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Clostridium difficile in sheep and lambs in Australia

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Executive summary

Recently, *Clostridium difficile* has been isolated from a wide variety of animals, particularly production animals, mainly cattle and pigs. 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. Interestingly, sheep and lambs appear not to have been investigated for carriage/colonisation with *C. difficile*. The aim of this project was to determine the prevalence of carriage of *C. difficile* in sheep and lambs in Australia by culturing faecal samples. A total of 300 sheep and lamb faecal samples were received in 4 batches from 3 different geographic areas in eastern Australia. The overall rate of detection in sheep and lambs was low, however, carriage/colonisation in lambs (4.2%) was statistically significantly higher than in sheep (0.6%) ($p=0.04$). In terms of toxin profiles, four isolates were A+B+Cdt-, with a single isolate each of A-B+Cdt+, A-B+Cdt- and A+B+Cdt+, thus showing remarkable diversity for such a small group of isolates. Seven different ribotyping patterns were generated, none of which matched any of the nearly 100 reference strains available for comparison. This low rate of carriage/colonisation suggests that sheep and lambs are unlikely to be a major source/reservoir of human infections, however, certain patient groups such as the elderly or immunosuppressed are always going to be at greater risk. Lastly, workers in the industry, particularly abattoir workers who might be exposed to gut contents and who are taking antimicrobials that perturb their gut flora, will be at increased risk of infection with *C. difficile*.

Contents

1 Background

A highly virulent strain of *Clostridium difficile* (called PCR ribotype 027 in Europe and NAP1 in the USA) has emerged in North America and Europe over the last 10 years (1-3). 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* infection (CDI) 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 CDI was caused by binary toxin producers. However, the significance of binary toxin 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).

This "new" organism produces more toxin A and B than other strains (7) as do many strains of *C. difficile*. 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, tcdR* and *tcdE.* The *tcdC* gene is a putative down regulator of toxin A and B production. Strains of C. difficile that produce more toxins have a deletion in the *tcdC* gene resulting in it no longer down regulating and these strains produce toxin throughout log phase of growth instead of just stationary phase. Non-toxigenic strains lack the PaLoc. Perhaps more important is the fact that ribotype 027 is resistant to fluoroquinolone antibiotics, and excessive fluoroquinolone use appears to be a contributing factor in the recent hospital outbreaks in humans (8). Another significant finding from overseas is an apparent increase in community-acquired CDI in the absence of classic risk factors such as antibiotic exposure, leading to suggestions that all patients with community-acquired diarrhoea should be tested for

C. difficile. Assertions that community-acquired *C. difficile* infection is a new disease are not correct — it has been recognised in Australia for over 25 years but is under-diagnosed (8). Therefore, it is difficult to determine whether this increase is a true increase or rather reflects better case ascertainment. Nonetheless, the suggestion that *C. difficile* infection should be considered more than just a hospital problem is valid, and general practitioners need to be aware of this change in epidemiology. The importance of community onset CDI was highlighted recently by a report of severe CDAD in previously healthy persons and peripartum women (9).

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 (10-12). Although it is not yet completely clear, it is likely 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. *C. difficile*, including the epidemic ribotype 027, has been isolated from both calves (13) and retail meat samples (14) 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* is still unknown, however, the possibility of food-borne transmission of *C. difficile* is being explored in the literature (15) and therefore food production industries in Australia need to understand the risks. Currently, there are no data on the prevalence of *C. difficile* carriage in Australian sheep and lambs.

2 Project objectives

The objectives of this project were three fold:

1) To undertake a survey of Australian sheep and lambs for *C. difficile* presence and determine the prevalence and concentration in 3 geographic regions.

2) To type *C. difficile* isolates recovered to see if there is any relationship with human isolates in Australia.

3) To assess any risk of food-borne transmission of *C. difficile* from contamination.

3 Methodology

3.1 Samples

A total of 300 sheep and lamb faecal samples were received in 4 batches from 3 different geographic areas in eastern Australia.

3.2 Culture for *C. difficile*

Attempts to isolate *C. difficile* were made based on our previously described methods (16) with some modifications. Faecal samples were cultured both directly on cycloserine cefoxitin fructose agar (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 h incubation, all enrichment broths were sub-cultured onto CCFA 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) (17). A PCR assay for the 16S rRNA gene of *C. difficile* was also used for confirmation (18). Amplification was performed in reaction volume of 50 µL with 1× reaction buffer (GeneAmp; 10 mM Tris-HCL [pH 8.3], 50 mM KCL), 3 mM $MgCl_2$, 200 µM of each dNTP, 0.3 µM of each primer (16S-PG48 and 16S-B), 0.1 mg/mL BSA, 2 units AmpliTaq Gold® DNA polymerase, and 5 µL of DNA template. The PCR program consisted of an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C, followed by a final extension step of 10 min at 72°C.

3.4 Toxin profiling

The toxigenic status of isolates was determined by a PCR assay, previously described by Kato *et al*. (19). One fragment from the non-repeating region of toxin B was amplified while two fragments from toxin A were amplified from both the non-repeating and repeating (rep) regions of the gene. Isolates that yielded a product of 250 bp from the *tcdA* primers NK3-NK2 and an approximately 1300 bp product from the *tcdA* rep primers NK11-NK9 were designated toxin A-positive. Isolates that did not produce a 250 bp *tcdA* product were designated toxin A-negative. Those that produced a smaller *tcdA* rep product, or no *tcdA* rep product at all were further

investigated. Four *C. difficile* reference strains were used as controls for the toxin gene PCR assays: VPI 10463 (*tcdA*$^+$ *tcdB*$^+$ *cdtAB*$^-$), 48489 (*tcdA*$^+$ *tcdB*$^+$ *cdtAB*$^+$), SE 844 (*tcdA*$^+$ *tcdB*$^+$ *cdtAB*$^+$) and 1470 (*tcdA*$^-$ *tcdB*$^+$ *cdtAB*$^-$).

The reaction mixes (total volume 20 µL) consisted of 10 mM Tris-HCl (pH 8.3) and 50 mM KCl (supplied in 1x reaction buffer, GeneAmp® II), 2.5 mM (*tcdA* rep) or 2 mM of $MgCl_2$ (*tcdA*, *tcdB*, *cdtA*, *cdtB*), 0.2 µM of each primer, 50 µM of each dNTP, 0.75 units AmpliTaq Gold® DNA polymerase and 0.1 mg/mL BSA. The reaction mixes were cycled on the following program: initial denaturation step of 95°C for 10 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 90s, with a final extension step of 72°C for 7 min. A negative control consisting of sterile water, primers, dNTP pool, reaction buffer, $MgCl_2$, and *Taq* polymerase was included in each round.

Products for the *tcdA* rep assay were separated through 0.8% agarose with 0.5 µg/mL of ethidium bromide for 40 min at 7.33 V/cm. Products from the *tcdA*, *tcdB*, *cdtA*, and *cdtB* assays were fractionated in 2.5% agarose for approx. 18 min at 12 V/cm. A 100 bp DNA ladder was included on all gels for normalisation. Products were visualised under UV light and digitally photographed with a Gel Doc 2000 system (Bio-Rad).

3.5 PCR ribotyping

The method described by O'Neill *et al*. (20) and Stubbs *et al*. (21) was used to type isolates. Using primers complementary to the 3' end of the 16S rRNA gene, and the 5' end of 23S rRNA gene, the intergenic-spacer-region (ISR) was amplified. DNA was extracted using the Chelex method. The reaction mixes (total volume 50 µL) consisted of 10 mM Tris-HCl (pH 8.3) and 50 mM KCl (supplied in 1x reaction buffer GeneAmp® II), 4 mM $MgCl_2$, 100 µM of each dNTP, 3.75 units of AmpliTaq Gold® DNA polymerase, 0.4 µM of each primer and 10 µL of DNA extract. The mixes were cycled on the following program: initial cycle of 95°C for 10 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, and a final cycle of 72°C for 7 min. Before separation by agarose gel electrophoresis, products were concentrated to approximately 20 µL by heating at 75°C for 1 h. They were then run on 3% agarose gels with EthBr 0.5 mg/L for 4.5 h, at 5 V/cm and 5°C. A 100 bp DNA ladder was included for normalisation at 5 lane intervals. DNA fragments were visualised under UV light and photographed with a Gel Doc 2000 (Bio-Rad) imaging system. Ribotyping patterns were analysed with Bionumerics v. 6.5 (Applied Maths).

4 Results and discussion

4.1 Prevalence of carriage

Samples were received in four batches and the number of samples from which *C. difficile* was cultured is shown in Table 1.

Table 1: Rates of detection of *C. difficile* in sheep and lambs.

Faecal samples	Sheep (n & % positive)	Lambs (n & % positive)
Batch 1 (n=50)	1/27 (3.7%)	3/23 (13%)
Batch 2 (n=100)	0/47	1/53 (1.8%)
Batch 3 (n=50)	0/24	0/26
Batch 4 (n=100)	0/58	2/42 (4.2%)
Total (n=300)	1/156 (0.6%)	6/144 (4.2%)

The overall rate of detection was 2.3% and carriage/colonisation in lambs (4.2%) was statistically significantly higher than in sheep (0.6%) (Chi-squared test, p=0.04) (Table 1).

4.2 Toxin profiles

The toxin profiles of all *C. difficile* isolates are shown in Table 2.

Table 2: Toxin profiles of all *C. difficile* isolates.

Code number	Isolate number	Sheep/lamb	Toxin profile
AI 192	Sheep 18	Sheep	A-B+Cdt+
AI 193	Sheep 44	Lamb	A-B+Cdt-
AI 194	Sheep 45	Lamb	A+B+Cdt-
AI 195	Sheep 6	Lamb	A+B+Cdt-
AI 196	F11	Lamb	A+B+Cdt-
AI 202	Lamb 61	Lamb	A+B+Cdt+
AI 203	Lamb 90	Lamb	A+B+Cdt-

Four isolates were A+B+Cdt-, with a single isolate each of A-B+Cdt+, A-B+Cdt- and A+B+Cdt+, thus showing remarkable diversity for such a small group of isolates.

4.3 Ribotyping

Understandably, this diversity was also seen in the ribotyping patterns generated and shown in Figure 1. None of the patterns matched any of more than 100 reference strain patterns checked.

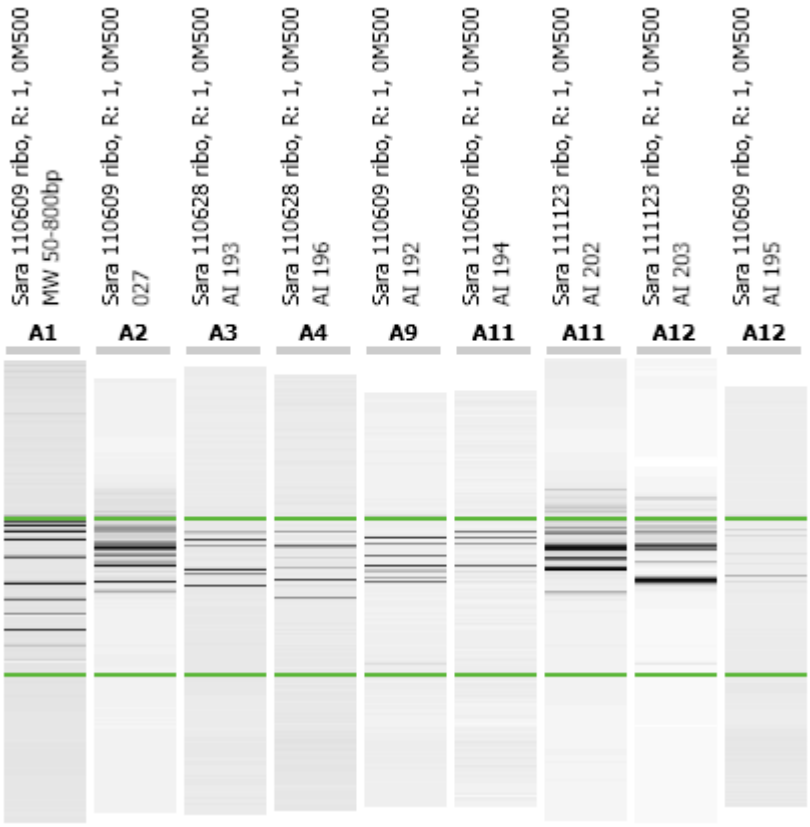

Figure 1: Ribotyping patterns of sheep and lamb isolates of *C. difficile*. Lane A1, molecular weight standard; lane A2, ribotype 027 reference strain; other lanes, various sheep and lamb isolates.

4.4 Discussion

In two previous studies conducted for MLA, we investigated the prevalence of *C. difficile* in Australian cattle. In the first of these (A.MFS.0124), 158 samples of gastrointestinal contents and 151 samples of carcass washings were processed. *C. difficile* was not isolated from any sample, either on direct culture or by enrichment culture. This study was undertaken in WA and, because of the possibility of regional variation, a second study (A.MFS.0157) was undertaken in Eastern Australia utilising samples collected by Food Science Australia who were conducting a national survey of cattle for STEC. Of 280 faecal samples processed, *C. difficile* was isolated from 5

(1.8%), a relatively low prevalence. All the samples from these two studies were collected from adult animals and various papers suggest that colonisation/carriage of *C. difficile* in young animals, particularly neonates, is much higher. For example, Rodriguez-Palacios *et al*. (13) studied faecal samples from 144 calves with diarrhoea and 134 control calves. *C. difficile* was isolated from 31 of 278 calves: 11 (7.6%) of 144 with diarrhoea and 20 (14.9%) of 134 controls (p = 0.009). Our own studies of neonatal piglets in WA showed an even higher prevalence of *C. difficile*, 62% (114/185), albeit from a single farm (Squire and Riley, unpublished).

In the present study both sheep and lambs were investigated. The overall rate of detection was low (2.3%) and the rate of detection in lambs (4.2%) was significantly higher than in sheep (0.6%). Given that a lamb may be aged up to 12 months, it is highly likely that neonatal lambs, like other neonates, will have an even higher rate of carriage/colonisation. Interestingly, this appears to be the first study in the world on the carriage of *C. difficile* by sheep and lambs. Only one previous study has looked for *C. difficile* in retail meat from sheep and found none, although the sample size (n=7) was very small (22).

The isolates of *C. difficile* obtained in this study were quite interesting. For a relatively small group of isolates (7), there was significant diversity in toxin profile and ribotype. Of the 6 possible combinations of the toxin genes, A, B and Cdt (binary toxin), 4 were represented in the 7 isolates (Table 2). These were A+B+Cdt- (4 isolates), with a single isolate each of A-B+Cdt+, A-B+Cdt- and A+B+Cdt+. As a corollary to this finding, 7 different ribotyping patterns were seen (Figure 1). The toxin profile A-B+Cdt+ is extremely rare in *C. difficile* from animals outside Australia, however, all 114 isolates from neonatal piglets mentioned above (Squire and Riley, unpublished) had this pattern and belonged to a new ribotype, 237. The fact that the ribotyping patterns generated did not match any of our more than 100 reference strain patterns, combined with the toxin profiles, suggests that these may be new ribotypes.

A recent collaborative study with workers in the UK (23) has added further weight to the idea that Australia contains many unique strains of *C. difficile*. The major ribotype of *C. difficile* isolated from animals outside Australia is ribotype 078 that infects approximately 80% of cattle and 90-100% of pigs (24). This ribotype is now causing significant disease in humans in Europe and is now the 3rd most common human isolate in Europe (25). Ribotype 078 has never been isolated from an animal in Australia and is rarely isolated from humans. It is very distantly related to the other major clades of *C. difficile* circulating in the world as shown in Fig. 2 (26). However, many of our isolates in Australia, particularly animals isolates, belong to the same multi-locus sequence

type (MLST) as ribotype 078 (ST 11) and may in fact predate ribotype 078 in an evolutionary sense.

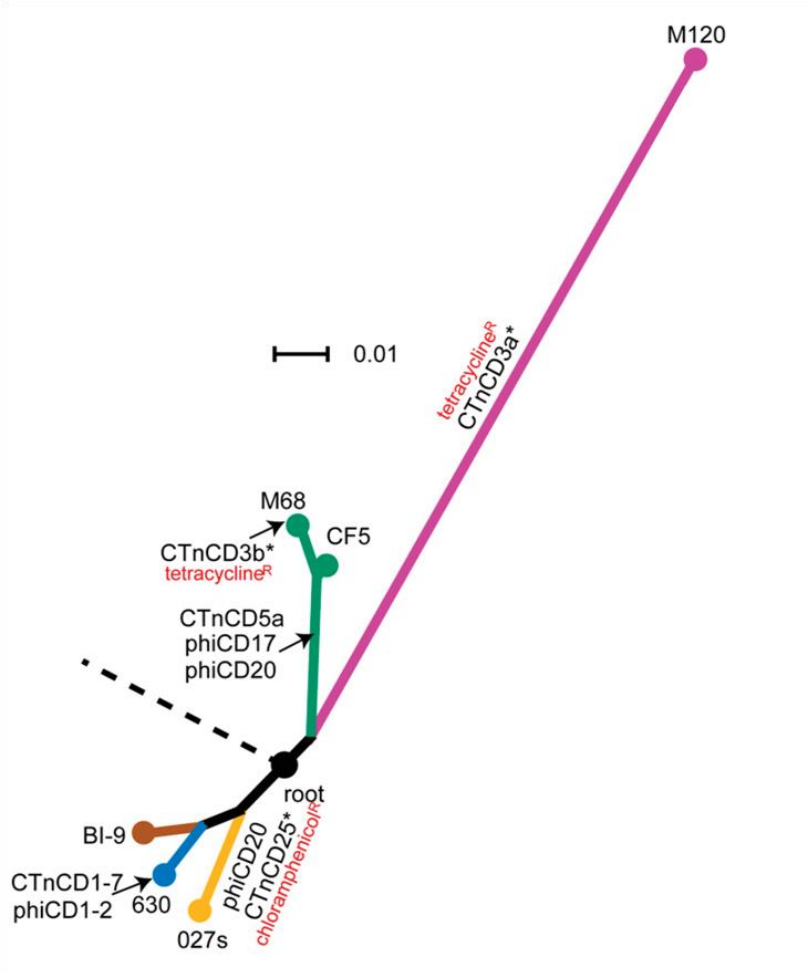

Figure 2. Phylogenetic tree of *C. difficile* based on whole-genome sequences
Deep-branching phylogeny illustrating the relationships between different lineages. Colour coding: brown, ribotype 001 (A^+B^+); blue, ribotype 012 (A^+B^+); green, ribotype 017 (A^-B^+, toxinotype VIII); pink, ribotype 078 (A^+B^+, toxinotype V). The root connects to *C. bartletti* and *C hiranonis*, fellow members of phylogenetic clade XI of the *Clostridium*. Arrows denote insertion events. Scale bar indicates number of substitutions per site. Image modified from He *et al*. (26).

5 Conclusions and recommendations

The low rate of carriage/colonisation found in this study suggests that sheep and lambs are unlikely to be a major source/reservoir of human infections, however, certain patient groups such as the elderly or immunosuppressed are always going to be at greater risk from exposure. Little is known about the infective dose of *C. difficile*.

Workers in the industry, particularly abattoir workers who might be exposed to gut contents and who are taking antimicrobials that perturb their gut flora, will be at increased risk of infection with *C. difficile*.

Given that some carriage/colonisation does appear to exist, the industry should not encourage further spread or expansion of *C. difficile* by injudicious use of antimicrobials, particularly cephalosporins, in animals. In addition, slaughtering practices that might lead to contamination of meat should be monitored.

Because some contamination of meat may not be preventable in the longer term if *C. difficile* becomes more established in production animals, some consideration should be given to investigating the minimum requirements for killing *C. difficile* spores during the cooking process and/or other ways of protecting meat and meat products from contamination.

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