final report

Project code: A.MFS.0157

Prepared by: Thomas V Riley, Stan Fenwick

University of Western Australia,
Murdoch University

Date submitted: June 2009

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

Clostridium difficile in beef in Australia

Part II

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.

This publication is published by Meat & Livestock Australia Limited ABN 39 081 678 364 (MLA). Care is taken to ensure the accuracy of the information contained in this publication. However MLA cannot accept responsibility for the accuracy or completeness of the information or opinions contained in the publication. You should make your own enquiries before making decisions concerning your interests. Reproduction in whole or in part of this publication is prohibited without prior written consent of MLA.

Contents

1 Background

There is great concern world-wide about a new infectious diseases threat following the recent emergence in Canada,[1] the USA,[2] and now Europe,[3] of a highly virulent strain of *Clostridium difficile* (called PCR ribotype 027 in Europe and NAP1 in the USA). Rates of detection of *C. difficile* have risen dramatically, *C. difficile* disease has been more severe, and attributable mortality was >10% in those aged >60 years[1]. *C. difficile* is the most commonly diagnosed cause of infectious hospital-acquired diarrhoea in developed countries. The majority of patients with *C. difficile*-associated diarrhoea (CDAD) have been exposed to antimicrobials that reduce 'colonisation resistance' of the large intestine allowing subsequent infection with *C. difficile.* Acquisition of *C. difficile* is facilitated by its ability to form spores that are resistant to many disinfectants allowing it to remain viable in the hospital environment for long periods of time. Toxigenic isolates of *C. difficile* usually produce two toxins, toxin A and toxin B, and these are thought of as the major virulence factors.[4]

Some strains of *C. difficile* produce an additional toxin, binary toxin (actin-specific ADP-ribosyltransferase, CDT), first reported in 1988 but not considered important until now[1,2,5]. Binary toxin producers make up the majority of strains isolated in the large outbreaks of disease overseas.[1,2] Barbut *et al.*[5] showed a correlation between binary toxin production and severity of diarrhoea, and more community-acquired CDAD was caused by binary toxin producers. However, the significance of binary toxin clearly needs further investigation. Although supernatants from $A^-B^-CDT^+$ strains of *C. difficile* caused fluid accumulation in a rabbit ileal loop after concentration and trypsinisation, challenge of clindamycin-treated hamsters with these strains resulted in colonisation but not diarrhoea or death.[6]

A second important feature of this "new" organism is that it produces more toxins A and B than other strains. Production of these toxins in *C. difficile* is encoded by the 8.1 kb *tcdA* and 7.9 kb *tcdB* genes, respectively. These two genes form part of a highly stable 19.6 kb pathogenicity locus (PaLoc) which also includes *tcdC, tcdD* and *tcdE.* Toxin A variant strains fail to produce detectable toxin A by enzyme immunoassay (EIA) because of a deletion in the *tcdA* gene. The *tcdC* gene is a putative down regulator of toxin A and B production. The PCR ribotype 027/NAP1 strain has a deletion in the *tcdC* gene resulting in it no longer down regulating and strains produce toxin throughout log phase of growth instead of just stationary phase.[7] Non-toxigenic strains lack the PaLoc.

The third important feature of these strains is that they are resistant to fluoroquinolone antibiotics, and excessive fluoroquinolone use appears to be a contributing factor in the recent outbreaks.[8] Another significant finding from the outbreaks reported overseas is the marked variation in CDAD rates among different age groups. While the elderly have always been at increased risk of CDAD, due primarily to decreased host defences, rates in persons ≥65 years of age have increased dramatically since 2000.[9] One possible novel risk factor is exposure to gastric acid suppressants such as histamine-2 receptor inhibitors or proton pump inhibitors. These agents have been more commonly prescribed in recent years and may be associated with increased rates of CDAD in the community,[10] although some case-control studies with hospital patients show no association.[1,8] The importance of community onset CDAD was highlighted recently by a report of severe CDAD in previously healthy persons and peripartum women.[11]

The new quinolone antimicrobials have significantly better anti-anaerobe activity than ciprofloxacin and are likely therefore to have a greater impact on colonisation resistance.[12] As mentioned above, it is possible that this issue, as well as increasing resistance of *C.difficile*

strains to the quinolones, is contributing to the significant increase in *C. difficile* diarrhoea worldwide.[13] The recent reports from Canada and the USA suggest that a strain of *C. difficile* has emerged that is both resistant to quinolones and a hyper-producer of toxins A and B, as well as producing binary toxin.[2,14]

One possible source of *C. difficile* in the community is animals. *C. difficile* has been associated with enteric disease in a variety of animals, including horses, pigs, cats and dogs.[15-17] Although it is not yet completely clear, it is possible that in all these situations excessive antibiotic exposure is driving the establishment of *C. difficile* in animals, in a manner analogous to human infection, rather than the organism just being normal flora of the animal gastrointestinal tract. Of great significance to Meat & Livestock Australia (MLA) are recent reports that *C. difficile*, including the epidemic ribotype 027, has been isolated from both calves[18] and retail meat samples[19] in Canada. *C. difficile* was isolated from 20% of 60 retail meat samples collected over a 10 month period in 2005. Clearly these meat samples were contaminated by *C. difficile* present in the bovine gastrointestinal tract. What risk such contamination poses for food-borne transmission of *C. difficile*, and the role of antibiotics in animal carriage of *C. difficile*, is unknown.

Some more recent developments are even more alarming. A second highly virulent strain of *C. difficile*, PCR ribotype 078, has emerged as a significant threat to human health. Ribotype 078 also produces more toxins A and B, is binary toxin positive but remains susceptible to fluoroquinolone antimicrobials. The association between ribotype 078 and animals is much stronger than for 027, and ribotype 078 strains have started to be isolated from humans in the USA.[20] In a survey of retail meat products in the USA undertaken in 2007, but only published in 2009, over 40% of 88 products contained *C. difficile*. More than 50% of beef products were contaminated, the majority with ribotype 078.[21] In The Netherlands, since 2005, there has been an increase in prevalence of human *C. difficile* infection with ribotype 078 strains. These infections were in a younger population and more frequently community acquired. In the eastern part of The Netherlands where >90% of pig farms are located, >20% of human isolates are now ribotype 078, and human and pig strains of *C. difficile* are highly genetically related.[22]

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 the previous investigation (A.MFS.0124) that looked at *C. difficile* prevalence on carcase and gut content samples of cattle. Food Science Australia has undertaken a national survey of cattle for STEC. This project will utilize the same faecal samples that were collected for the STEC project to study the prevalence of *C. difficile.*

2 Study aims

1. To undertake a survey of Australian cattle for *C. difficile* and determine the prevalence and concentration using faecal samples collected by Food Science Australia.

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 Methods

Bacteria

An isolate of *C. difficile* PCR ribotype 027 was obtained from Dr Luis Arroya at the University of Guelph, Canada. This and a fluoroquinolone resistant local isolate of *C. difficile* (WA15) were used as controls.

Specimens

Samples of adult cattle faeces (approx. 50 g) were collected by Food Science Australia from various cattle properties throughout Australia. Samples were coded so as the sites of collection were not known to the investigators. Samples were transported to Perth as soon as possible and processed within 48 hours.

Culture for *C. difficile*

The method to isolate *C. difficile* was based on our previously described methods[23] 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.

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).[24]

Toxin gene B PCR assay

Some faecal samples were tested directly for the presence of toxin B gene DNA by a PCR assay, based on that previously described by Kato *et al*.[25] One fragment from the non-repeating region of toxin B was amplified by real time PCR.

Toxin typing 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.[25,26] Organisms were also PCR ribotyped[27] (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.

4 Results

1) Faecal samples

A total of 280 faecal samples were cultured. *C. difficile* was isolated from 5 samples (approx. 2%). Details are shown in Table 1.

Table 1. Positive samples giving data on sampling date, location, feed type and toxigenic status of isolates.

Code #	Isolate #	Abattoir Code	Feed type	State	Sampling date	Toxin status	tcdE
AI25	29	F	Grass	NSW	1/10/2008	A-B-Cdt-	-
AI26	41	I	Grain	QLD	9/10/2008	A-B-Cdt-	-
AI27	48	J	Grass	NSW	9/10/2008	$A^{-}B+Cdt+$	-
AI28	58	L	Grass	QLD	8/10/2008	$A+B+Cdt^{-}$	+
AI34	245	S	Grass	VIC	5/02/2009	A-B-Cdt-	-

2) Toxin gene B PCR assay

All direct toxin B gene PCR assays were negative.

3) Isolate typing

Ribotyping patterns for Australian cattle isolates of *C. difficile*, compared to known ribotypes, are shown in Fig. 1. All ribotyping patterns were different to each other and none matched any of the common ribotypes found in Australia. None was ribotype 027 nor 078.

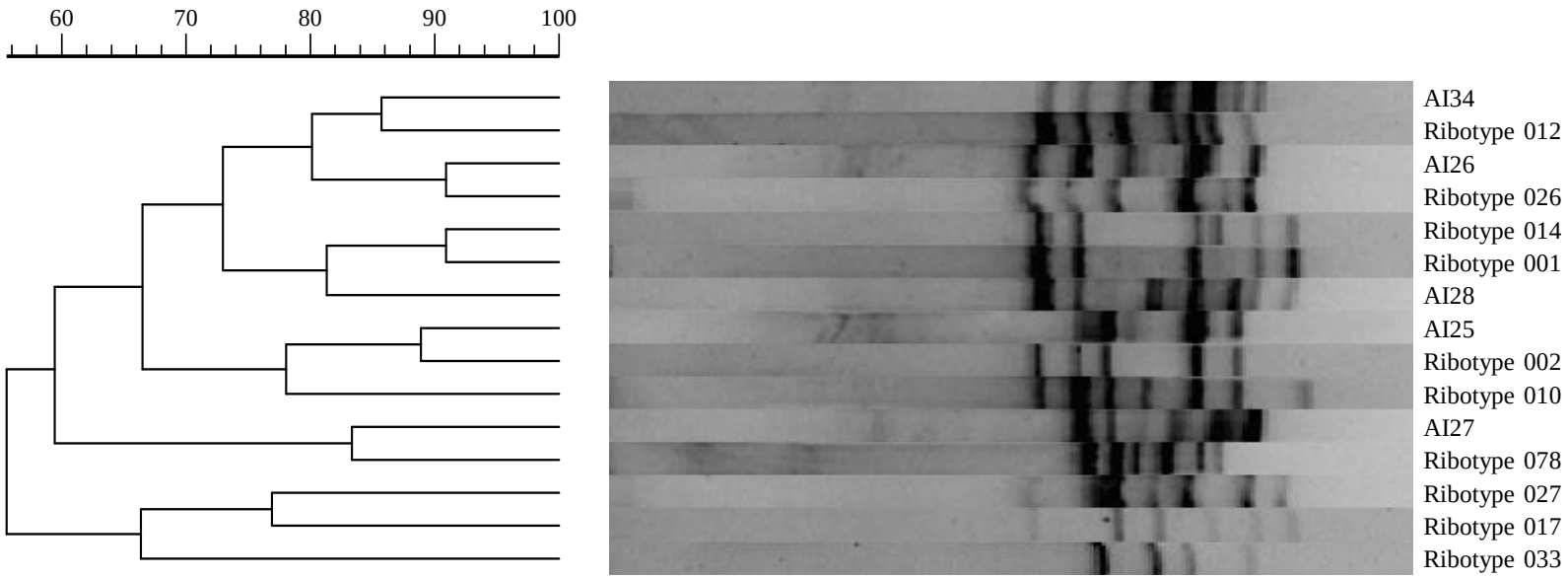

Fig. 1. Ribotyping patterns for Australian cattle isolates of *C. difficile*, compared to known ribotypes.

5 Conclusions

There is a growing body of evidence that many neonatal or infant animals are colonized with *C. difficile*, including cattle.[28] Whether such colonization continues beyond the infant period may well depend on exposure to antimicrobials. The results of this study represent the first time, to our knowledge, that *C. difficile* has been isolated from Australian cattle. While the overall prevalence was low (approx. 2%) this is nonetheless an important observation. It was interesting that none of the 5 isolates matched any of the ribotypes commonly found in cattle and retail meat products[21] overseas. It may be worthwhile trying to follow-up these positive animals to see if they had been given any antimicrobials during their life and, if so, what antimicrobials. The question we still need to ask is what risk does this low prevalence of *C. difficile* in Australian cattle pose to the consumer, and possibly workers in the industry. Given our previous investigation (A.MFS.0124) failed to find any *C. difficile* in 150 carcase washings and 150 gut content samples from WA it would appear that little contamination of Australia meat is occurring, probably as a result of better slaughtering practices in this country compared to the USA. Thus the risk to the consumer is likely to be extremely low. As with many infectious disease issues, *C. difficile* in animals is an evolving situation. Ten years ago there was little evidence that *C. difficile* was as widespread in animals as it currently appears to be. Clearly, if *C. difficile* becomes established in animal populations in Australia, as appears to be the case in pigs in The Netherlands,[22] this may pose a risk to humans, not necessarily through consumption of food but more likely through environmental contamination. The lower population density in this country may lessen this risk. The amplification of *C. difficile* in humans and animals is driven by antimicrobial use. Australia's conservative policies thus far regarding fluoroquinolone use in humans and animals may offer us some protection against epidemic strains of *C. difficile* circulating in North America and Europe, however, if cephalosporins are driving *C. difficile* infection in animals, additional effort may be required to target cephalosporin use in veterinary medicine.

6 References

1. Loo VG, Poirier L, Miller MA. et al. A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. *N Eng J Med* 2005; 353: 2442-2449.
2. McEllistrem MC, Carman RJ, Gerding DN, et al. A hospital outbreak of *Clostridium difficile* disease associated with isolates carrying binary toxin genes. *Clin Infect Dis* 2005; 40: 265-272.
3. Kuijper Ed J, van den Berg RJ, Debast S, et al. *Clostridium difficile* ribotype 027, toxinotype III, the Netherlands. *Emerg Infect Dis* 2006; 12: 827-830.
4. Riley TV. Nosocomial diarrhoea due to *Clostridium difficile*. *Curr Opinions Infect Dis* 2004; 17: 323-327.
5. Barbut F, Deere D, Lalande V, et al. Clinical features of *Clostridium difficile*-associated diarrhoea due to binary toxin (actin-specific ADP-ribosyltransferase)-producing strains. *J Med Microbiol* 2005; 54: 181-185.
6. Geric B, Carman RJ, Rupnik M, et al. Binary toxin-producing, large clostridal toxin-negative *Clostridium difficile* strains are enterotoxic but do not cause disease in hamsters. *J Infect Dis* 2006; 193: 1143-1150.
7. Warny M, Pepin J, Fang A, et al. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *Lancet* 2005; 366: 1079-1084.
8. Pepin J, Saheb N, Coulombe M-A, et al. Emergence of fluoroquinolones as the predominant risk factor for *Clostridium difficile*-associated diarrhea: a cohort study during an epidemic in Quebec. *Clin Infect Dis* 2005; 41: 1254-1260.
9. McDonald LC, Owings M, Jernigan DB. *Clostridium difficile* infection in patients discharged from US short-stay hospitals, 1996-2003. *Emerg Infect Dis* 2006;12: 409-415.
10. Dial S, Delaney JAC, Barkun AN. et al. Use of gastric acid-suppressive agents and the risk of community-acquired *Clostridium difficile*-associated disease. *JAMA* 2005; 94: 2989-2995.
11. Centers for Disease Control and Prevention. Severe *Clostridium difficile*-associated disease in populations previously at low risk – four States, 2005. *MMWR Morb Mortal Wkly Rep* 2005; 54: 1201-1205.
12. Hernandez F, Khorana AA, Scott JD, Feuerstein S, Forrest A, Freer JP. Incidence of *Clostridium difficile* colitis in patients treated with antibiotics in a community hospital setting, with emphasis on trovafloxacin. SHEA abstract 1999.
13. Gerding D. Clindamycin, cephalosporins, fluoroquinolones, and *Clostridium difficile*-associated diarrhea: this is an antimicrobial resistance problem. *Clin Infect Dis* 2004; 38: 646-8.
14. Pepin J, Valiquette L, Alary ME, Villemure P, Pelletier A, Forget K, Pepin K, Chouinard D. *Clostridium difficile*-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. *CMAJ* 2004; 171: 466-72.
15. Baverud, V. *Clostridium difficile* diarrhea: infection control in horses. *Vet Clin Equine* 2004; 20: 615-630.
16. Songer JG, Post KW, Larson DJ, Jost BH, Glock RD. Infection of neonatal swine with *Clostridium difficile*. *Swine Health Prod* 2000; 4: 185-189.
17. Riley TV**,** Adams JE, O'Neill GL, Bowman RA. Gastrointestinal carriage of *Clostridium difficile* in cats and dogs attending veterinary clinics. *Epidemiol. Infect.* 1991: 107: 659-665.
18. Rodriguez-Palacios A, Stampfli H, Duffield T, et al. *Clostridium difficile* PCR ribotypes in calves, Canada. *Emerg Infect Dis* 2006; 12: 1730-6.

19. Rodriguez-Palacios A, Stampfli H, Duffield T, Weese JS. *Clostridium difficile* in retail ground meat, Canada. *Emerg Infect Dis* 2007; 13: 485-7.
20. Jhung MA, Thompson AD, Kilgore GE, et al. Toxinotype V *Clostridium difficile* in humans and food animals. *Emerg Infect Dis* 2008; 14: 1039-44.
21. Songer JG, Trinh HT, Killgore GE, et al. *Clostridium difficile* in retail meat products, USA, 2007. *Emerg Infect Dis* 2009 ; 15 : 819-21.
22. Goorhuis A, Bakker D, Corver J, et al. Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. *Clin Infect Dis* 2008; 47: 1162-70.
23. Bowman RA, Riley TV. The laboratory diagnosis of *Clostridium difficile*-associated diarrhoea. *European J Clin Microbiol Infect Dis* 1988; 7: 476-484.
24. Bowman RA, Arrow SA, Riley TV. Latex particle agglutination for detecting and identifying *Clostridium difficile. J Clin Pathol* 1986; 39: 212-214.
25. 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. *J Clin Microbiol* 1998; 36:2178-82.
26. Stubbs S, Rupnik M, Gibert M, et al. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. *FEMS Microbiol Lett* 2000; 186: 307-312.
27. 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. *J Clin Microbiol* 1999; 37: 461-463.
28. Rupnik M. Is *Clostridium difficile*-associated infection a potentially zoonotic and food-borne disease? *Clin Microbiol Infect* 2007; 13: 457-9.