final report

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Title:	**Determining the prevalence of *Clostridium difficile* in Australian calves**
Prepared by:	Mr Daniel Knight and 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
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	Meat & Livestock Australia Limited Locked Bag 991 NORTH SYDNEY NSW 2059

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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. Concurrently, 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 clearly raises a serious public health risk. To assess the situation in Australia two previous studies (A.MFS.0124 and A.MFS.0157) looked at the prevalence of *C. difficile* in cattle and found low levels (~2%) of carriage suggesting cattle are unlikely to be a major source/reservoir of human infections. In this study, we investigated the prevalence and genetic diversity of *C. difficile* in Australian calves at slaughter. Faeces from veal calves aged up to 7 days old were collected from abattoirs across five Australian states. Selective culture was performed and isolates characterised by PCR for toxin A, B and binary toxin genes, and PCR ribotyping. *C. difficile* prevalence was 72% (63/88) in faeces from 7 day old calves and 3.8% (1/26) in 2-6 month old calves. Three PCR ribotypes (126, 127 and 033) comprised 61 (95%) of 64 isolates. These ribotypes are genetically related to epidemic strain 078 and have all been isolated from humans with disease in Australia.

Contents

1 Background

C. difficile is a recognized enteric pathogen in a variety of animals including companion animals (cats, dogs, horses) and food animals (cattle, sheep, goats, pigs)[1,2]. 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. For this reason predisposing antibiotics may not be required for development of CDI in young animals although there is a worrying trend in Australia toward routine use of extended-spectrum cephalosporins in production animals. This is particularly concerning in the pork industry where gross contamination of facilities with *C. difficile* spores is commonplace. *C. difficile* can be isolated from the faeces of piglets 1 hour after birth, presumably ingested from their environment. Within 48 hours 100% of piglets had acquired *C. difficile* of the same molecular type that was found in the piggery environment[3]. A 2011 Australian study showed contamination with toxigenic *C. difficile* increased from 0%-61% of sites within a swine facility only one month after occupation with pigs[4]. Airborne *C. difficile* spores can be found up to 20 metres from the pig facility[5]. The predominant genotype isolated from food production animals outside Australia is PCR ribotype 078, toxinotype V, NAP 7/8, REA group BK[6]. This ribotype is now the third most common European human ribotype[7].

Meat products, seafood, ready-to-eat salads, salad leaves and vegetables are also contaminated with *C. difficile*, predominantly ribotype 078-like strains[2, 8-10]. Contamination may occur through spillage of gut contents at slaughter or direct contamination by food handlers during processing or retailing. Environmental contamination may also play a role. *C. difficile* spores survive in treated piggery effluent, the by-products of which are then applied to agricultural land, used in retail compost manufacture, or recycled within the swine facility[11].

Currently, there are few data on the prevalence of *C. difficile* carriage in Australian cattle. What risk such contamination poses for food-borne transmission of *C. difficile* is unknown. This project is a continuation of two previous investigations that looked at *C. difficile* prevalence on carcasses and in gut contents (A.MFS.0124) and in faeces (A.MFS.0157) from Australian cattle. In the second of those two studies (A.MFS.0157), *C. difficile* was isolated from 1.8% of faecal samples from adult cattle. Given that carriage is likely to be higher in younger cattle, the present study targeted calves of various ages but predominantly "bobby" calves.

2 Project objectives

The objectives of this project were three fold:

1. To undertake a survey of Australian calves at slaughter for *C. difficile* presence and determine the prevalence and concentration in two geographic regions.

2. *C. difficile* isolates recovered would be typed to see if there is any relationship with humans isolates in Australia.

3. Based on the findings to assess any risk of food-borne transmission of *C. difficile* from contamination.

3 Methodology

3.1 Samples

Samples of faeces from calves aged <7 days at slaughter (n=88) were collected by Food Science Australia in two 2-day periods in March and April 2012 from two abattoirs in Warrnambool, VIC and Gleneagle, QLD respectively. Older calves were also sampled from QLD; 2 months of age (n=5), 4 months of age (n=4) and 6 months of age (n=17). Sampling in March comprised 4 different lots (1 large lot of 25 calves and 3 smaller lots totalling 25 calves). Sampling in April comprised 13 different lots (10 lots of 7 day old calves and 3 lots of older calves) totalling 64 calves. Each lot had originated from a separate veal farm. All samples were transported to The University of Western Australia, stored at 5ºC and processed within 24 hours.

3.2 Culture for *C. difficile*

The method to isolate *C. difficile* was based on our previously described methods[12] with some modifications. Faeces were cultured both directly on CCFA and in an enrichment broth. All plates were incubated in an anaerobic chamber (Don Whitley Scientific Ltd.) at 37°C, in an atmosphere containing 80% nitrogen, 10% hydrogen and 10% carbon dioxide. Three control strains were used to monitor anaerobiosis; *P. aeruginosa* ATCC 27853, *C. difficile* ATCC 43593, and *M. luteus* ATCC 4698. After 48 hours incubation, all enrichment broths were alcohol shocked and sub-cultured onto CCFA containing sodium cholate to enhance spore germination and incubated as above.

3.3 Identification of *C. difficile*

C. difficile was identified on the basis of characteristic colony morphology (yellow, ground glass appearance) and odour (horse dung smell). The identity of doubtful isolates was confirmed by Gram stain and a latex agglutination test kit (Oxoid).[13]

3.4 Toxin profiling and ribotyping of *C. difficile*

The genes for toxin A, toxin B, and binary toxin (both *cdtA* and *cdtB* and the repetitive region of toxin A) were detected in isolates by PCR.[14,15] Organisms were also PCR ribotyped[16] (PCR amplification of ribosomal intergenic regions results in specific banding patterns that can be used to genetically fingerprint *C. difficile*) and a method of determining strain relatedness. Dendrogram and cluster analysis of PCR ribotyping band patterns were performed using the Dice coefficient within BioNumerics software package v.6.5 (Applied Maths, Saint-Martens-Latem, Belgium). Isolates that could not be identified with the available reference library were designated with internal nomenclature.

3.5 Statistical analysis

A t-test was used to compare the prevalence of *C. difficile* among the sampled abattoirs and to analyse the effect of age and geographic distribution on the number and types of ribotypes identified.

4 Results and discussion

4.1 Prevalence of carriage

The prevalence of *C. difficile* in veal calves is presented in Table 1. From veal calves (aged <7 days of age), a total of 88 faecal samples were collected and processed, of which *C. difficile* was isolated from an overall total of 63 (72%) of samples. Of the 26 older calves aged 2 months (n=5), 4 months (n=4) and 6 months (n=17) *C. difficile* was isolated from a single calf (aged 2 months from the Victorian abattoir) by enrichment culture. The age of the animal appeared to significantly affect the numbers of positive cultures obtained (7 days old versus 2 - 6 month old (P=<0.0001).

The overall prevalence of *C. difficile* in faecal samples from the abattoir in Victoria (72%) and Queensland (71%) was similar. From Victoria, 36 of 50 samples were positive by enrichment

culture, none was positive by direct culture methods. *C. difficile* was cultured from 27 samples from Queensland, 12 (44%) by direct culture and the remainder (n=15, 55%) from enrichment culture.

Table 1. Isolation of *C. difficile* from Australian calves at slaughter.

Source	Age	Location	n	Isolation of *C. difficile (n/%)*
Faeces	<7 days	Warrnambool, Victoria	50	36 (72.0)
Faeces	<7 days	Gleneagle, Queensland	38	27 (71.1)
Faeces	2 months	Gleneagle, Queensland	5	1 (20.0)
Faeces	4 months	Gleneagle, Queensland	4	0 (0.0)
Faeces	6 months	Gleneagle, Queensland	17	0 (0.0)
Total			**114**	**64 (56.1)**

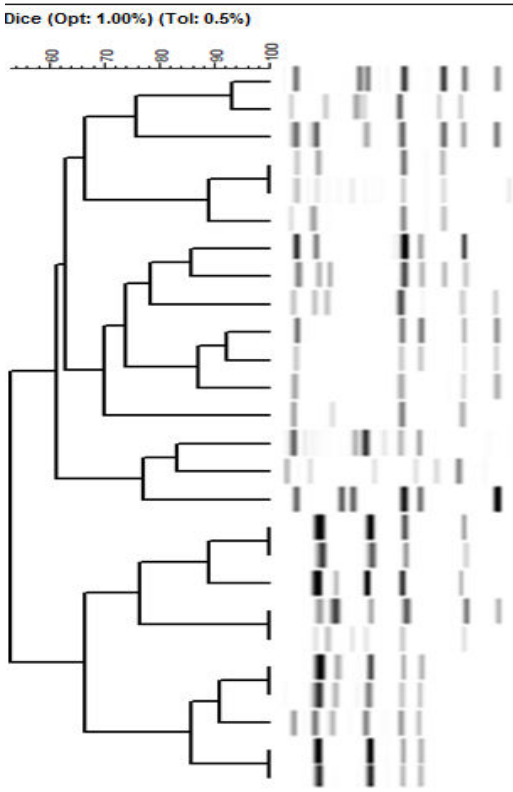
4.2 Toxin gene profiles

Of the 64 isolates of *C. difficile* recovered from adult and calves, 54 (84%) were positive for *tcdA* and *tcdB* (A^+B^+), of which 52 (96%) were also positive for binary toxin genes (CDT^+). Nine isolates (14%) were negative for both *tcdA* and *tcdB* but CDT^+ ($A^-B^-CDT^+$) and one isolate (1.6%) was a variant strain ($A^-B^+CDT^+$). Toxin gene profiles for all isolates along with demographic distributions of toxin genes between abattoirs and between age groups are summarised in Fig. 1.

4.3 Ribotyping

Multiple PCR ribotypes were identified (Fig.1). Of the 64 isolates obtained from calves, 88% (n=61) were assigned one of six ribotypes; 033, 126, 127, AU260, AU261 and AU262. None was ribotype 027 nor 078. PCR ribotype 127 ($A^+B^+CDT^+$) was the most common ribotype found overall representing 59.4% (41/64) of isolates and was more common in Victoria (n=31, 76%) than in Queensland (n=10, 24%), (P=<0.0001). PCR ribotype 126 ($A^+B^+CDT^+$) represented 15.9% (11/64) of isolates and was found exclusively in Queensland. PCR ribotype 033 ($A^-B^-CDT^+$) was present in 13.0% (9/64) of isolates and there was no significant difference in its prevalence between Victoria and Queensland.

Figure 2. Comparison of *C. difficile* PCR ribotypes from representative veal calf isolates (n=6), all adult cow isolates from previous MLA study A.MFS.0157 (n=5) compared with reference strains (n=15). Also shown are isolate demographics and detection of *tcdA*, *tcdB* and binary toxin genes.



PCR Ribotype	Toxin profile			n (%)	Age	Specimen type	Abattoir (n)
	tcdA	*tcdB*	*cdtA/cdtB*				
AU258	+	+	-	1 (1.4)	Adult cow	Faeces	Q5
AU259	-	-	-	1 (1.4)	Adult cow	Faeces	V4
UK103R	+	+	-				
UK002R	+	+	-				
UK010R	-	-	-				
AU255	-	-	-	1 (1.4)	Adult cow	Faeces	N1
AU261	+	+	-	1 (1.4)	Veal calf	Faeces	Q12
UK005R	+	+	-				
UK244R	+	+	+				
UK014R	+	+	-				
UK020R	+	+	-				
UK001R	+	+	-				
AU256	-	-	-	1 (1.4)	Adult cow	Faeces	Q4
UK070R	+	+	-				
AU257	-	+	+	1 (1.4)	Adult cow	Faeces	N2
AU260	+	+	-	1 (1.4)	Veal calf	Faeces	V5
UK033	-	-	+	9 (13.0)	Veal calf	Faeces	Q12 (6) / V5 (3)
UK033R	-	-	+				
AU262	-	+	+	1 (1.4)	Veal calf	Faeces	V5
UK027R	+	+	+				
UK054R	+	+	-				
UK126	+	+	+	11 (15.9)	Veal calf	Faeces	Q12
UK126R	+	+	+				
UK078R	+	+	+				
UK127	+	+	+	41 (59.4)	Veal calf	Faeces	Q12 (10*) / V5 (31)
UK127R	+	+	+				

R - reference strain, * - one isolate from 2 month old calf

abattoir code locations: Victoria V4 (A.MFS.0157) +V5 (this study), New South Wales N1+N2 (A.MFS.0157), Queensland Q4 and Q5 (A.MFS.0157) and Q12 (This study).

4.4 Discussion

To our knowledge this is the first time that *C. difficile* has been isolated from Australian veal calves. Prevalence of *C. difficile* in veal calves reported here (73%) was significantly higher than in similar studies; in Canada, 11.2% (31/278)[17] and 32% - 51%[18], the United States 9% (18/50)[19], Slovenia 9% (4/42)[20] and Switzerland 0.5% (1/204)[21]. We also found a higher proportion of *C. difficile* isolates from veal calves with at least one toxin gene present (100%) than reported elsewhere (7%)[19].

Differences in slaughter age may well explain the contrasting prevalence of *C. difficile* obtained prior to slaughter in this current study and similar studies overseas. In North America, reports of *C. difficile* prevalence in calves include data from calves up to 21 weeks of age at which point they are slaughtered. The calves sampled in this study were slaughtered at 7 days of age. The observed decline in prevalence with increasing age supports studies reported elsewhere[18]. This age related affect, where prevalence decreases as age increases, has also been reported in pigs[3]. As is the case with pigs, the decline is most likely a result of an increase in the gut microflora responsible for colonization resistance. Neonatal animals have underdeveloped intestinal micro flora and *C. difficile* is better able to colonize, proliferate and produce toxins[17]. The prevalence of *C. difficile* in the gut at the age at which the calf is slaughtered will ultimately affect the likelihood of *C. difficile* making its way in to retail food and eventually humans. Season was also reported as a factor affecting *C. difficile* prevalence in veal calves, with the highest prevalence being in winter[22]. Sampling of veal calves in the present study took place in late summer (March and April) suggesting we may see a higher prevalence in colder months of the year.

It was interesting that none of the ribotypes detected was the same as ribotypes commonly found in cattle and retail meat products overseas[6]. Of the ribotypes detected in this study, 127 and 126 are both $A^+B^+CDT^+$ and, together with ribotype 033 ($A^-B^-CDT^+$) belong to sequence type (ST) 11 (by MLST) which falls into clade 5, the same clade as ribotype 078. Ribotype 078 ($A^+B^+CDT^+$) is the most common animal ribotype worldwide[23] and has similar hypervirulent attributes to the epidemic strain PCR ribotype 027, but a much stronger association with animals in the Northern Hemisphere[23]. Ribotype 078 is prevalent in veal calves in Canada (67%)[18] and the United States (94%)[24]. This ribotype also appears to be infecting humans in the United States[25] and it is the 3rd most common human isolate in

European hospitals[7]. Strains belonging to ribotypes 033, 126 and 127 have all been isolated from humans with disease in Australia in the last decade (unpublished).

There is a growing body of evidence that many neonatal or infant animals are colonized with *C. difficile*, including cattle[25.] Whether such colonization continues beyond the infant period may well depend on exposure to antimicrobials. We suspect that there has been a shift in antibiotic prescribing practices by Australian veterinarians in recent years, particularly in livestock. Availability of once-daily antimicrobial agents like ceftiofur could be the driving force for amplification of *C. difficile* in production animal populations leading to an outbreak of community-acquired CDI in Australia.

It would be beneficial to follow up this investigation with a larger study of Australian veal calves at slaughter, specifically aiming to understand the use of antimicrobials in calves prior to slaughter. Sampling of greater numbers of calves in different areas of Australia and over a longer time frame would also be beneficial given the small number of animals sampled in the present study. Further information regarding volumes and destinations for domestic and exported meat consumption, environmental contamination sources and animal health prior to slaughter would be of interest. Whether *C. difficile* from veal calves is making its way into retail meat in Australia is unclear as to-date no *C. difficile* has been found in Australian retail meats, however, the high prevalence of *C. difficile* in Australian veal calves is a concern and a potentially poses a threat to consumers, workers in the industry and, ultimately, Australia's biosecurity[26].

5 Conclusions

The high rate of carriage/colonisation found in this study suggests that veal calves (unlike older cattle) are potentially a major source/reservoir of *C. difficile* known to cause disease in humans.

The current study presents a case for the potential for toxigenic *C. difficile* to be contaminate food (both directly and indirectly) for human consumption both in Australia and to nations where food, particularly meat, is exported.

The amplification of *C. difficile* in humans is driven by antimicrobial use and this is likely to be the same in animals, particularly young animals. Whether such colonization or indeed disease in calves continues beyond the neonatal period may well also depend on exposure to antimicrobials. The industry should not encourage further spread or expansion of *C. difficile* by injudicious use of antimicrobials, particularly cephalosporins.

In addition, slaughtering practices that might lead to contamination of meat should be monitored. Workers in the industry, particularly abattoir workers who might be exposed to faeces and who are taking antimicrobials that perturb their gut flora, may be at increased risk of infection with *C. difficile*.

6 References

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