* and *tcdB* (A^+B^+), of which 37 (41%) were also positive for binary toxin genes *cdtA/B* (CDT^+). Six isolates (5.9%) were negative for both *tcdA* and *tcdB* but CDT^+ ($A^-B^-CDT^+$) and the remaining six isolates (5.9%) were non-toxigenic ($A^-B^-CDT^-$). Toxin gene profiles for all isolates are summarised in Figure. 3.

4.5 Ribotyping

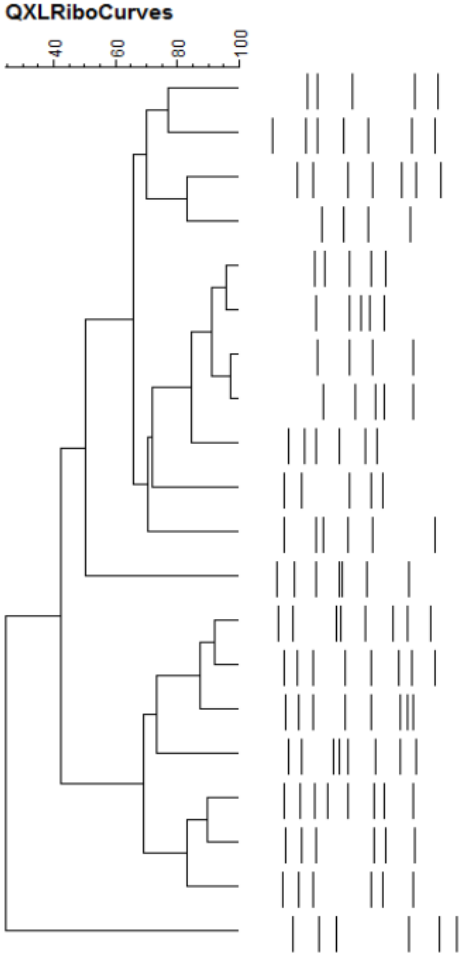
Multiple RTs were identified (Table 4, Figure 3). Of the 102 isolates of *C. difficile* recovered from calves, 62.7% (n=64) were assigned one of 10 internationally recognised RTs; 033, 052, 053, 056, 070, 103, 126, 127, 137 and 251. A number of isolates gave ribotyping banding patterns that we were unable to match to our available reference library and these were designated with internal nomenclature, QX; 006, 104, 141, 150, 231, 232, 233 and 234. UK 127 was most prominent ribotype comprising 33.3% of isolates followed by QX 150 (28.4%) which was found almost exclusively in the first batch of abattoir I (Table 4) and UK

137 (14.7%). Strains associated with *C. difficile* community-acquired outbreaks in Australia (RT244 and RT251) were not recovered, neither were strains implicated in outbreaks overseas (RT 027, RT 078).

Table 4. Distribution of *C. difficile* PCR ribotypes.

PCR Ribotype	Abattoir (Batch ID)				N (%)
	I (A)	I (F)	II (B,C)	III (D,E)	
UK 127	7		17	10	34 (33.3)
QX 150	26	1	2		29 (28.4)
UK 137	1	5	7	2	15 (14.7)
UK 033	1		4	1	6 (5.9)
UK 126			2		2 (2.0)
QX 006			2		2 (2.0)
QX 233				2	2 (2.0)
UK 056				2	2 (2.0)
QX 104	1				1 (1.0)
QX 234		1			1 (1.0)
QX 232			1		1 (1.0)
UK 052			1		1 (1.0)
QX 141	1				1 (1.0)
QX 231			1		1 (1.0)
UK 251			1		1 (1.0)
UK 070				1	1 (1.0)
UK 053	1				1 (1.0)
UK 103				1	1 (1.0)
Total	**38**	**7**	**38**	**19**	**102**

Figure 3. Summary of *C. difficile* PCR ribotypes and toxin profiles.



PCR Ribotype	Toxin Profile			N (%)
	tcdA	*tcdB*	*cdtA/B*	
QX 141	-	-	-	1 (1.0)
UK 027[R]	+	+	+	
UK 103	+	+	-	1 (1.0)
QX 150	+	+	-	29 (28.4)
UK 126	+	+	+	2 (2.0)
UK 127	+	+	+	34 (33.3)
UK 033	-	-	+	6 (5.9)
QX 233	-	-	-	2 (2.0)
UK 078[R]	+	+	+	
UK 070	+	+	-	1 (1.0)
UK 251	+	+	+	1 (1.0)
QX 231	+	+	-	1 (1.0)
UK 056	+	+	-	2 (2.0)
QX 006	+	+	-	2 (2.0)
UK 052	+	+	-	1 (1.0)
QX 104	-	-	-	1 (1.0)
UK 053	+	+	-	1 (1.0)
QX 234	-	-	-	1 (1.0)
UK 137	+	+	-	15 (14.7)
QX 232	-	-	-	1 (1.0)

Dendrogram created using the Dice coefficient (optimization, 1.00%; tolerance, 0.5%). [R] - Reference strain

4.6 Discussion

This study follows up three previous MLA projects focused on determining the prevalence and genotypic characterisation of *C. difficile* in Australian veal calves at slaughter. In addition, this study provides the first estimate of viable *C. difficile* concentration both in the faeces and on the carcases of Australian veal calves. The prevalence of *C. difficile* in veal calve faeces (60%) is consistent with the findings of a previous MLA study (A.MFS.0254) (15). There was a difference in prevalence between abattoirs (range 30.0-90.0%), however, the numbers of samples of this type were small (n=30) as they were specifically collected only to determine a gastrointestinal concentration. The prevalence of *C. difficile* recorded was higher than reported elsewhere: Canada, 11.2% (31/278) (17), the United States 9% (18/50) (12), Slovenia 9% (4/42) (26) and Switzerland 0.5% (1/204) (27). As well as differences in animals selected, season etc, there are methodological differences which could account for these discrepancies such as the use of toxin ELISAs in many of these studies which are known to have low sensitivities and have not been validated for animal faeces.

The concentration of viable spores in faeces was also high (mean 2.8 x 10^5 cfu/mL), confirming our previous finding that the faeces of bobby veal calves at slaughter contain *C. difficile*. To our knowledge this is the first time that *C. difficile* has been isolated from carcases of Australian veal calves. The prevalence of *C. difficile* on carcases was 25% and the majority of isolates (67/82) were recovered by direct culture. The concentration of viable spores on carcases ranged up to 33 cfu/cm^2 with a median count of 3 cfu/cm^2 (Figure 2).

Toxin profiling and PCR ribotyping revealed a heterogeneous population of *C. difficile* strains. Consistent with what we found in an earlier study (15), there was a high proportion of *C. difficile* isolates from veal calves with at least one toxin gene (94%). As before, RT127 was the most common RT comprising 33% of isolates; however prevalence of RT033 and RT126 were lower than we have previously reported. Strains belonging to ribotypes 033, 126 and 127 have all been isolated from humans with disease in Australia in the last decade (unpublished). Interestingly, we noted the presence of a novel RT (QX 150) that comprised 28% of isolates and was found almost exclusively in the first batch of abattoir I. RT127, 126 and 033 belong to sequence type (ST) 11 (by MLST) in clade 5, the same as RT078, the most common animal ribotype worldwide (1). Given the epidemiological link with animals, it is possible that QX 150 also groups within ST11 (although it is CDT negative), and further research is warranted in this area.

It is interesting both that none of the ribotypes detected was the same as ribotypes commonly found in cattle and retail meat products overseas, and that strains associated with *C. difficile* community-acquired outbreaks in Australia (RT244,RT251) and overseas (RT 027, RT 078) were not recovered.

5 Conclusions

- Overall, the prevalence of *C. difficile* in the faeces and on the carcases of bobby calves at slaughter was high. Plate counts revealed a high concentration of viable *C. difficile* in faeces (most probably as spores).

- *C. difficile* was found in animals from all abattoirs sampled and prevalence rates varied widely.

- Multiple RTs were identified, and whilst not predominating to the extent seen in previous studies, ST 11 strains 126, 127 and 033 were seen in high numbers.

- The epidemiology of *C. difficile* in Australian calves contrasts with studies in the northern Hemisphere where a single well-described RT (078) predominates. This epidemiology appears unique and requires further investigation, both from a disease phenotype and molecular epidemiology perspective.

- The faeces of a large proportion of the Australian calves sampled are colonised with strains of *C. difficile* known to infect humans, albeit with relatively low numbers of cases currently in Australia.

6 Acknowledgements

We are grateful to Mr Chris Sentance (Food Safety Services, Lyndoch, SA) for the collection of samples. We thank Dr Briony Elliott, Miss Papanin Putsathit and Miss Anita Williams for assistance with sample processing. Finally, we are indebted to Ms Julie Balan and Mr Ian Jenson from Meat & Livestock Australia for their continued support throughout this project.

7 References

1. Rupnik M, Widmer A, Zimmermann O, Eckert C, Barbut F. Clostridium difficile toxinotype V, ribotype 078, in animals and humans. Journal of Clinical Microbiology. 2008;46:1963–4.
2. Bauer MP, Veenendaal D, Verhoef L, Bloembergen P, Dissel JTv, Kuijper EJ. Clinical and microbiological characteristics of community-onset Clostridium difficile infection in The Netherlands. Clinical Microbiology and Infection. 2009;15(12):1087-82.
3. Rupnik M, Wilcox MH, Gerding DN. Clostridium difficile infection: new developments in epidemiology and pathogenesis. Nature Reviews Microbiology. 2009;7:526-36.
4. Wiegand PN, Nathwani D, Wilcox MH, Stephens J, Shelbaya A, Haider S. Clinical and economic burden of Clostridium difficile infection in Europe: a systematic review of healthcare-facility-acquired infection. Journal of Hospital Infection. 2012;81:1-14.
5. Hensgens MP, Keessen EC, Squire MM, Riley TV, Koene MG, Boer Ed, et al. Clostridium difficile infection in the community: a zoonotic disease? Clinical Microbiology and Infection. 2012;18(7):635–45.
6. Keel MK, Songer JG. The comparative pathology of *Clostridium difficile*-associated disease. Veterinary Pathology. 2006 May;43(3):225-40. PubMed PMID: 16672570. Epub 2006/05/05. eng.
7. Rupnik M, Songer JG. *Clostridium difficile:* Its Potential as a Source of Foodborne Disease. Advances in Food Nutrition and Research. 2010;60C:53-66. PubMed PMID: 20691953. Epub 2010/08/10. Eng.
8. Squire MM, Carter GP, Mackin KE, Chakravorty A, Norén T, Elliott B, et al. Novel Molecular Type of Clostridium difficile in Neonatal Pigs, Western Australia. Emerging Infectious Disease. 2013;19:790-2.
9. Knight DR, Riley TV. Prevalence of Clostridium difficile gastrointestinal carriage in Australian sheep and lambs. Applied Environmental Microbiology. 2013;Jul 12. [Epub ahead of print] doi:10.1128/AEM.01888-13.
10. Thean S, Elliott B, Riley TV. Clostridium difficile in horses in Australia-a preliminary study. Journal of Medical Microbiology. 2011;60:88-92.
11. Rupnik M. Is *Clostridium difficile*-associated infection a potentially zoonotic and foodborne disease? Clinical Microbiology and Infection. 2007 May;13(5):457-9. PubMed PMID: 17331126. Epub 2007/03/03. eng.
12. Houser BA, Soehnlen MK, Wolfgang DR, Lysczek HR, Burns CM, Jayarao BM. Prevalence of Clostridium difficile toxin genes in the feces of veal calves and incidence of ground veal contamination. Foodborne Pathogens and Disease. 2012;9(1):32-6.
13. Rodriguez-Palacios A, Stampfli HR, Duffield T, Weese JS. Clostridium difficile in retail ground meat, Canada. Emerging Infectious Disease. 2007;13:485-7.
14. Squire MM, Lim SC, Foster NF, Riley TV. Detection of Clostridium difficile after treatment in a two-stage pond system. 2011 Australasian Pig Science Association Conference Adelaide, Australia2011.
15. Knight DR, Thean S, Putsathit P, Fenwick S, Riley TV. Cross-Sectional Study Reveals High Prevalence of Clostridium difficile Non-PCR Ribotype 078 Strains in Australian Veal Calves at Slaughter. Applied Environmental Microbiology. 2013;79:2630-5.
16. Hopman NEM, Keessen EC, Harmanus C, Sanders IMJG, Leegoed LAMGv, Kuijper EJ, et al. Acquisition of Clostridium difficile by piglets. Veterinary Microbiology. 2011;149:186-92.
17. Rodriguez-Palacios A, Stämpfli HR, Duffield T, Peregrine AS, Trotz-Williams LA, Arroyo LG, et al. Clostridium difficile PCR ribotypes in calves, Canada. Emerging Infectious Disease. 2006;12(11):1730-6.
18. (AQIS) AQaIS. Carcase Microbiological Monitoring Program (ESAM). Notice Number MEAT 2000/092000.

19. Bowman RA, Riley TV. The laboratory diagnosis of Clostridium difficile-associated diarrhoea. European Journal of Clinical Microbiology & Infectious Diseases. 1988;7:476-84.
20. Carroll SM, Bowman RA, Riley TV. A selective broth for Clostridium difficile. Pathology. 1983;15(2):165-7.
21. Brazier JS. The diagnosis of Clostridium difficile-associated disease. Journal of Antimicrobial Chemotherapy. 1998;41:29-40.
22. Kato N, Ou CY, Kato H, Bartley SL, Brown VK, Jr VRD, et al. Identification of toxigenic Clostridium difficile by the polymerase chain reaction. Journal of Clinical Microbiology. 1991;29:33-7.
23. Kato H, Kato N, Watanabe K, Iwai N, Nakamura H, Yamamoto T, et al. Identification of toxin A-negative, toxin B-positive Clostridium difficile by PCR. Journal of Clinical Microbiology. 1998;36(8):2178-82.
24. Stubbs SL, Rupnik M, Gibert M, Brazier JS, Duerden BI, Popoff MR. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of Clostridium difficile. FEMS Microbiology Letters. 2000;186:307–12.
25. Stubbs SL, Brazier JS, O'Neill GL, Duerden BI. PCR targeted to the 16S-23S rRNA gene intergenic spacer region of Clostridium difficile and construction of a library consisting of 116 different PCR ribotypes. Journal of Clinical Microbiology. 1999;37:461-3.
26. Avbersek J, Janezic S, Pate M, Rupnik M, Zidaric V, Logar K, et al. Diversity of Clostridium difficile in pigs and other animals in Slovenia. Anaerobe. 2009;15:252-5.
27. Hoffer E, Haechler H, Frei R, Stephan R. Low occurrence of Clostridium difficile in fecal samples of healthy calves and pigs at slaughter and in minced meat in Switzerland. Journal of Food Protection. 2010;73(5):973-5.