Aspects of *Clostridium difficile* infection in pigs

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*This thesis is presented in fulfilment of the requirement for the degree of Doctor of Philosophy at The University of Western Australia*

School of Biomedical Sciences

Faculty of Health and Medical Sciences

2017
THESIS DECLARATION

I, Peter Moono, certify that:

This thesis has been substantially accomplished during enrolment in the degree.

This thesis does not contain material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution.

No part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of The University of Western Australia and where applicable, any partner institution responsible for the joint-award of this degree.

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The research involving animal data reported in this thesis was assessed and approved by The University of Western Australia Animal Ethics Committee, Approval #: RA/3/500/75 and RA/3/100/1481.

The research involving animals reported in this thesis followed The University of Western Australia and national standards for the care and use of laboratory animals.
The work described in this thesis was funded by an Australian Government Research Training Program (RTP) Scholarship and the Australian Research Council.

Technical assistance was kindly provided by Dr Aleksandra Debowski and Ms Melissa Andrade for the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) assay Chapter 8; Dr Briony Elliott provided training for the ribotyping techniques described in Chapter 3.

This thesis contains published work and/or work prepared for publication, some of which has been co-authored.

Signature: Peter Moono
Date: 7.11.2017
ABSTRACT

Clostridium difficile infection (CDI) is a well-known cause of gastrointestinal tract disease in humans undergoing antimicrobial therapy. C. difficile colonises the gut after disruption of the endogenous microbiota by antimicrobials, proton pump inhibitors, use of an endoscope, and stress. Recently, CDI has been recognised as a cause of neonatal enteritis in food animals such as pigs. Piglets with CDI have a stunted growth, delayed weaning, and increased mortality, including colitis which is a common clinical outcome in ostriches and horses. C. difficile is a strictly anaerobic, spore-forming bacterium, which produces two major toxins, A (TcdA) and B (TcdB), as its main virulence factors. The majority of strains isolated from animals produce an additional binary toxin (C. difficile transferase, CDT) that is associated with increased virulence. C. difficile is ubiquitous in the
environment and has a wide host range. This project summarises the epidemiology, clinical presentations, risk factors and potential zoonotic transmission of CDI, particularly in relation to pigs. Furthermore, the findings of this thesis highlight the urgent need for increased collaborative efforts on surveillance of CDI among different disciplines that include veterinarians, medical practitioners and public health workers to prevent zoonotic transmission of *C. difficile*.

Although zoonotic transmission of *C. difficile* has not been proved, there is good circumstantial evidence that transmission of *C. difficile* from animals or animal products to humans is occurring. Following on from an initial study that identified *C. difficile* ribotype (RT) 237 in a piggery in Western Australia, I conducted cross-sectional and cohort studies to determine if RT 237 had persisted in the piggery and whether there had been a temporal change in *C. difficile* diversity. *C. difficile*
carriage in litters with and without diarrhoea was investigated, as was the acquisition of *C. difficile* over time. Rectal swabs were obtained from piglets aged 1-10 days to determine the prevalence of *C. difficile* carriage and samples were obtained from 20 piglets on days 1, 7, 13, 20, and 42 of life to determine the duration of shedding. Isolation of *C. difficile* from faeces was achieved by selective enrichment culture. All isolates were characterised by standard molecular typing. Antimicrobial susceptibility testing was performed on selected isolates (n = 29). Diarrhoeic piglets were more likely to shed *C. difficile* than the non-diseased (*p* = 0.0124, $\chi^2$). In the cohort study, *C. difficile* was isolated from 40% of samples on day 1, 50% on day 7, 20% on day 13, and 0% on days 20 and 42. All isolates were RT 237 and no antimicrobial resistance was detected. The decline of shedding of *C. difficile* to zero has public health implications because at the slaughter age for pigs (> 3
months) there is little likelihood of spreading *C. difficile* to consumers via pig meat.

Although *C. difficile* is a well-established hospital pathogen, it is increasingly being detected in patients without hospital contact. Given this rise in community-associated infections with *C. difficile* (CA-CDI), it was hypothesised that the environment could play an important role in the transmission of spores. Lawn samples (n = 311) collected in public spaces in the metropolitan area of Perth, Western Australia, from February to June 2016 were cultured for *C. difficile*. *C. difficile* was isolated from the samples by selective enrichment culture and characterised by standard molecular methods using toxin gene PCR and ribotyping. The overall prevalence of *C. difficile* was 59%, and new lawn was twice as likely as old lawn to test positive (OR = 2.3; 95%CI 1.16-4.57, *p*=0.015) and 35 *C. difficile* RTs were identified with RT
014/020 (39%) predominating. These results provide an important finding that lawn in Perth, Western Australia, harbour toxigenic *C. difficile*. The source of lawn contamination is likely related to the modern practice of producing “roll-out” lawn grown (in Western Australia) on pig manure. Further work should focus on identifying specific management practices that lead to *C. difficile* contamination of lawn to inform prevention and control measures.

Animal manure is widely used in agriculture and landscaping, despite public health concerns that manure could harbour pathogenic microorganisms. The standard for relatively safe manure is generally determined by the absence or low prevalence of indicator microorganisms such as enterotoxigenic *Escherichia coli* (ETEC) and *Salmonella*. The use of these indicator microorganisms as a measure of biosafety of manure could be misleading.
This is because of high variation of these microorganisms in faeces within and between farms. Moreover, even manure without detectable indicator microorganisms can harbour other pathogenic microorganisms. Farm soils with or without exposure to manure, pig farm influent (raw manure), and effluent from seven farms representing 4 states in Australia were tested for *C. difficile*. In addition, soil conditioning products that included manure, compost, and human biosolids from retail shops were studied. The farm level of *C. difficile* prevalence was 100%. The overall prevalence of *C. difficile* was 72.5% from 40 environmental samples representing seven farms. The prevalence of *C. difficile* in the soil (effluent irrigated) was 90.9%. The prevalence of *C. difficile* in gardening products varied from 0% to 75%. RTs 014/020 and 237 were recovered from effluent at pig farms and in retail compost, manure, and human biosolids suggesting that transmission of *C. difficile* to humans could be through
contact with manure. These results suggest that animal manure could be a vehicle for *C. difficile* dispersal in the environment and could contribute significantly to CA-CDI.

To break the transmission and infectious cycle of *C. difficile*, a toxoid vaccine comprising the main virulence factors of *C. difficile* was developed. Progeny of sows vaccinated against CDI had a lower *C. difficile* prevalence (15.9%) compared to control piglets (33%). The serum IgG-anti-TcdA in vaccinated gilts was significantly higher than control gilts (*p* < 0.05). High serum IgG against TcdA in humans has been associated with less severe disease. This appeared to be the case in piglets, as vaccinated gilts conferred passive immunity to piglets, reducing colonisation by *C. difficile*.

There are three major public health implications of the work reported in this thesis. First, identifying farms
without \textit{C. difficile} is an important step in preventing human exposure to \textit{C. difficile} from animal manure. Second, screening manure for \textit{C. difficile} and composting \textit{C. difficile} in free manure will prevent accumulation of spores in manure intended for vegetables or landscaping. Third, vaccinating pigs has potential to reduce shedding of spores in faeces and, consequently, contamination of vegetables and/or lawns.
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AUTHORSHIP DECLARATION: CO-AUTHORED PUBLICATIONS

This thesis contains work that has been published and work which has been prepared for publication. In all the journal articles listed below, authorship contribution was based on the guidelines described in the National Health and Medical Research Council/Australian Vice-Chancellors’ Committee guidelines. This includes all the work where I made significant contribution such as i) study conceptualisation, study design, sample collection, sample processing, ii) data analysis and interpretation and iii) preparation and revising manuscript for submission to a journal. In all the manuscripts listed below, editorial support was provided by my co-authors.
Published articles

Chapter 2

1. Moono P, Foster NF, Hampson DJ, Knight DR, Bloomfield LE, Riley TV. *Clostridium difficile* infection in production animals and avian species: a review. Foodborne Pathogens and Disease 2016; 3:647-655.

*My contribution to this work (80%): designed manuscript outline, data collection, manuscript writing and critical reviewing.*

Chapter 4

My contribution to this work (80%): study design, sample collection, sample processing, data analysis and manuscript writing.

Chapter 7


My contribution to this work (80%): study design, sample collection, sample processing, data analysis and manuscript writing.
Papers submitted for publications

1. Moono P, Jenkin S, Foster NF, Hampson DJ, Riley TV. Outbreak of *Clostridium difficile* infection in suckling piglets in Eastern Australia.

   *My contribution to this work (80%): study design, sample processing (culture, molecular typing), data analysis and manuscript writing.*

2. Lim SC, Moono P, Riley TV. *Clostridium difficile* found in gardening products: innocent bystander or the cause of community-acquired *C. difficile* infection through contamination of foods and environments?

   *My contribution to this work (50%): study design, sample processing (culture, molecular typing), data analysis and manuscript writing.*

3. Moono P, Androga GO, McGovern AM, Roshan N, Hampson DJ, Riley TV. Passive immunotransfer
reduces *Clostridium difficile* carriage in suckling piglets.

*My contribution to this work (80%): Conceptualisation, study design, vaccine development, sampling (serum, colostrum and faeces), sample processing (culture, ELISA, SDS-PAGE), data analysis and manuscript writing.*

4. Lim SC, Androga GO, Knight DR, Moono P, Foster NF, Riley TV. Antimicrobial susceptibility of *Clostridium difficile* from diverse environmental sources.

*My contribution to this work (10%): Sample processing (culture), and manuscript editing.*

**Conference poster presentations**

1. Moono P, Putsathit P, Squire MM, Hampson DJ, Foster NF, Riley TV. Persistence of *Clostridium difficile* PCR ribotype 237 in a Western Australian commercial
piggery (Conference paper, 5th International *Clostridium difficile* Symposium, Bled, Slovenia, 19-21 May 2014, abstract number 51).

2. Lim SC, Moono P, Riley TV. *Clostridium difficile* found in gardening products: innocent bystander or the cause of community-acquired *C. difficile* infection through contamination of foods and environments? (Poster presentation at the Australian Society for Microbiology, 44th Annual Scientific Meeting, Perth July 2016, abstract number 333).

3. Moono P, Jenkin S, Foster NF, Hampson DJ, Riley TV. Outbreak of *Clostridium difficile* infection in suckling piglets in Eastern Australia. (Poster presentation at the Australian Society for Microbiology, 44th Annual Scientific Meeting, Perth, July 2016, abstract number 245).

I, Professor Thomas V. Riley certify that the student statements regarding their contribution to each of the works listed above are correct

Coordinating supervisor signature:
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APS</td>
<td>Ammonium per-sulfate</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BA</td>
<td>Blood agar</td>
</tr>
<tr>
<td>BHIB</td>
<td>Brain heart infusion broth</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CA-CDI</td>
<td>Community associated <em>C. difficile</em> infection</td>
</tr>
<tr>
<td>CDI</td>
<td><em>Clostridium difficile</em> infection</td>
</tr>
<tr>
<td>CDT</td>
<td>Binary toxin</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Deionised water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPA</td>
<td>US Environmental Protection Agency</td>
</tr>
<tr>
<td>ESCMID</td>
<td>European Society for Clinical Microbiology and Infectious Disease</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ETEC</td>
<td>Enterotoxigenic <em>E. coli</em></td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
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<tr>
<td>GEE</td>
<td>Generalised estimating equations</td>
</tr>
<tr>
<td>GDH</td>
<td>Glutamate dehydrogenase</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MLST</td>
<td>Multi-locus sequence typing</td>
</tr>
<tr>
<td>MLVA</td>
<td>Multilocus Variable-Number Tandem Repeat Analysis</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PaLoc</td>
<td>Pathogenicity locus</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffer saline in Tween</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMC</td>
<td>Pseudomembranous colitis</td>
</tr>
<tr>
<td>PS</td>
<td>Peptone saline solution</td>
</tr>
<tr>
<td>PSL</td>
<td>Public space lawn</td>
</tr>
<tr>
<td>PST</td>
<td>Peptone saline in Tween</td>
</tr>
<tr>
<td>RT</td>
<td>Ribotype</td>
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Aspects of *Clostridium difficile* infection in pigs

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline-Tween</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TMB</td>
<td>3, 3', 5, 5'-tetramethylbenzidine</td>
</tr>
<tr>
<td>μL</td>
<td>Microlitre(s)</td>
</tr>
<tr>
<td>μM</td>
<td>Micromole(s)</td>
</tr>
<tr>
<td>WA</td>
<td>Western Australia</td>
</tr>
<tr>
<td>WGS</td>
<td>Whole genome sequencing</td>
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CHAPTER 1: INTRODUCTION

*Clostridium difficile* is a Gram-positive anaerobic bacillus and the leading cause of antibiotic-associated diarrhea in humans and animals (Bartlett, 2008). Many outbreaks associated with *C. difficile* infection (CDI) have been reported in diverse regions of the world in hospitalised patients (van Beurden *et al.*, 2016; Loo *et al.*, 2005; Muto *et al.*, 2005) and in animals (chapter 4; Weese *et al.*, 2003; Waters *et al.*, 1998). *C. difficile* colonises the large intestine and the caeca when the endogenous microbiota has been disrupted (Lyras *et al.*, 2009; Jank and Aktories, 2008; Keel and Songer, 2007). *C. difficile* produces two major toxins, A (TcdA) and B (TcdB), as its main virulence factors (Jank and Aktories, 2008). Neonatal pigs with CDI may develop enteritis resulting in stunted growth, delays in weaning, and
mortality (Songer and Uzal, 2005). Other animals, including horses and large birds such as ostriches, can have colitis (Cooper et al., 2013).

The major risk factors for CDI in humans are (1) exposure to the *C. difficile* spores and (2) the use of antimicrobials that are known to disrupt the resident microbiota (Hurley and Nguyen, 2002). Conversely, in animals, the epidemiologic link between antibiotic exposure and CDI is not as clear. This has largely been confounded by practices such as metaphylaxis, where whole herds (or flocks) of animals are treated with antimicrobials (McEwen and Fedorka-Cray, 2002). More recently, the association between antibiotic exposure and *C. difficile* shedding has been reported in some animal species including veal calves, horses, captive ostriches, zebras and pumas (Magistrali et al., 2015; Álvarez-Perez et al., 2013; Silva et al., 2013; Arroyo
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*et al.*, 2005). On the other hand, the main known risk factor for CDI in animals is young age and high environmental contamination rates by *C. difficile* spores (Squire and Riley, 2013). This could explain the high *C. difficile* carriage rates and disease in neonatal pigs (Squire and Riley, 2013). The prevalence of *C. difficile* is reduced drastically as animals grow older, due to the establishment of resident microbiota.

Human neonates can have high rates of *C. difficile* carriage (~35%), but do not exhibit clinical disease (Pant *et al.*, 2016; Jangi and Lamont, 2010). This is because children are postulated to possess no receptors for *C. difficile* toxins and, in addition, colostral IgG is thought to play a protective role in children (Jangi and Lamont, 2010). However, within certain age groups of children (median age 6 years), mortality associated with CDI was reported as 1.42% in the exposed compared to 0.66% in the control group (*p* < 0.001,
Fisher exact $\chi^2$) (Sammons et al., 2013). However, others suggest that CDI in children is often confounded by co-infections because most of the studies do not actively look for pathogens apart from *C. difficile* (de Graaf et al., 2015). Similar to human neonates, not all neonatal animals colonised with *C. difficile* experience diarrhoea. The lack of disease manifestation in some neonatal animals has been attributed to the protective role of the humoral and innate immunity (Squire et al., 2013). For example, a study found that pregnant hamsters vaccinated with a *C. difficile* toxoid vaccine successfully transferred protective humoral immunity to their neonates (Kim et al., 1987). Serum antibodies such as IgG against toxin A have been associated with protection against clinical disease in asymptomatic people (Kyne et al., 2000). Another study found that an initial response to toxin A in infected patients during the first
episode of diarrhoea was associated with protection against
*C. difficile* recurrence (Kyne *et al.*, 2001). Overall, it appears
that developing a vaccine against *C. difficile* could be the
most cost-effective infection control and prevention strategy.

**Aims of the thesis**

The primary objective of this PhD project was to
investigate the epidemiology of *C. difficile* infection in pigs
and to determine the immunotherapeutic potential of a *C.
difficile* toxoid vaccine. To determine the vaccine targets, a
cross-sectional study was conducted with the following aim:
i) To determine if *C. difficile* RT 237 had persisted in a
piggery or whether there had been a temporal change in *C.
difficile* diversity.

Furthermore, to understand the natural infection of *C.
difficile* in piglets, a prospective cohort study of neonatal
piglets was conducted. The aims of this study were:
i) To investigate the epidemiology of *C. difficile* acquisition/shedding in a piggery in Western Australia.

ii) To determine factors which were significantly associated with *C. difficile* transmission within the piggery, including variables such as age, litter size, mortality, and diarrhoea.

In chapter 5, a *C. difficile* outbreak occurring in neonatal pigs in Eastern Australia was characterised. This was the first report of a *C. difficile* outbreak in a piggery in Australia. The aims of this study were to identify drivers for this outbreak and to provide preventive measures to minimise the possible future occurrence of CDI outbreaks.

The overarching objective of this report was to investigate the zoonotic potential of *C. difficile*. The prevalence of *C. difficile* in animal manure, compost, garden products obtained from retail shops and public space lawns was investigated. Furthermore, *C. difficile* spore
Aspects of *Clostridium difficile* infection in pigs contamination in pig environments, and farmland amended with pig manure, was characterised and quantitated. Second, the efficacy of mesophilic treatment of effluent in reducing *C. difficile* contamination by comparing *C. difficile* prevalence in treated effluent to the influent at pig farms was determined.

Furthermore, to determine the distribution of *C. difficile* in the environment, the prevalence of *C. difficile* in commercially produced lawns in public space was investigated. In this study, it was hypothesised that suburban expansion of Perth city was possibly responsible for the increase in the community-associated (CA)-CDI through the introduction of commercially-farmed lawns that used contaminated animal manure or compost.

The aim of this study was to i) determine the prevalence of *C. difficile* in newly established (NL) and older (OL) lawns
Aspects of *Clostridium difficile* infection in pigs

in public spaces in Perth and to characterise any *C. difficile* isolates by phenotypic and genotypic techniques, ii) investigate factors such as the location and size of the lawn to see if they could predict *C. difficile* status, and iii) quantitate the *C. difficile* viable in the lawn samples. This report demonstrates the importance of a “One animal health dialogue” to prevent zoonotic transmission of *C. difficile*.

Structure of the thesis

Chapter 2 describes the current literature on CDI in animals. An overview of the historical perspective of CDI in food and companion animals is provided. This review summarises the epidemiology, clinical presentations, risk factors and laboratory diagnosis of CDI in animals and describes the importance of collaborative work among public health workers and the livestock industry in preventing zoonotic transmission of *C. difficile*. Finally, this chapter
Aspects of *Clostridium difficile* infection in pigs provides an overview of methods for controlling environmental contamination and potential therapeutics available.

Chapter 3 describes the materials required and the general methods for *C. difficile* recovery and identification from faecal and environmental samples. In addition, this chapter describes *C. difficile* viable counts from soil samples exposed to animal effluent or manure and lawn samples. Phenotypic and genotypic methods for characterising *C. difficile* isolates are described. Furthermore, phenotyping techniques for antimicrobial susceptibility tests for clinical *C. difficile* isolates are also described. Finally, this chapter describes the protocol for producing a possible *C. difficile* vaccine including assessment of the antibody response using an indirect ELISA.
Chapter 4 describes two observational studies, a cross-sectional study and a prospective cohort study in a piggery in Western Australia. This chapter examines the relationship between \textit{C. difficile} colonisation and disease in various progenies of sows with varied parities. The prospective cohort study examines the relationship between age and \textit{C. difficile} shedding over time.

Chapter 5 describes the characterisation of a CDI outbreak in a piggery from the Eastern state in Australia. This chapter raises important issues such as recognising \textit{C. difficile} as an important pre-weaning infectious pathogen for piglets in Australia. This chapter also examines risk factors for CDI outbreaks in piglets and offers recommendations for infection control.

Chapter 6 examines the prevalence of \textit{C. difficile} in a pig farm environment: in farm soils amended with pig
Aspects of *Clostridium difficile* infection in pigs

effluents, and in compost and manure from various animal farms. This chapter provides recommendations to prevent contamination of farmland and/or public spaces with *C. difficile* spores.

Chapter 7 explores *C. difficile* contamination of lawn in public spaces within the metropolitan area of Perth. Since manure is used as a fertiliser in landscaping in many countries, it is possible that farmed lawns are fertilized with contaminated manure. Furthermore, this chapter examines the potential role played by lawns in occurrence of CA-CDI. Sampling was in suburban areas which have seen a high incidence of CA-CDI. Therefore, this chapter explores the epidemiological link between the environment and human CDI.

Chapter 8 examines the potential of passive immunotherapy using a toxoid vaccine for CDI in gravid
gilts. In the previous chapter, the environmental sources of CDI at pig farms, including soils amended with effluent, compost, and manure, were discussed. The prevalence of *C. difficile* infection is quite high (> 50%) in piglets. A plausible control method for CDI in animals and by extension in humans is a vaccination strategy in pigs. In this chapter, the development of a *C. difficile* toxoid vaccine that has potential to reduce *C. difficile* shedding in piglets is described. Vaccination, applied in combination with appropriate disinfection protocols, might break the infectious cycle of *C. difficile* in piggeries. In the second part of this report, the anamnestic response of gilts to the *C. difficile* toxoid vaccine compared to the controls is examined.

Chapter 9 summarises major findings in this report. This chapter brings together a general discussion and elaborates how chapters are interlinked and provides some
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in-depth interpretations of the results presented. Finally, this chapter describes limitations of the research and provides future research directions.
CHAPTER 2: LITERATURE REVIEW

2.1 INTRODUCTION

*Clostridium difficile* is a strictly anaerobic Gram-positive bacillus which is the leading cause of antibiotic-associated diarrhoea in humans (Bartlett, 2008) and an emerging animal pathogen (Keel and Songer, 2006). An important property of the bacterium is that it forms highly resistant spores that survive indefinitely on contaminated surfaces (Maillard, 2011; Kramer et al., 2006). *C. difficile* infection (CDI) is transmitted by the faecal-oral route through the ingestion of spores. The clinical presentation of CDI in humans and livestock varies from asymptomatic carriage to mild diarrhoea, severe diarrhoea and, sometimes, pseudomembranous colitis in humans (Britton and Young, 2014; Keessen et al., 2011a). The disease manifests, in large part, because of the activity of two exotoxins TcdA and
TcdB that are expressed in the gut by toxigenic strains of *C. difficile*. The presence of toxin receptors is required for toxin uptake by colonocytes (Keel and Songer, 2006). Different animal species vary in the type of toxin receptors present in the gut (Keel and Songer, 2006), but this does not correlate with disease severity (Best *et al.*, 2012). Additionally, some *C. difficile* strains, especially those commonly found in animals, produce a binary toxin (*C. difficile* transferase, CDT) that has been associated with enhanced virulence in human disease (Schwan *et al.*, 2009). Some strains also differ in the nucleic acid composition of *tcdC*, a toxin regulatory gene, and this is believed to result in increased toxin production (Merrigan *et al.*, 2010).

The incidence and severity of CDI in humans have increased over recent years. The epidemic or hypervirulent strain RT 027 is also known as North American pulsed-field...
Aspects of *Clostridium difficile* infection in pigs

gel electrophoresis type 1 (NAP1) or restriction endonuclease analysis group BI based on typing method. In Europe (Norén *et al.*, 2004), North America (Kuntz *et al.*, 2011), and Australia (Slimings *et al.*, 2014), this change has been attributed to the emergence since the early 2000s of a “hypervirulent” strain of *C. difficile*, ribotype (RT) 027 (NAP1/BI) that is fluoroquinolone resistant (Loo *et al.*, 2005). In addition, RT 078, a predominantly animal strain, is increasingly responsible for human infection in Europe (Goorhuis *et al.*, 2008; Bauer *et al.*, 2011). *C. difficile* RT 027 and RT 078 both produce all three toxins, TcdA, TcdB, and CDT (Bauer *et al.*, 2011; Merrigan *et al.*, 2010) and some strains have reduced susceptibility to various antibiotics used for treatment such as metronidazole (Álvarez-Perez *et al.*, 2013). Countries outside Europe and North America, including Australia, have seen a similar but
more recent rise in the incidence of CDI (Slimings et al., 2014). Furthermore, there have been reports of CDI in people who have not been exposed to traditional CDI risk factors such as antibiotics, hospitalisation, and living in an aged care facility (Khanna et al., 2013).

Strains of *C. difficile* recovered from different animal species and humans are indistinguishable by conventional molecular typing techniques including PCR ribotyping, multilocus sequence typing (MLST), and multilocus variable tandem repeat analyses (MLVA) (Knetsch et al., 2014; Bakker et al., 2010). More recently, this genetic overlap was further confirmed by whole genome sequencing and core genome single nucleotide polymorphism (SNP) typing, which showed that pigs and pig farmers were colonised by identical strains of *C. difficile* (Knetsch et al., 2014). These findings have raised concerns that cases of CDI could arise
by zoonotic transmission. Transmission could occur by direct contact with live animals or their environment, and during or after slaughter since *C. difficile* has been isolated from animals at slaughterhouses (Knight *et al.*, 2016; Knight *et al.*, 2013) and from retail meat (Lund and Peck, 2015). It is possible that meat and meat products could play a role in human CDI, although zoonotic transmission has not yet been conclusively proven.

### 2.2 Host susceptibility to CDI

As in humans, the intestinal microbiota is likely to play an important role in the susceptibility of animals to CDI. The intestinal microbiota prevents overgrowth of *C. difficile* and other enteric pathogens by competing for nutrition or acting as a mechanical blockade of enterocytes in a process that is often referred to as colonisation resistance (Britton and Young, 2014). Furthermore, the gut microbiome plays a role
in the deconjugation of taurocholate to chenodeoxycholate, a key component in inhibiting spore germination in the small intestine and cecum (Britton and Young, 2014; Giel et al., 2010), and the biosynthesis of secondary bile salts such as deoxycholate, that inhibit vegetative cell growth in the colon (Giel et al., 2010). When the commensal intestinal microbiota has been disrupted, there is increased production of cholate from bile salts that promotes spore germination (Giel et al., 2010). This was recently demonstrated in a murine model and expanded to human studies (Buffie et al., 2015). Mice treated with clindamycin developed an altered gut microbiome with a reduced ability to convert primary bile salts into secondary bile salts that correlated with susceptibility to infection by *C. difficile* (Buffie et al., 2015; Britton and Young, 2014).
Disruption of the normal gut microbiota by antibiotics is the best-known predisposing mechanism leading to *C. difficile* colonisation of the large intestine (Buffie *et al.*, 2015; Slimings and Riley, 2014), in particular, the second-, third-, and fourth-generation cephalosporins, penicillins, carbapenems, clindamycin, trimethoprim/sulphonamides and fluoroquinolones (Slimings and Riley, 2014). Mice treated with a single dose of clindamycin or a cocktail of antibiotics exhibited significant disruption of the microbiota and were susceptible to CDI up to 21 days post exposure to clindamycin (Buffie *et al.*, 2015). Hamsters are exquisitely susceptible to the infection and die very quickly after antibiotic and *C. difficile* exposure (Best *et al.*, 2012; Britton and Young, 2014) suggesting that they are not a particularly good model of human disease. An experimental study demonstrated that calves injected with either TcdA or TcdB,
or both, without antibiotics developed lesions consistent with CDI on histology (Hammitt et al., 2008). Recently, an epidemiological study in calves found an association between antibiotic exposure and *C. difficile* shedding (Magistrali et al., 2015). However, the association between antibiotic exposure and CDI has not been commonly reported in livestock. This is complicated by the likelihood that animals can develop diarrhoea associated with antibiotic therapy that is unrelated to CDI.

Apart from disrupting the normal gut microbiota, antibiotics play other key roles in spreading CDI. In one study, apparently healthy mice that were challenged with *C. difficile* spores became asymptomatic carriers shedding low numbers of spores (Lawley et al., 2009). When these asymptomatic carriers were treated with clindamycin, the concentration of *C. difficile* spores in their faeces increased.
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In turn, this amplification led to environmental contamination by spores and encouraged the transmission of infection to naïve mice (Lawley *et al.*, 2009).

Animals that rely on fermentation in the hindgut, known as pseudomonogastric animals (hamsters, horses, guinea pigs and rabbits), are highly dependent upon commensal bacterial populations for digestion of fibre. Interestingly, most pseudomonogastric animals are more susceptible to severe colitis and death associated with CDI compared with true monogastric animals (Keel and Songer, 2006). The evidence so far suggests antibiotics may play a significant role in precipitating colonisation of livestock by *C. difficile* and that antibiotics may escalate numbers of CDI in animals without clinical disease. It is possible that the impact of antibiotics on the normal gut microbiota for
pseudomonogastric such as hamsters is more sudden than for true monogastric animals.

2.3 Epidemiology of *C. difficile* in production animals

Although diarrhoea is common in neonatal livestock, there are potentially many pathogens that may be involved apart from *C. difficile*, such as enterotoxigenic *Escherichia coli* (ETEC), *C. perfringens*, *Coccidia* sp., *Cryptosporidium* sp., *Giardia* sp. and rotavirus, amongst others. Studies in Australia (Squire *et al.*, 2013), Europe and North America (Hammitt *et al.*, 2008; Yaeger *et al.*, 2002) have found that it is rare for other pathogens to be present with *C. difficile* in faecal samples, suggesting that *C. difficile* was associated with diarrhoea. However, the importance of screening for other pathogens when undertaking *C. difficile* surveys should not be ignored.
2.4 *C. difficile* in pigs

The earliest published report of natural infection with *C. difficile* in swine was that of two piglets diagnosed with enterocolitis in the 1980s (Jones *et al.*, 1983). A decade later there was a major outbreak of CDI at a swine farm in Canada with a weekly mortality rate in the range 7% to 58% in piglets aged 1-14 days (Waters *et al.*, 1998). *C. difficile* was isolated from faeces, and toxins were detected, although strain types were not determined. Post-mortem findings consistent with CDI, such as mesocolonic oedema and typhlocolitis, were common. The significance of CDI in piglets became prominent after a 5-month surveillance study of enteric pathogens in neonatal pigs at the Iowa Veterinary Diagnostic Laboratory (Yaeger *et al.*, 2002). This study showed a decline in the relative frequency of traditional enteric pathogens such as transmissible gastroenteritis virus,
Aspects of *Clostridium difficile* infection in pigs

*E. coli*, and *C. perfringens* type C from 70% to 21%, and an increase in *C difficile* (Yaeger *et al*., 2002).

The prevalence of *C. difficile* in piglets aged 1-2 weeks is reported in the range of 50% to nearly 100% (Table 2.1) (Rodriguez *et al*., 2016; Weese *et al*., 2010a; Norman *et al*., 2009; Keel and Songer, 2006). This high rate is followed by a gradual decline as piglets grow older (Weese *et al*., 2010a; Norman *et al*., 2009).

Piglets with CDI can be underweight by 10-15% and have an extended weaning time (Songer and Uzal, 2005). Squire *et al*. (2013) reported a mortality rate of 14% in piglets. Even though sporadic outbreaks of CDI in adult pigs are rare, they can have significant consequences because adult pigs can also die from infection (Kiss and Bilkei, 2005).
Table 2.1 Prevalence of *C. difficile* in piglets and adult pigs at various production stages in various countries

<table>
<thead>
<tr>
<th>Country</th>
<th>Age</th>
<th>Percentage positive samples (n)</th>
<th>PCR ribotype (s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>1-7 days old</td>
<td>67.2 (154/229)</td>
<td>014/020</td>
<td>Knight <em>et al.</em>, 2015</td>
</tr>
<tr>
<td>Australia</td>
<td>Neonates</td>
<td>62 (114/185)</td>
<td>237</td>
<td>Squire <em>et al.</em>, 2013</td>
</tr>
<tr>
<td>Austria</td>
<td>Slaughter age</td>
<td>3.3 (2/61)</td>
<td>126</td>
<td>Indra <em>et al.</em>, 2009</td>
</tr>
<tr>
<td>Belgium</td>
<td>Slaughter age</td>
<td>1 (1/100)</td>
<td>078</td>
<td>Rodrigue <em>z et al.</em>, 2013</td>
</tr>
<tr>
<td>Belgium</td>
<td>Less than 15 days</td>
<td>78.3 (18/23)</td>
<td>078</td>
<td>Rodrigue <em>z et al.</em>, 2012</td>
</tr>
<tr>
<td>Canada</td>
<td>1 day old</td>
<td>93 (28/30)</td>
<td>078</td>
<td>Hawken <em>et al.</em>, 2013</td>
</tr>
<tr>
<td>Canada</td>
<td>188 days old</td>
<td>3.8 (1/26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>Slaughter</td>
<td>6.9 (30/436)</td>
<td>078</td>
<td>Weese <em>et al.</em>, 2011</td>
</tr>
<tr>
<td>Canada</td>
<td>Day 2</td>
<td>74 (90/121)</td>
<td>078</td>
<td>Weese <em>et al.</em>, 2010a</td>
</tr>
<tr>
<td></td>
<td>Day 7</td>
<td>56 (66/117)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 30</td>
<td>40 (45/113)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 44</td>
<td>23 (23/101)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 62</td>
<td>3.7 (2/54)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sows prior to farrowing</td>
<td>40 (4/10)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Aspects of Clostridium difficile infection in pigs

<table>
<thead>
<tr>
<th>Country</th>
<th>Stage</th>
<th>Outcome</th>
<th>Isolate(s)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germany</td>
<td>0-1 day</td>
<td>68.2 (30/44)</td>
<td>078</td>
<td>Schneberg et al., 2013</td>
</tr>
<tr>
<td></td>
<td>2-14 days</td>
<td>93.5 (101/108)</td>
<td>126</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15-77 days</td>
<td>32.7 (16/49)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Japan</td>
<td>Finishing (13-27 weeks)</td>
<td>0.8 (2/250)</td>
<td>Not reported</td>
<td>Asai et al., 2013</td>
</tr>
<tr>
<td>Slovenia</td>
<td>Litters</td>
<td>50.9 (247/485)</td>
<td>029, 066, SI 010, SI 011</td>
<td>Avbersek et al., 2009</td>
</tr>
<tr>
<td>Slovenia</td>
<td>Litters</td>
<td>51.8 (133/257)</td>
<td>Not reported</td>
<td>Pirs et al., 2008</td>
</tr>
<tr>
<td>Spain</td>
<td>Day 8</td>
<td>70 (14/20)</td>
<td>078</td>
<td>Álvarez-Pérez et al., 2013</td>
</tr>
<tr>
<td></td>
<td>Day 15</td>
<td>85 (17/20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 22</td>
<td>15 (3/20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 29</td>
<td>0 (0/20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 36</td>
<td>0 (0/20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 43</td>
<td>5 (1/20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 50</td>
<td>30 (6/20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 57</td>
<td>0 (0/20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spain</td>
<td>Non-diarrhoeic</td>
<td>29.6 (76/257)</td>
<td>Not reported</td>
<td>Álvarez-Pérez et al., 2009</td>
</tr>
<tr>
<td></td>
<td>Diarrhoeic piglets</td>
<td>22.8 (58/254)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweden</td>
<td>Neonatal pigs</td>
<td>67 (45/67)</td>
<td>046,</td>
<td>Norén et al., 2014</td>
</tr>
<tr>
<td>Switzerland</td>
<td>Slaughter</td>
<td>0 (0/165)</td>
<td>Not applicable</td>
<td>Hoffer et al., 2010</td>
</tr>
<tr>
<td>The</td>
<td>Slaughter</td>
<td>0 (0/100)</td>
<td>Not</td>
<td>Koene et al., 2003</td>
</tr>
</tbody>
</table>
### Aspects of *Clostridium difficile* infection in pigs

<table>
<thead>
<tr>
<th>Location</th>
<th>Setting</th>
<th>Incidence</th>
<th>Application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Netherlands</td>
<td>In clinic</td>
<td>36 (9/25)</td>
<td>005, 023, 078</td>
<td>Keessen et al., 2011</td>
</tr>
<tr>
<td>The Netherlands</td>
<td>Slaughter</td>
<td>8.6 (58/677)</td>
<td>013, 014, 078</td>
<td>Hopman et al., 2011</td>
</tr>
<tr>
<td>The Netherlands</td>
<td>Slaughter</td>
<td>28 (14/50)</td>
<td>015,</td>
<td>Rodriguez-Palacios et al., 2014</td>
</tr>
<tr>
<td>USA</td>
<td>16-20 weeks</td>
<td>0.67 (1/150)</td>
<td>Not reported</td>
<td>Susick et al., 2012</td>
</tr>
<tr>
<td>USA</td>
<td>Conventional farm</td>
<td>34 (120/350)</td>
<td>Toxinotype V</td>
<td>McNamara et al., 2011</td>
</tr>
<tr>
<td>USA</td>
<td>Antibiotic free farm</td>
<td>23 (56/244)</td>
<td>Not reported</td>
<td>Thitaram et al., 2011</td>
</tr>
<tr>
<td>USA</td>
<td>Farm pigs</td>
<td>1.8 (1/56)</td>
<td>Not reported</td>
<td>Norman et al., 2011</td>
</tr>
<tr>
<td>USA</td>
<td>Slaughter age</td>
<td>15.9 (55/345)</td>
<td>Not reported</td>
<td>Norman et al., 2011</td>
</tr>
<tr>
<td>USA</td>
<td>Farrowing</td>
<td>24.9 (175/702)</td>
<td>078</td>
<td>Norman et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Nursery</td>
<td>5.1 (14/274)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Breeding</td>
<td>4.3 (26/604)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Finishing</td>
<td>2.7 (37/1370)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Aspects of *Clostridium difficile* infection in pigs

<table>
<thead>
<tr>
<th>Location</th>
<th>Group Description</th>
<th>Prevalence</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA</td>
<td>Free ranging feral pigs</td>
<td>4.4 (7/161)</td>
<td>Not reported</td>
<td>Thakur <em>et al.</em>, 2011</td>
</tr>
<tr>
<td>USA</td>
<td>During farrowing</td>
<td>73 (183/251)</td>
<td>Not reported</td>
<td>Thakur <em>et al.</em>, 2010</td>
</tr>
<tr>
<td>USA</td>
<td>Post farrowing</td>
<td>47 (32/68)</td>
<td>Not reported</td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Diarrhoeic neonates; Large, integrated system</td>
<td>17.3 (57.7/333)</td>
<td>Not reported</td>
<td>Baker <em>et al.</em>, 2010</td>
</tr>
<tr>
<td></td>
<td>Smaller regional farms</td>
<td>15.1 (27.2/180)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USA</td>
<td>Suckling piglets</td>
<td>50 (61/122)</td>
<td>Not reported</td>
<td>Norman <em>et al.</em>, 2009</td>
</tr>
<tr>
<td></td>
<td>Nursery</td>
<td>8.4 (10/119)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grow finishing</td>
<td>3.9 (15/382)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Breeding sows/boars</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Other farrow</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.4.1 Risk factors for CDI in pigs

Few studies have adequately investigated the risk factors contributing to CDI in pigs. Pig age is the most commonly reported factor associated with risk of CDI. The prevalence of *C. difficile* is typically highest in piglets up to 7 days postpartum (chapter 4; Weese *et al.*, 2010a; Norman *et al.*, 2009). Hopman *et al.* (2011) showed that piglets were colonised with *C. difficile* within 48 hr after farrowing. In the same study, Hopman and colleagues showed that if piglets were delivered by caesarian section, they were less likely to be infected with *C. difficile*, and if they did, this infection would almost certainly come from the environment (Hopman *et al.*, 2011). However, piglets delivered normally may well be exposed as they pass through the birth canal, as well as potential exposure in the farrowing environment (Hopman *et al.*, 2011). A longitudinal study found higher *C.*
Aspects of *Clostridium difficile* infection in pigs

difficulte prevalence in cooler months (16.2%) than in warmer months (10.3%) in a vertically integrated pig farm (Norman *et al.*, 2009). Whether the seasonal variation in CDI seen in swine in North America is due to temperature, humidity, or other seasonal factors, and whether it does so through impacting exposure or host susceptibility, is unclear.

Vermin may play a role in the spread of *C. difficile* on pig farms. A survey on a pig farm in Utrecht, The Netherlands, was undertaken to determine whether mice (*Mus musculus linnaeus*) were competent vectors for *C. difficile* (Burt *et al.*, 2012). Mice on the farm were trapped and their skin, muscles, and gut contents aseptically sampled for *C. difficile*. Additionally, dead insects (drain flies, lesser house flies and yellow meal worms) and birds were sampled. The external body surface of mice had a culture positive prevalence rate of 51-66% compared to 8% for the
gastrointestinal contents, with the predominant strain of \textit{C. difficile} being RT 078. Although Burt \textit{et al.} (2012) did not sample pigs in this study; the finding of RT 078 in vermin is significant because it is a well-established animal pathogen. Given that the contamination rate of the body surfaces was higher than the gut, mice may be more likely to spread \textit{C. difficile} mechanically in the environment than through the faecal route. The prevalence of \textit{C. difficile} in wild bird droppings was 4\%, in dead sparrows 66\%, and in various insects 56-100\% (Burt \textit{et al.}, 2012). \textit{C. difficile} has since been isolated from urban rats, further highlighting the role vermin could play in the dissemination of \textit{C. difficile} in the environment (Himsworth \textit{et al.}, 2014).

2.5 \textit{C. difficile} in cattle

\textit{C. difficile} was first isolated from cattle in the early 1980s (Princewell and Agba, 1982). Pre-weaning neonatal
calf enteritis and mortality is a common problem in the cattle industry (Gunn, 2003). Although there are many pathogens associated with neonatal calf enteritis, *C. difficile* was only described as a potential causative agent in the early 2000s (Hammitt *et al.*, 2008). Additionally, a study found a poor recovery rate of pathogens from faeces of diarrhoeic calves; 25% to 45% samples did not yield any pathogen (Gunn, 2003). This study by Gunn (2003) was limited by the small number of pathogens covered in the surveillance program and, in particular, there was no *C. difficile* detection protocol in place. Rodriguez-Palacios *et al.* (2006) reported a prevalence rate of *C. difficile* (by culture) of 7.6% (11/144) in diarrhoeic calves and 15% (20/134) in control calves. Toxins were more likely to be detected in diarrhoeic calves 39.6% (57/144) compared to 20.9% (28/134) in controls. This study further demonstrated that CDI could play a role in
calf enteritis (Rodriguez-Palacios et al., 2006). In a subsequent study, Rodriguez-Palacios et al. (2007) did not find an association between infected calves with *C. difficile* and disease. Hammit et al. (2008) reported a *C. difficile* prevalence of 25.3% (64/253) in faeces of diarrhoeic calves compared to non-diarrhoeic calves 13% (7/53). Furthermore, the investigation revealed that 22.9% (58/253) of specimens were toxin positive from diarrhoeic calves compared to 30.2% (16/53) non-diarrhoeic calves (Hammitt et al., 2008). Although idiopathic enteritis in calves aged 1-14 days is well described in the literature, very few studies have screened for *C. difficile* and those that did some have found a correlation between *C. difficile* colonisation and disease.

Longitudinal studies of veal calves have shown that younger animals were colonised soon after birth, and the prevalence gradually declined as the calves grew older.
Aspects of *Clostridium difficile* infection in pigs

(Magistrali *et al.*, 2015; Zidaric *et al.*, 2012; Costa *et al.*, 2011). Zidaric *et al.* (2012) showed a high diversity of *C. difficile* strains in veal calves (RTs 078, 126, 012, 045, 010, and 033), similar to Costa *et al.* (2011), with this diversity diminishing as they grew older. A cross-sectional study conducted in Australia found a *C. difficile* prevalence of 56% with three predominant RTs (126, 033, and 127) amongst 7-day-old veal calves (Knight *et al.*, 2013). These RTs belong to clade 5 of *C. difficile*, as do RTs 078 and 237, and are frequently isolated from livestock and occasionally from human cases of CDI (Tsai *et al.*, 2016; Magistrali *et al.*, 2015). The high prevalence of *C. difficile* in calves could increase the risk of meat contamination at the abattoir.

### 2.5.1 Risk factors for CDI in cattle

Putative risk factors for CDI in cattle include younger age and antibiotic use in veal calves. Magistrali *et al.* (2015)
found that veal calves aged 13-28 days were twice as likely to shed *C. difficile* than those aged 36-45 days (odds ratio 4.57 vs 2.79). Elsewhere, calves reached a peak of *C. difficile* shedding by at least 14-18 days of age (Zidaric *et al.*, 2012; Costa *et al.*, 2011). Antimicrobial use appears to be a common practice in veal production in Europe (Magistrali *et al.*, 2015; Zidaric *et al.*, 2012) and USA (Thitaram *et al.*, 2016; Costa *et al.*, 2011). The use of multiple antimicrobials, (more than four classes of antibiotics), polymyxin E, or a beta-lactam antimicrobial, was highly associated with *C. difficile* shedding in veal calves (odds ratio 5.83) (Magistrali *et al.*, 2015). Interestingly, a longitudinal study in the USA demonstrated no association between *C. difficile* shedding by calves and housing type (Costa *et al.*, 2011). However, this field
requires further investigation as production systems for veal calves vary immensely within and among countries.

2.6 C. difficile in goats and sheep

There are a few published studies describing C. difficile epidemiology in sheep and fewer for goats (Avberšek et al., 2014; Rahimi et al., 2014; Knight and Riley, 2013; Romano et al., 2012; McNamara et al., 2011). Rieu-Lesme and Fonty (1999) undertook a C. difficile carriage study in sheep with a view to understanding environmental reservoirs for humans. In that study, the prevalence of C. difficile in sheep was 1%. In a larger study, Knight and Riley (2013) reported a 4% prevalence of C. difficile in sheep with lambs having 6%. In another study, Avberšek et al (2014) reported a C. difficile prevalence of 5.7% (n = 105) in sheep and 9.2% (n = 109) in goats. Recently, Rahimi et al. (2014) reported a C. difficile prevalence of 2% (n = 660) meat samples, 3.3% (n = 92) in
Aspects of *Clostridium difficile* infection in pigs

raw goat meat and 0.9% (n = 150) in raw sheep meat. Given that sheep are similar to goats in many ways in terms of general physiology and diet, it is likely that the epidemiology of *C. difficile* colonisation is similar. The lower prevalence of *C. difficile* in sheep and goats may suggest that they could play a less significant role in *C. difficile* transmission to humans.

2.6.1 Risk factors for CDI in goats and sheep

There are few studies that have described risk factors for *C. difficile* acquisition in sheep and goats, although the study conducted by Knight and Riley (2013) showed that *C. difficile* detection was higher in lambs (6%) than adult sheep (4%), suggesting that age could be a risk factor. Age as a risk factor for *C. difficile* colonisation was confirmed in the study conducted by Avberšek *et al.* (2014), as *C. difficile* prevalence was highest in neonatal goats and lambs less than
Aspects of *Clostridium difficile* infection in pigs

16 days of age. On the other hand, in a farm level prevalence study, Romano *et al.* (2012) did not find age as a risk factor of *C. difficile* colonisation, possibly due to the study design and uneven distribution of animals in different age groups. A study reported a zero prevalence of *C. difficile* from samples obtained from goats (McNamara *et al.*, 2011). McNamara *et al.* (2011) did not find evidence of zoonotic transmission of *C. difficile* between livestock and human populations. There is paucity in the epidemiology of *C. difficile* in small ruminants.

2.7 *C. difficile* in avian species

Prevalence studies show that poultry can be colonised with *C. difficile* (Simango and Mwakurudza, 2008; Zidaric *et al.*, 2008; Simango, 2006). In a cross-sectional study of poultry in Zimbabwe, the *C. difficile* culture prevalence was reported as 29% and, of the strains isolated, 90% were
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toxigenic (Simango and Mwakurudza, 2008). However, these prevalence studies did not state the age of chickens sampled and neither was there evidence of enteritis. In the Netherlands, Burt *et al.* (2012) detected *C. difficile* in dead house sparrows at a piggery. Additionally, CDI has been reported in captive ostriches (Shivaprasad, 2003; Frazier *et al.*, 1993). Poultry or indeed other bird species could be CDI reservoirs for CDI for other animal species and possibly humans by direct contact or by environmental contamination with faeces.

2.7.1 Risk factors for CDI in birds

CDI in avians has been reported, in particular, captive ostriches (ratites) aged 9-19 days, similar to other animal species (Shivaprasad, 2003; Frazier *et al.*, 1993). In one study, 19-day old captive ostrich chicks treated with amikacin, piperacillin and enrofloxacin were diagnosed with
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CDI (Shivaprasad, 2003). In another study, an outbreak of CDI in 9-day old ostrich chicks treated with sulfamerazine in North America was reported (Frazier et al., 1993). This is consistent with literature that has found the association of antibiotic therapy and CDI in humans and some animal species. Although C. difficile colonisation has been reported in poultry (Simango et al., 2008; Simango, 2006), there are no reports of enteritis associated with C. difficile colonisation. What is clear though is that cephalosporin antibiotics associated with CDI amplification in humans are widely used off-label in poultry production in North America (Webster, 2009). The relationship between exposure to antibiotics in poultry and C. difficile shedding needs further investigation.
2.8 *C. difficile* in horses

The epidemiology of CDI in horses is similar to that reported in humans, although there are more reports suggesting that community acquired is as common as hospital acquired infections (Schoster and Staempfli, 2016). As opposed to older foals, young foals (< 1 month) experience enteritis, while adult horses experience enterocolitis, and sometimes typhlitis (Diab *et al.*, 2013; Uzal *et al.*, 2012; Jones *et al.*, 1987). Newborn foals can experience watery or bloody diarrhoea, sometimes resulting in death (Diab *et al.*, 2013). Possible co-infection with *C. perfringens* type C and *C. difficile* has been suggested, with severe necrotic mucosa, pseudomembrane, haemorrhage, and vascular thrombosis in the small intestine and colon (Uzal *et al.*, 2012; Weese *et al.*, 2001).
C. difficile has been detected in faeces of diarrhoeic and non-diarrhoeic horses (Schoster et al., 2012; Weese et al., 2001), and in horses not administered antibiotics in Australia (Thean et al., 2011) and abroad (Diab et al., 2013). The infection can occur sporadically or during an outbreak. The role of antibiotics in CDI in horses was demonstrated in vivo using a horse infection model (Gustafsson et al., 2004). A study found that hospitalised horses (n= 73) with clinical diarrhoea had a lower carriage rate of C. difficile compared to horses without clinical presentation (12.2% vs 15.6%) (Rodriguez et al., 2014). In the same study, C. difficile carriage was highest in hospitalised horses treated with antibiotics (20.5%). They also reported overall C. difficile prevalence in horses of 13.7% (10/73) and foals were more likely to have CDI. These findings are in agreement with Weese et al. (2001) who reported that foals and adult horses
without colitis were less likely to have \textit{C. difficile} carriage.

Additionally, a small retrospective study conducted in Canada did not find an association between antimicrobial therapy and CDI in hospitalised horses (Weese \textit{et al.}, 2006). This is not surprising because the pathogenesis of CDI is complicated by the fact that host factors are important in disease manifestation.

\textbf{2.9 \textit{C. difficile} in companion animals}

Dogs and cats can be colonised by \textit{C. difficile} (Álvarez-Perez \textit{et al.}, 2015; Hussain \textit{et al.}, 2015; Schneeberg \textit{et al.}, 2012; Clooten \textit{et al.}, 2008; Riley \textit{et al.}, 1991). While the role of pets in disseminating CDI in private homes is yet to be determined, some pet animals in the community are carriers of toxigenic \textit{C. difficile} without clinical disease (Clooten \textit{et al.}, 2008). Álvarez-Perez \textit{et al.} (2015) detected toxigenic strains of \textit{C. difficile} such as RTs, 020, 056, and 078 in dogs.
Schneeberg et al. (2012) reported the detection of toxigenic RTs 014/020 and 045 in cats and dogs in an animal shelter. RT 056 has been associated with complicated disease outcomes in hospitalised human patients in Europe (Davies et al., 2016; Bauer et al., 2011). RT 014/020 is one of the most detected RTs in medical settings in continental Europe (Bauer et al., 2011). Companion animals could be a reservoir for community acquired-CDI in humans, as studies suggest (Orden et al., 2017; Stones et al., 2016).

2.10 Risk factors for CDI in horses and companion animals

CDI in adult horses is commonly associated with antimicrobial use, particularly prior to hospitalisation (Diab et al., 2013; Gustafsson et al., 2004). CDI is common in young horses (Rodriguez et al., 2012) but not in healthy older horses in the absence of antibiotic exposure or
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hospitalisation (Gohari *et al.* 2014; Diab *et al.*, 2013; Slovis *et al.*, 2013; Thean *et al.*, 2011; Weese *et al.*, 2006; Båverud *et al.*, 2003). The majority of antimicrobials implicated in horse CDI are beta-lactams, trimethoprim-sulfonamides, clindamycin, erythromycin, rifampicin, and gentamicin (Schoster *et al.*, 2016; Knetsch *et al.*, 2014, Rodriguez *et al.*, 2012; Magdesian *et al.*, 2002). Other risk factors associated with CDI in horses are stress, transportation, change in diet, starvation, nasogastric intubation, and gastrointestinal surgery (Diab *et al.*, 2013; Båverud, 2004). These putative risk factors were associated with perturbation of the gastrointestinal microbiome of the horses increasing the risk of CDI (Diab *et al.*, 2013). Horses can also acquire infection from the environment, or potentially from humans (Rodriguez *et al.*, 2013). The other potential source of *C. difficile* could be cross-faecal contamination between mare
Aspects of *Clostridium difficile* infection in pigs

and foal (Magdesian and Leutenegger, 2011). Horses that are subclinically shedding *C. difficile* may experience CDI through the proliferation of spores when appropriate factors are present (Båverud, 2002).

Like other animals, young dogs (puppies) can be colonised by *C. difficile*, and peak prevalence is attained within 14 days of birth (Álvarez-Perez *et al.*, 2015). Hospitalisation is also a risk factor for *C. difficile* acquisition by dogs and cats in veterinary hospitals (Clooten *et al.*, 2008; Riley *et al.*, 1991). Clooten *et al.* (2008) showed that the longer an animal stayed in a hospital, the greater its likelihood of being colonised. Furthermore, animals that were administered immunosuppressive drugs and antibiotics had an increased risk of acquiring CDI. Diarrhoea was associated with colonisation by a toxigenic strain in dogs.
Aspects of *Clostridium difficile* infection in pigs
during hospitalisation but clinical status was not reported for
cats.

Although no zoonotic transmission has been reported
for CDI, a study showed that dogs visiting hospitalised
patients became culture positive after having an initial faecal
culture negative of *Clostridium difficile* (Lefebvre *et al.*, 2009). Overall, dogs visiting health care facilities were twice
as likely to be culture positive than the control (Lefebvre *et
al.*, 2009).

**2.11 C. difficile in wildlife**

Wildlife could be an important reservoir for CDI
(Bondo *et al.*, 2015; Álvarez-Pérez *et al.*, 2014; Jardine *et
al.*, 2013; Silva *et al.*, 2013; Thakur *et al.*, 2011). Bondo and
colleagues (2015) reported a *C. difficile* prevalence of 9% in
racoons near piggeries in Canada. This study together with
others suggests that wildlife could play a role in *C. difficile*
dissemination in the environment. In Australia, there is a wide range of fauna such as the marsupials, and feral swine, but their role if any in *C. difficile* transmission to the farming industry and people is unknown. In Australia, RT 126 a toxigenic *C. difficile* strain has been detected in a marsupial (D.R. Knight and T. V. Riley, unpublished) which is a common RT detected in cattle in Australia and humans (Knight *et al.*, 2013). It is unclear whether this could be as a result of interspecies transmission or common source acquisition. More studies are required for source attribution and control of *C. difficile* transmission.

### 2.11.1 Risk factors for CDI in captive wildlife

Like humans and livestock, captive wildlife is at risk of CDI if they are exposed to antimicrobials and stress. Reports of CDI in zoo animals after antimicrobial therapy is available. For example, Álvarez-Perez *et al.* (2013) report
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CDI in a captive zebra after exposure to metronidazole.

Other reports are for wildlife housed at rehabilitation animal sanctuaries (Arroyo *et al.*, 2005; Silva *et al.*, 2013). The wildlife and livestock interface is a potential risk factor for interspecies pathogen transmission (Thakur *et al.*, 2011).

**2.12 C. difficile in food**

*C. difficile* has been detected in both livestock products and by-products, wildlife and seafood (Knight *et al.*, 2016; Esfandiari *et al.*, 2015; Rahimi *et al.*, 2014; Pasquale *et al.*, 2012; Songer *et al.*, 2007). Although the prevalence in food animal products is very low (Rahimi *et al.*, 2014), in a recent study, *C. difficile* was detected on veal calf carcases immediately after processing in an abattoir (Knight *et al.*, 2016). The source of the *C. difficile* on veal carcases was attributed to evisceration techniques and environmental contamination within the abattoir. Similarly, *C. difficile* has
been detected in edible bivalve molluscs (Pasquale et al., 2012) and zooplankton, clams and mussels (Pasquale et al., 2011).

Apart from meat products, \textit{C. difficile} has been isolated from unwashed raw vegetables [carrots, cucumbers, mushrooms, onion, and radish] (Al Saif and Brazier, 1996) and ready to eat vegetables (Eckert \textit{et al.}, 2013; Bakri \textit{et al.}, 2009). All these studies show \textit{C. difficile} is widely distributed in the environment and potentially a source for CA-CDI.

\textbf{2.13 Pathogenesis of CDI}

\textit{C. difficile} causes disease through the expression of two main virulence factors, TcdA and TcdB (Merrigan \textit{et al.}, 2010). The corresponding genes, \textit{tcdA} and \textit{tcdB}, are located on the chromosome alongside three accessory genes, \textit{tcdR}, \textit{tcdC} and \textit{tcdE}, that together form a 19.6-kb pathogenicity
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locus [PaLoc] (Britton and Young, 2014). The *tcdC* gene is suggested to have a modulatory effect on production of toxins A and B rather than acting as a strict down-regulator (Merrigan *et al.*, 2010). Some studies show that *C. difficile* strains with truncations in *tcdC* produce more toxins A and B (Carter *et al.*, 2011; Merrigan *et al.*, 2010) leading to increased disease severity, but others have not found this association (Goldenberg and French, 2011). Recently, a study found that transcription of TcdA and TcdB is also regulated by *cdtR* in addition to *tcdR* (Lyon *et al.*, 2016). Most toxigenic *C. difficile* isolates possess *tcdA* and *tcdB*; however, some variant isolates are negative for *tcdA* (Squire *et al.*, 2013) and others are missing the PaLoc altogether, instead of having a 115-base pair sequence.

TcdA has been described as an enterotoxin because it causes exudative colitis. TcdB is cytotoxic and causes
epithelial cell collapse, apoptosis, and cell death. TcdA and TcdB are large molecular weight toxins (308 kDa and 269.6 kDa, respectively) belonging to the large clostridial toxins family (Jank and Aktories, 2008). Both toxins have an N-terminal enzymatic domain, a carboxyl-terminal receptor binding domain, and a central membrane translocation domain. Internalisation of toxins into host cells is promoted by the membrane translocation domain subsequently binding the carboxyl-terminal domain to non-proteinaceous surface receptors. The catalytic domain is responsible for glycosylation of the Rho-Rac family of small GTPases leading to the destruction of the actin cytoskeleton, exudative enteropathy, acute inflammation and necrosis of colonic mucosa. The tight junctions of epithelial cells lining the gut are destroyed (Jank and Aktories, 2008).
Some strains of *C. difficile*, particularly those associated with livestock, produce a binary toxin (CDT), the function of which remains speculative even though it has been associated with so-called “hypervirulence” (Cowardin *et al.*, 2016; Kuehne *et al.*, 2014). CDT comprises two separate proteins, one of which is responsible for host cell binding (CDTb) and the other for toxic enzymatic activity (CDTa) (Schwan *et al.*, 2009). CDT is biologically similar to binary toxins produced by *C. botulinum* (C2 toxin), *C. perfringens* (iota toxin), and *C. spiroforme*. It is thought to enhance microtubule protrusion from gut epithelial cells leading to the formation of a network of mesh around the bacterial cells and resulting in adhesion (Schwan *et al.*, 2009).

### 2.13.1 Pathology of CDI

Gross pathological findings include mild to severe ascending mesocolonic oedema, and an inflamed dilated
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colon and sometimes typhlitis in pigs and ostriches (Songer and Anderson, 2006). Pathologic presentation varies with species, but generally, the colon and caecum are affected in most but not all species like the piglets and ostriches (Steele *et al.*, 2010; Keel and Songer, 2006; Waters *et al.*, 1998; Frazier *et al.*, 1993). Pleural effusion, lung consolidations, ascites and, occasionally, hydrothorax have been reported at post-mortem in pigs and ostriches (Steele *et al.*, 2010; Shivaprasad, 2003; Frazier *et al.*, 1993). However, ostriches show lesions in the proventriculus which is the equivalent of the porcine stomach. Other organs may be involved; for example, localised atelectasis, and interstitial thickening of lungs with no evidence of pneumonia have been described. Non-specific lesions in the liver and spleen have also been reported (Steele *et al.*, 2010; Frazier *et al.*, 1993). However, the gross pathology of CDI in poultry is less clear.
Histopathological findings include infiltration of polymorphonuclear cells into the colonic epithelium and mucosal injury caused by \textit{C. difficile} toxins in piglets (Steele \textit{et al.}, 2010) and ostriches (Frazier \textit{et al.}, 1993). In a study conducted using gnotobiotic piglets, acutely infected piglets showed severe neutrophilic inflamed mucosa and submucosa (Steele \textit{et al.}, 2010). The colonic lumen was filled with mixed cells, including neutrophils, bacteria, and a necrotic layer forming the characteristic pseudomembrane associated with CDI. Severe erosions of the colonic mucosa with a “volcanic eruption” type of lesions are uncommon in pigs (Steele \textit{et al.}, 2010; Waters \textit{et al.}, 1998). As with pigs, ostriches have been diagnosed with acute, necrotising typhlitis and colitis, with large numbers of Gram-positive bacilli seen within glands and in the intestinal lumen (Frazier \textit{et al.}, 1993). Additionally, lymphoid tissue necrosis has been
reported in ostriches. Tissue sections of the ostrich chick liver revealed multifocal coagulation necrosis of hepatocytes in association with multifocal granulomatous inflammation (Shivaprasad, 2003). Furthermore, necrosis and an increased number of degranulating heterophils surrounded by multinucleated giant cells, lymphocytes, and macrophages were common (Shivaprasad, 2003).

2.13.2 Clinical signs of CDI

Intestinal colonisation with *C. difficile*, and consequent disease, is common in neonatal piglets within 7 days of farrowing (Weese *et al.*, 2010a; Norman *et al.*, 2009). The most common clinical sign for CDI in livestock is diarrhoea, which may be acute or chronic; however, many neonatal animals remain without clinical disease. CDI may be self-limiting, intermittent, or continuous in nature. Piglets infected with *C. difficile* may present with a yellow pasty or
watery, non-haemorrhagic diarrhoea. Ostrich chicks often experience anorexia, weight loss (Shivaprasad, 2003), acute diarrhoea, and sudden death (Frazier et al., 1993). Obstipation and constipation, scrotal oedema and dyspnoea occur uncommonly in piglets (Steele et al., 2010).

2.13.3 Laboratory diagnosis of CDI

The diagnostic tests available for detection of C. difficile in humans can broadly be classified into three categories (Crobach et al., 2016). First, there are tests such as toxigenic culture to isolate toxin-producing C. difficile. Second, tests that detect C. difficile products such as aromatic fatty acids, glutamate dehydrogenase (GDH) and toxins A and/or B, and last, tests that detect C. difficile genes.

Although toxigenic culture for C. difficile is labour intensive with a long turnaround time, it is still regarded as
one of the gold standards for diagnosis of human and animal CDI (Crobach et al., 2016). Faecal samples can be transported at ambient temperature within 24 h to the laboratory or stored frozen. Toxigenic culture involves isolating *C. difficile* from faeces by using selective culture media and determining if the isolate is toxin-producing (Lund and Peck, 2015; Burnham et al., 2013). The methods for isolating *C. difficile* from faeces either by direct plating on selective media and/or selective enrichment in broth, followed by plating on selective media, have been extensively reported (Lund and Peck, 2015). By direct culture, chromogenic agar (bioMérieux, Marcy l'Etoile, France) gives a shorter turnaround time (24 h) compared to pre-reduced cycloserine-cefoxitin-fructose agar with added sodium taurocholate (Carson et al., 2013). Presumptive *C. difficile* colonies on blood agar are identified by chartreuse
fluorescence under UV light (~360 nm wavelength), colonial morphology (ground glass appearance), and horse dung odour. Identification of uncertain isolates can be achieved by Gram staining and detection of L-proline aminopeptidase (Knight et al., 2014), or more recently MALDI-TOF-MS (92.5-100% sensitivity) (Kim et al., 2016).

Other assays for diagnosing CDI include commercially-available enzyme immunoassays (EIA) (Crobach et al., 2016). Despite the limitations associated with these tests (Burnham and Carroll, 2013, Tenover et al., 2010), they are popular in laboratories because they are easy to use, relatively cheap, and have a short turnaround time. Some EIAs are designed to detect GDH in faeces, the “common antigen” on C. difficile strains, in addition to TcdA and TcdB, even though there are reports of reduced sensitivity for these tests (Tenover et al., 2010). EIAs that target GDH
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were initially said to have a higher sensitivity than those that only target TcdA or TcdB (Crobach *et al.*, 2016). Furthermore, some studies have suggested that EIAs vary in their ability to detect certain RTs of *C. difficile* in human disease (Tenover *et al.*, 2010).

Additionally, while there is no correlation between strain type, toxin in faeces and disease severity both in humans and animals (Yaeger *et al.*, 2002), a large study by Planche *et al.* (2013) showed that the presence of toxins in faeces predicted poorer outcomes in humans. Although EIAs that target GDH, and PCR methods that detect toxin genes, have relatively high sensitivity, they lack specificity for the disease. Therefore, a two-step diagnostic algorithm has been suggested that involves re-testing positive samples with a toxin EIA which increases specificity and positive predictive value. A complete diagnosis of CDI in pigs or indeed other
animal species will include a clinical history, toxigenic culture of \textit{C. difficile} and detection of free toxins in faeces or detection of toxin genes or enzyme in isolates. Further, the European Society for Clinical Microbiology and Infectious Disease (ESCMID) recommend testing faeces that are not formed and are negative for other enteropathogens (Crobach \textit{et al.}, 2016). Currently, no single standalone diagnostic test for CDI with suitable sensitivity and specificity is available (Bloomfield and Riley, 2016).

Most of the CDI diagnostic tests available on the market have been validated for human medicine and these perform sub-optimally on animal samples. For example, some human commercial molecular diagnostic assays showed low sensitivity in the range of 25\% to 50\% on animal samples (Knight \textit{et al.}, 2014). The reason for sub-optimal performance on animal samples is unclear and
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requires further research. Better diagnostic tools are crucial for the early detection of many veterinary pathogens including C. difficile.

2.14 C. difficile and antibiotic resistance in livestock

The relationship between treatment with antimicrobials and increase in antimicrobial resistance in animals (Thitaram et al., 2016; Knetsch et al., 2014; Costa et al., 2011; Bakker et al., 2010) and humans has been extensively studied (Tang et al., 2017; Knight et al., 2015; Loo et al., 2005). The interspecies transfer of antimicrobial resistance genes has also been extensively studied for various bacteria including C. difficile (Knight et al., 2015; Knetsch et al., 2014; Bakker et al., 2010). The increase in antimicrobial resistance in infectious organisms other than C. difficile in humans has been reported as a factor driving CDI by the Centers for Disease Control and Prevention (CDC, 2013). Of particular
interest is the reduction of *C. difficile* susceptibility to some antibiotics such as metronidazole (Álvarez-Perez *et al*., 2013) and fluoroquinolones (Loo *et al*., 2005). Similar studies in veal calves showed high resistance of *C. difficile* to tetracycline (MIC ≥ 16 µg/ml) (Costa *et al*., 2011), macrolides, and fluoroquinolones (Thitaram *et al*., 2016). By molecular methods, the presence of tetracycline resistance genes such as *tet* (M), *tet* (O) and *tet* (W) was detected in *C. difficile* isolates (Costa *et al*., 2011). There is a likelihood that similar resistance to tetracycline observed in human strains of *C. difficile* (Bakker *et al*., 2010) may occur on a larger scale in veterinary medicine, and could be driving the development of CDI in animals. A recent report describes isolates of RT 078 from pigs and farmers in the Netherlands sharing transposable elements and associated antibiotic resistance genes for tetracycline (*tetM; Tn 6190*) and
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streptomycin (aphA1; Tn 6235) (Knetsch et al., 2014). On the other hand, there is a high likelihood that resistance genes can be acquired by humans from animals (Knight et al., 2015). Webster (2009) observed that widespread use of off-label antibiotics in livestock could be a driver of antibiotic resistance in Canada. The judicious use of antimicrobials in both livestock and hospitals can slow development of antibiotic resistance.

2.15 Control of C. difficile in the veterinary environment

C. difficile spores can persist in the environment for prolonged period of time (Maillard, 2011; Kramer et al., 2006). Like other pathogenic organisms, lower temperatures (4-5°C), high humidity, and quantity of inoculum have been suggested as potential causes of persistence (Kramer et al., 2006). Although still controversial, some studies have shown that epidemic strains of C. difficile have a higher sporulation
capacity than non-epidemic strains and may persist in the environment longer (Merrigan et al., 2010). However, a study conducted by Robinson and colleagues (2014) did not find a difference in sporulation capacity between hypervirulent strains and a non-hypervirulent strain. The thymidine synthase gene in RT 027 strains could confer a growth advantage, and, hence, it has a more substantial role in competitive fitness (Robinson et al., 2014). The majority of factors that enhance higher fitness among epidemic strains of C. difficile are unknown.

2.16 Potential role of the environment in community-acquired C. difficile infection

Initially, C. difficile-associated disease was recognised as a nosocomial infection (Leffler and Lamont, 2012; McFarland et al., 1989). However, because of its ubiquity outside the hospital environment, and with an increase in the
incidence of community-acquired *C. difficile* infections (CA-CDI) (Eyre *et al.*, 2015; Slimings *et al.*, 2014; Kuntz *et al.*, 2011), food sources have emerged as a potential reservoir of *C. difficile*. This paradigm is supported by studies that have reported detection of *C. difficile* in animals (Keessen *et al.*, 2013), meat (Knight *et al.*, 2016; Houser *et al.*, 2012), root vegetables (Eckert *et al.*, 2013), and leaf vegetables (Bakri *et al.*, 2009). However, the prevalence of *C. difficile* in foods is low (Limbago *et al.*, 2012). Although the foodborne CDI paradigm is plausible, there is a shift towards the environment as a more important source of CA-CDI (Bauer and Kuijper, 2015). This is evident from many studies where novel clones of *C. difficile* have detected from the environment including the hypervirulent strains (RT 027 045, 078, 117 and 126) (Alam *et al.*, 2014; Romano *et al.*, 2012; Zidaric *et al.*, 2010).
Since the 2000s, hypervirulent clones of RT 027 dominated hospital outbreaks in North America and Europe (Rupnik et al., 2009). Hypervirulent RT 027 has been associated with hospital-acquired infections whereas RT 078 predominates in community-associated infection in many countries (Khanna et al., 2012; Goorhuis et al., 2008). A report has shown a decline of RT 027 in the United Kingdom, with the increase of other RTs (RT 078, 002, 005, 014, and 015) (Fawley et al., 2016). In Australia, a similar shift has been observed although RT 078 has not been detected from animals nor the environment (Cheng et al., 2016; Collins and Riley, 2016; Eyre et al., 2015; Knight et al., 2015).

The rate of community-acquired CDI in adults has been reported as 20-50% (Collins and Riley, 2016; Foster et al., 2014). The transmission pathways of C. difficile in the
hospital environment have been extensively studied (Ziakas et al., 2015; McFarland et al., 2007). However, the sources of community-acquired strains are less clear. In the USA, 50% of all biosolids produced each year are used in agriculture and landscaping (EPA, 2012; Iranpour et al., 2004). Outbreaks with infectious organisms (other than C. difficile) associated with manure have been reported (Gerba and Smith, 2005; Mac Kenzie et al., 1994). C. difficile has been isolated from human biosolids (Xu et al., 2014; Romano et al., 2012), tap water (Kotila et al., 2013), and treated animal effluent (Squire et al., 2011). The treatment process of biosolids does not reduce the spore load of C. difficile (Nikaeen et al., 2015; Xu et al., 2015; Romano et al., 2012).

Animal manure is widely used in agriculture and landscaping although there are public health concerns about
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manure harbouring pathogenic microorganisms (Fry *et al.*, 2014; Létourneau *et al.*, 2010; Carrington *et al.*, 1988). The standard for relative safe manure is generally determined by the absence or low prevalence of indicator microorganisms such as enterotoxigenic *Escherichia coli* (ETEC) and *Salmonella* sp. (McCarthy *et al.*, 2013). The use of a limited number of indicator microorganisms as a measure of biosafety of manure can be misleading. This is because the variation in numbers of these microorganisms in faeces among farms has been documented (McCarthy *et al.*, 2013). Moreover, even manure without detectable indicator microorganisms has been found to harbour pathogenic microorganisms (Suslow *et al.*, 2003). Similar conclusions have been reached by researchers examining water contamination (Wu *et al.*, 2011). Wu *et al.* suggested that an absence of indicator pathogens does not necessarily mean an
absence of pathogenic microorganisms or their presence does not correlate with increased risk of infection. Despite all this, some researchers favour increasing the number of indicator microorganisms (Schijven et al., 2003; Sartory et al., 1998). Of great importance, others have suggested the use of specific indicators for specific situations (Yates, 2007).

Composting is the procedure applied to manure to increase the bioavailability of plant nutrients and reduce the load of pathogenic microorganisms. There are no universal standards for composting manure. For example, in Canada, it is voluntary to follow national guidelines for composting (Guan and Holley, 2003). In the USA, the department of agriculture recommends composting of manure intended for crops by using minimum temperature 65-74°C for one hour (USDA, 2016). Other recommended assessment for manure
that meets a minimum standard of treatment is that it should have $10^3$ faecal coliforms and or 3 most probable number of *Salmonella*. There is no minimum application interval required when untreated raw manure is applied in a manner that does not contact covered produce during or after application (FDA, 2015). In the USA, foodborne illnesses associated with contaminated vegetables and fruits have increased over the years (Berry *et al.*, 2015; Gould *et al.*, 2013; Painter *et al.*, 2013; Doyle and Erickson, 2008; Sivapalasingam *et al.*, 2004; Guan and Holley, 2003). Although there is no proof of zoonosis, *C. difficile* has been isolated from unwashed raw vegetables (carrots, cucumbers, mushrooms, onion, and radish) (Al Saif and Brazier, 1996), meat products (Songer *et al.*, 2009) and contaminated tap water in Finland (Kotila *et al.*, 2013).
Wind transmission of pathogenic organisms to crops from livestock or feral animal faeces has been reported (Berry et al., 2015; Jay et al., 2007). Wind transmission of C. difficile spores within a piggery has been reported (Keessen et al., 2011b). Keessen et al. (2011b) detected C. difficile in air 20 m from pig sheds. Berry et al. (2015) demonstrated that crops planted within 180 m of a feedlot had low E. coli contamination compared to crops that were 60 m. In an experimental study of pig slurry inoculated with E. coli, the pathogen was detected in air samples at 180 m from the slurry spraying gun (Hutchison et al., 2008). The contamination of produce by E. coli also increased with increase in dry weather. Studies have also found that proximity of a residential area to a livestock farm (Dieters et al., 2015; Carrel et al., 2014) or crop farm that applied animal manure (Casey et al., 2013) was a good predictor of
methicillin-resistant *Staphylococcus aureus* infection. Altogether, these studies suggest that storage or processing manure near crops has a high risk of airborne transmission of pathogens.

Another risk factor for fresh produce being vehicles of pathogenic microorganism has been attributed to the method by which manure is applied to crops. Suslow and colleagues (2003) suggest that contamination of vegetables with pathogenic microbes is more likely to occur if crops are irrigated with aerial spray close to harvest. The risk of vegetable contamination with pathogens is increased if water used for irrigation has a higher concentration of pathogens (FDA, 2015). Irrigation of vegetables with manure near the point of harvest should be avoided or if not, then the use of withholding period should be implemented (FDA, 2015).
Although many infectious outbreaks are reported from leafy vegetables contaminated with manure (Beuchat and Ryu, 1997), it is possible that other routes could be important. For example, a study in rural Canada found a high incidence of gastrointestinal tract infections in children living in regions practising intensive animal farming (Levallois et al., 2014). Other infections such as community associated methicillin-resistant *Staphylococcus aureus* have been found to be more common in people living close to farms that applied pig manure than those further away (Casey et al., 2013). A recent study in Asia found a correlation between increased animal intensification and a high rate of zoonotic diseases (Richter et al., 2015). Richter and colleagues (2015) suggest that animal manure runoff could be the vehicle for most of the gastrointestinal infections in humans living near farms and water catchment
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areas. This is supported by a study that found a high prevalence of *C. difficile* (54%-60%) in the estuarine environment in the UK (Hargreaves *et al.*, 2013).

*C. difficile* has been detected from river water (Zidaric *et al.*, 2010), sea water (Al Saif and Brazier, 1996), sewerage effluent (Xu *et al.*, 2014; Romano *et al.*, 2012), treated animal effluent (Squire *et al.*, 2011) and soil (Simango, 2006). Norman *et al.* (2011) reported the detection of *C. difficile* from human and swine compost sewage samples from a closed integrated human and swine population in the USA. More recently, Usui and colleagues (2016) have shown that pig manure based compost which has been treated mesophilically had viable *C. difficile* spores. Therefore, these studies suggest the need to increase surveillance of soil and water sources that receive animal or
human biosolids effluent to inform prevention and control of pathogenic microbes.

The major problem which is often overlooked in applying animal manure or biosolids to crops is the consideration of the duration of persistence of certain pathogens on a plant (Suslow et al., 2003). For many years, researchers have incorrectly used surrogate pathogens such as *E. coli* as representative of all other pathogens. Toxigenic *E. coli* is a well-known pathogen of calves, piglets and other animals, but the prevalence varies within and between species (McCarthy et al., 2013; Berry and Wells, 2010).

### 2.17 Treatment options for *C. difficile* infection

CDI is associated with increased health care burden in hospitalised patients in the USA (Lessa et al., 2015) and an emerging pathogen in commercial farms (Songer and Anderson, 2006). The current treatment approach includes
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the use of metronidazole for a less severe form of disease and vancomycin for severe cases (Kociolek and Gerding, 2016; Jarrad *et al.*, 2015). Recently, fidaxomicin and tigecycline have emerged as alternative antimicrobials because they have fewer negative effects on the microbiome. Up to 30% of human patients receiving antimicrobials experience CDI recurrence (Kociolek and Gerding, 2016; Ivarsson *et al.*, 2015; Jarrad *et al.*, 2015; Bassetti *et al.*, 2012). Although fidaxomicin has less effect on the gastrointestinal microbiome, it is very expensive. The cost of antimicrobials is high and the fact that *C. difficile* shows high resistance to most antimicrobials implies that alternative therapies should be explored.

Many patients treated with antibiotics for CDI experience a recurrence of infection within 30 days of first treatment. This highlights the urgent need to identify
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alternative treatment regimens, including possible therapy that uses faecal microbiota transplantation (FMT) (Britton and Young, 2014; Petrof and Khoruts, 2014). In a recent study, Buffie and colleagues (2015) demonstrated that there is a specific microbiome that assists colonisation resistance against infection by toxigenic strains of *C. difficile*. Harvey *et al.* (2006) showed that enterally fed neonatal pigs had better colonisation resistance against *C. difficile* than those parenterally fed. A study conducted at a poultry farm found that poultry colonised with *Acidaminococcus acini* were less likely to be colonised by *C. difficile* (Skraban *et al.*, 2013). Kim *et al.* (2014) showed that tigecycline altered microbiota balance of gnotobiotic piglets (increased Proteobacteria and reduced Firmicutes) but this did not predispose piglets to CDI. In contrast, other researchers have found that mice treated with tigecycline or hamsters (with clindamycin) were
susceptible to CDI despite showing a shift in microbiota similar to that in piglets (Bassis et al., 2014; Peterfreund et al., 2012). However, a recent study using metagenomics has shown that the underlying ecological dynamics of gut microbiome (i.e. intra and interspecies) communities are independent of host influence (Bashan et al., 2016). This could explain why FMT has been a successful therapy for CDI patients. Overall, it appears that no single community of microbiota determines the mechanism by which the gut mounts colonisation resistance (Theriot and Young, 2015). Studies have evaluated the potential benefits of probiotics (Arruda et al., 2015; Schoster et al., 2015) although they show varied performances against CDI in animals and humans (Floch et al., 2015; Collado et al., 2005). The major problem is that probiotics can be marketed without scientific proof of their claims (Hoffmann et al., 2013). In addition,
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Vaccines for humans are at various developmental stages, although none is currently available for livestock. The fact that the CA-CDI rates are increasing worldwide suggests that environmental decontamination in the hospitals and outside will not stop re-introduction of infection. Therefore, more economical approaches that target the host immunity could be more cost effective than antibiotics or disinfectants alone.

In a small clinical trial, zinc bacitracin was successful in treating enteritis associated with Clostridia cadaveris infection in equines (Staempfli et al., 1992). Feed formulations for pre-weaning piglets have high levels of copper sulphate (5ppm) and zinc oxide (3000ppm) to prevent enteritis (Jacela et al., 2010). For example, Holm and Poulsen (1996) demonstrated that zinc oxide could treat diarrhoea associated with toxigenic E. coli infection. However, Schoster et al. (2016) have reported that most C.
Clostridium difficile strains are universally resistant to zinc oxide. The use of high quantities of zinc oxide could lead to heavy metal soil contamination.

Studies have shown that high serum IgG response and secretory IgA to TcdA have been associated with less severe form of disease (Bauer et al., 2014; Kyne et al., 2000; Warny et al., 1994). Furthermore, a study using a hamster infection model showed that a high serum IgG prevented severe disease (Torres et al., 1995). Passive and active immunisation against CDI in hamsters and mice has been shown to confer protection against death and this has been dependent on the route of vaccine administration (Wang et al., 2016; Anosova et al., 2013; Torres et al., 1995; Bacon et al., 1994).

Human antibodies against CDI are prevalent (~60%) in the general population and patients (Kelly et al., 1992).
However, the mode by which antibodies neutralise toxins is not fully understood. Studies have shown that breast fed human neonates have lower rates of *C. difficile* carriage compared to formula fed babies (Tullus *et al.*, 1989; Cooperstock *et al.*, 1983; Cooperstock *et al.*, 1982). A similar study in neonatal hamsters fed with hyperimmune bovine colostrum were protected by passive immunity against diarrhoea compared to those fed with infant formula alone (Lyerly *et al.*, 1991). More recently, hyperimmune colostrum reduced CDI recurrence by 67% in a mice infection model (Hutton *et al.*, 2017). Piglets administered with equine origin antitoxins were protected against *C. difficile* infection (Ramirez *et al.*, 2014). Kim and colleagues (1987) showed that pregnant hamsters vaccinated with a toxoid *C. difficile* vaccine produced protective antibodies in colostrum against *C. difficile* infection associated disease in
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their off springs. All these studies suggest that immunotherapy could be important in preventing disease severity associated with CDI.

A study has shown that CDT could play a role in *C. difficile* colonisation (Schwan *et al.*, 2009). Many surface proteins of bacterial pathogens including *C. difficile* have been shown to play a role in anchoring bacteria to host gut epithelial mucosa during colonisation (Willing *et al.*, 2015). Many studies have shown that *C. difficile* cell wall proteins and the surface layer (S-layer) and the polymer PSII could play an important role in colonisation and proliferation in the gastrointestinal tract of mammals (Willing *et al.*, 2015; Merrigan *et al.*, 2013). Few studies have investigated bacterial proteins other than TcdA, TcdB and CDT as vaccine antigens (Senoh *et al.*, 2015a; Merrigan *et al.*, 2013). Despite this, leading potential *C. difficile* candidate vaccines
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are formulated to target toxin neutralisation and not reducing bacterial colonisation.

*C. difficile* toxoid vaccines using toxins (A and B) can confer protection against clinical disease (de Bruyn *et al.*, 2016; Anosova *et al.*, 2013), but will not eradicate spores which are a source of infection. Candidate vaccines that aim to prevent infection and colonisation are being investigated (Hong *et al.*, 2017; Bruxelle *et al.*, 2016; Pechine *et al.*, 2016; Kociolek and Gerding, 2016; Ghose *et al.*, 2015; Senoh *et al.*, 2015; Permpoonpattana *et al.*, 2011; Bacon *et al.*, 1994) but no vaccine is commercially available for animals.

2.18 Conclusions and outlook

*C. difficile* is an important pathogen of humans and animals. The fact that indistinguishable strains of *C. difficile* have been detected in humans, animals, animal products and
crops treated with manure, suggests that it could be acquired from a common source or zoonotically transmitted. The importance of developing tools that can accurately diagnose CDI in livestock is cardinal in improving our understanding of the evolving epidemiology of CDI and, thus, its control. However, the overarching issue is likely to be the use of antimicrobials in production animals. The research focus should be directed towards understanding the functionality of various host microbiomes as a treatment option for many infectious diseases including *C. difficile*.

The fact that *C. difficile* is ubiquitous in the environment and has wide host range suggests that a multifaceted approach of intervention is required to prevent the escalating rates of CA-CDI or nosocomial infection. Vaccination strategies have succeeded in reducing the incidence of zoonotic transmission of infectious pathogens.
which are widely distributed in the environment such as

*Bacillus anthracis* and *Clostridium tetani*. The same could be possible for CDI, because vaccinations are unlikely to lead to microbiota imbalance as do antibiotics. Developing a *C. difficile* vaccine is likely to be cost effective than the use of antimicrobials and contentious disinfection programmes in pig pens. A *C. difficile* vaccine in livestock could prevent *C. difficile* spore dissemination in the environment and possibly CA-CDI.
CHAPTER 3: MATERIALS AND METHODS

3.1 MATERIALS

This chapter opens with description and sources of various reagents and chemicals used in the experiments and commercial companies of origin. This is followed by protocols that were used in *C. difficile* isolation, phenotypic and molecular typing, and genotypic characterisation of *C. difficile* isolates. Finally, methods for the production of a toxoid vaccine are described and determination of seroconversion of the vaccinated pigs is described.

3.1.1 Reagents and chemicals

A list of primers, chemicals and reagents (with manufacturers) is shown in Tables 3.1 and 3.2.

3.1.2 Buffers and solutions

Buffers and solutions used in all the studies conducted in this project are listed. Peptone saline was commercially produced and supplied by PathWest Media which is the media section of PathWest Laboratory Medicine Western
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Australia (PathWest). Phosphate buffered saline (PBS) was commercially produced and supplied by PathWest. Solutions used in the preparation of PCR primer premixes were made with ultra-pure water supplied by Sigma-Aldrich. Other solutions were made with de-ionised water that was sterilised by autoclaving (121°C/15 psi for 15 min). Some buffers and solutions are described in specific sections.

12% Separating gel for SDS-PAGE for protein characterisation

dH₂O 3.35 mL
1.5 M Tris pH 8.8 2.5 mL

- 18.17 g Tris base (m.w. 121.10) was dissolved in 70 mL deionized or distilled water.
- Adjusted pH by slowly adding concentrated hydrochloric acid (HCl); monitoring with a pH meter.
- Add deionised or distilled water to make 100 mL total solution.
10% Sodium Dodecyl Sulfate (SDS) 0.1 mL

- Dissolve 10 g electrophoresis-grade SDS (m.w. 288.37) in 80 mL deionized water.
- Add deionized or distilled water to make 100 mL total solution.

Acrylamide/Bis-acrylamide (Acry/Bis), 30% solution

4.0 mL
- Acrylamide 30 g
- Bis-acrylamide 0.8 g

Acrylamide and bis-acrylamide were dissolved in 70 mL of distilled water and made up to 100 mL and stored in amber coloured bottles at 4°C.

10% Ammonium persulphate (APS)

0.05 m APS

- 100 mg/ml concentration
- Mix to dissolve

Tetramethylenediamine (TEMED) 5 µL

4% Stacking gel

dH₂O 3.213 mL

0.5M Tris pH 6.8 1.25 mL

6.05g of Tris was dissolved in 70 mL distilled water and pH adjusted to 6.8 with HCl and made the volume to 100 mL.

10% SDS 0.05 mL

30% Acryl/Bis 0.05 mL

10% APS 0.085 mL

Tetramethylenediamine (TEMED) 5 µL

10x Running buffer

Tris 30 g

Glycine 114 g

SDS 10 g

This was dissolved in 1 litre of dH₂O
Coomassie stain

Coomassie stain blue R-250 0.5 g

Destaining solution 500 mL

Coomassie blue destaining solution

- dH₂O 250 mL
- Methanol 200 mL
- Acetic acid 50 mL

Transfer buffer (electrophoresis) buffer

Tris base 3.03 g
Glycine 14.4 g
Methanol 200 mL

This was dissolved in 1 litre of dH₂O.
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**Tris Buffered Saline (Tris-NaCl pH 7.4) (TBS)**

Tris base 1.2 g

NaCl 8.7 g

This was made up to 1 litre of dH$_2$O.

**Saline (0.85% NaCl)**

Saline was obtained from PathWest Media.

**Tween 20, 0.1% (v/v) solution** was obtained from Sigma-Aldrich, Pty, Castle Hill, NSW, Australia.

**3.1.3 Antimicrobials**

Antimicrobials used in susceptibility testing were commercially prepared and used per manufacturer instruction as described in section 3.4.6 to 3.4.11.

**3.1.4 Culture media**

Blood agar (BA) plates (PathWest Media)
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*Clostridium difficile* Chromogenic ID ® (ChromID)

(bioMérieux, Marcy l'Etoile, France)

Selective brain heart infusion broth (BHIB-S) containing per L:

- BHIB base: 29.9 g
- Gentamicin: 5 mg
- Cefoxitin: 10 mg
- Cycloserine: 200 mg

Was prepared by PathWest Media.

### 3.2 Isolation of *C. difficile* from faeces

*C. difficile* was isolated as previously described, with minor modifications (Knight *et al.*, 2014). Briefly, rectal swabs were cultured directly on ChromID™ agar (bioMérieux, Marcy l'Etoile, France) and in a selective enrichment Robertson’s cooked meat broth containing cefoxitin (10 mg/L), cycloserine (200 mg/L), and gentamicin
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(5 mg/L) (PathWest Media). Following alcohol shock, when 1 mL of 48 h broth culture was mixed with 1 mL anhydrous ethanol (96%) and incubated at room temperature for 1 h, 0.01 mL of the mixture was cultured on ChromID™ agar. Effluent and treated water samples (10 μL) were cultured directly on ChromID™ agar or following broth enrichment. An aliquot of 1 mL of either effluent or treated water was transferred to the enrichment broth and processed as for faecal samples.

All plate cultures were incubated anaerobically (A35 anaerobic chamber, Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK) at 37°C, with an atmospheric gas composition of 80% N₂, 10% CO₂ and 10% H₂. One probable *C. difficile* colony on ChromID™ agar was cultivated on blood agar and identified based on a characteristic chartreuse fluorescence detected with UV light.
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(∼360 nm wavelength), colonial morphological characteristics (ground glass appearance), and horse dung odour. Identification of uncertain isolates was achieved by Gram staining and detection of L-proline aminopeptidase (Remel Inc., Lenexa, KS, USA).

3.3 Quantitation of C. difficile in effluent, and in soil amended with effluent

A 10-fold serial dilution of samples was performed as described previously with modifications (Knight et al., 2016; Housman et al., 2015; Rodriguez-Palacios et al., 2011; USEPA, 2001). Briefly, 1 g of soil or 1 mL of liquid effluent was added to 9 mL of sterile deionised water and a 1 mL aliquot used to make five 10-fold serial dilutions. Negative and positive controls (RT 027) were used in each assay to monitor for potential contamination. A 100 µL aliquot was obtained from the $10^{-2}$ to $10^{-4}$ dilutions and plated on C. diff
ChromID™ using hockey stick sterile spreaders (InterPath Services Pty Ltd, West Victoria, Australia) and incubated anaerobically. Colonies were enumerated from all the ChromID agar plates after 48 h incubation. When two or more dilutions had colonies, the ChromID agar plate with sparse growth (high dilution plate) was counted. The number of colonies in 100 µL was converted to CFU/mL and multiplied by the dilution factor to determine the concentration in the initial sample as shown in the formula below.

\[
\text{CFU/mL} = \frac{\text{Number of colonies formed}}{\text{Volume plated}} \times \text{dilution factor}
\]

Toxin gene PCR and ribotyping were performed on all enumerated plates to confirm that the colonies were \textit{C. difficile}.
3.4 Molecular characterisation of *C. difficile*

3.4.1 DNA extractions for ribotyping and toxin gene PCR

Chromosomal DNA was extracted and purified from a 48 h culture on BA. A loopful (10 µL) of culture was suspended in 100 µL of 5% Chelex® 100 resin freshly prepared in DepC-treated water (Table 3.2). The suspension was vortexed and heated at 100°C on a dry heating block for 12 min before centrifugation at 20,817×g (Eppendorf 5417C microfuge, Hamburg, Germany) for 12 min to pellet debris. A 50 µL aliquot of the supernatant (containing chromosomal DNA) was removed and stored at -20°C until use as template DNA in genotyping reactions (O’Neill *et al.*, 1996).

3.4.2 PCR toxin gene profiling

Reagents and chemicals

EDTA, 0.5M (pH 8.0)
Aspects of *Clostridium difficile* infection in pigs

Ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich)

93.03 g

Deionised water 400 mL

DEPC, 0.1% (v/v) 0.4 mL

NaOH was used to adjust the pH to 8.0. The 0.1% (v/v) DEPC was added, and the solution homogenised and incubated at 37°C overnight. It was, thereafter, autoclaved for 60 min at 15 psi.

**Tris-acetate-EDTA (TAE) buffer, 50**

Tris 121 g

Acetic acid, glacial 23.6 mL

EDTA, 0.5M (pH 8.0) 50 mL

This was dissolved in 500 mL of deionised water and stored at 4°C. TAE was used for electrophoresis of nucleic acids in agarose and polyacrylamide gels.
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**Ethidium bromide solution (10 mg/mL)**

1 g of ethidium bromide was dissolved in 100 mL of deionised water and solution stored at room temperature.

**Loading buffer**

Bromophenol blue 1 g

Sucrose 160 g

Bromophenol blue and sucrose were dissolved in 400 mL and stored at 4°C.

**Reactions**

All *C. difficile* isolates were characterised by PCR to determine the presence of genes for toxins A (*tcdA*) and B (*tcdB*), and binary toxin (*cdtA* and *cdtB*) (Kato *et al.*, 1991; Stubbs *et al.*, 2000). Briefly, 4 µL of DNA extract were added to a PCR mixture (16 µL to detect *tcdA*) that contained 2 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH
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8.3), 200 mM of dNTP, 0.2 mM of each primer NK2 and BE-tdcA-1 (FP: 5’ CAGTCACTGGATGGGAGATT -3’ (20bp) and BE-tdcA-2 (RP: 5’-AAGGCAATAGCGGTATCAG -3’ (19bp) [B. Elliott, unpublished data] (Table 3.1). Novel primers designated tdcA-1 and tdcA-2 were used to detect the tdcA repeating region A3 fragment. The novel primers were multiplexed with tdcA primers NK2 and NK3 (chapter 3; Kato *et al.*, 1991). Detection of both the tdcA1 and tdcA3 fragments was required for an isolate to be considered tdcA positive. To detect tdcB, 2 µL of DNA extract was added to a PCR mixture (18 µL), composition as described for tdcA, 0.2 mM of each primer NK104, NK 105 and 0.75 U of AmpliTaq Gold Taq DNA Polymerase. The final reaction volume was 20 µL. Amplifications were carried out in a 1700 Thermal Cycler (Applied Biosystems, Foster City, California, USA).
Initial denaturation of 94°C for 10 min, followed by 45 amplification cycles of 94°C for 30 s, 45°C for 30 s and 72°C for 60 s, with a final extension cycle of 72°C for 7 min. A negative control containing the reaction mix without template DNA and a positive control with DNA extracted from *C. difficile* RT 027 (SSCC28297) in each experiment. The lower and upper alignment markers used for toxin profiling were 15 bp and 5 kb. The alignment marker calibrates the migration of time variation in the twelve channels in the UltraFlux® 0.2 mL PCR 12-tube strips with domed caps (InterPath, Service, Vic, Australia).
3.4.3 PCR ribotyping (Amplification of the 16S-23S intergenic spacer region)

Reagents and chemicals

Chelex-100 solution 5% (w/v)

Chelex-100 resin 50 g
DEPC-treated water 1 L

The inhibitors in resins were removed by washing in DEPC-treated water three times within 7 days of mixing, and then resuspended.

Agarose gel 3% (w/v)

Agarose, Ultrapure-1000 2.1 g
TAE buffer, 50× plus Ethidium bromide
bromide (25 mg/mL) 1.4 mL
Aspects of *Clostridium difficile* infection in pigs

The gel was prepared by dissolving the ingredients in 70 mL of deionised water then heated for 1 h then decanted into a casting tray with 20-well comb. Gels were stored at 4°C.

**Reactions**

Reactions were prepared with 5 µL of 1 × reaction buffer II, 4mM MgCl₂, 0.4 mM of each primer, 0.4 mM of dNTP, 3.75 units AmpliTaq Gold *Taq* polymerase, 0.02% BSA and 10 µL template DNA (Table 3.2).

PCR was performed on a Gene Amp® PCR system 1700 thermocycler (Applied Biosystems, Foster City, CA, USA) with the following specifications: initial denaturation at 95°C for 10 min, followed by 25 amplification cycles at 72°C for 7 min. each. A negative control containing the reaction mix without template DNA and a positive control with DNA from *C. difficile* RT 027 (SSCC28297) was included in each
Aspects of Clostridium difficile infection in pigs

experiment (Table 3.1). The ribotyping is based on capillary electrophoresis.

### 3.4.4 Purification of reaction products

PCR amplification products were purified using a MiniElute PCR Products Purification Kit (Qiagen, Germantown, MD, USA) as per manufacturer’s instructions. Briefly, the principle of DNA purification is based on the fact that in the presence of high levels of chaotropic salts, DNA selectively binds to a silica membrane. Impurities such as proteins and metabolites do not bind to the membrane and are washed away. The DNA of *C. difficile* eluted using the protocol described below was approximately 15 µL.

In our laboratory, DNA was cleaned up for ribotyping using standard protocols. Briefly, an aliquot of phosphate buffer solution (250 µL) with pH ≤ 7.5 was added to a 1.5 mL DNase-free microcentrifuge tube. Then, 50 µL of the
PCR product was added to the 250 µL phosphate buffer and vortexed for 30 s. The 300 µL mixture was placed into a MinElute column with a 2 mL collection tube and centrifuged at 17900 × g for 60 s. A further 750 µL of buffer PE was added to the MinElute column and centrifuged for 60 s. The MinElute column was placed in a new collection tube, (discarding the old one with the flow-through), and centrifuged for 120 s. Then the MinElute column was placed in a further new collection tube, an Eppendorf snap-lock tube and then a 15 µL of Buffer EB aliquot was applied close to the centre of the column membrane without touching it, left for 60 s at ambient temperature, then centrifuging for 60 s. The Buffer PE concentrate was mixed with absolute ethanol before use. The buffer PB had an indicator added (1:250) volume to bring the pH to pH ≤ 7.5.
3.4.5 Analysis of ribotyping banding patterns

Cluster analysis of PCR ribotyping band profiles was performed by generating dendrograms using neighbour joining and Pearson correlation within BioNumerics™ software package v.7.5 (Applied Maths, Saint-Martens-Latem, Belgium). The upper and lower alignment markers used for ribotyping were 1 kb and 15 bp. The alignment marker calibrates the migration of time variation in the twelve channels in the UtraFlux® 0.2mL PCR 12-tube strips with domed caps (InterPath, Service, Vic, Australia). The DNA size marker used in ribotyping was 50-800 bp (Table 3.2). RTs were identified by comparing their banding patterns with those in our reference library of animal and human C. difficile strains including 15 reference strains from the European Centre for Disease Prevention and Control (ECDC) and the most prevalent PCR ribotypes currently
circulating in Australia [B. Elliott, T.V. Riley, unpublished data].
Table 3.1 Primers and control strains used in profiling *C. difficile* toxin genes and ribotyping by PCR

<table>
<thead>
<tr>
<th>Target gene (product)</th>
<th>Primer sequence (5’-3’)</th>
<th>Primer concentration (pmol ul⁻¹)</th>
<th>Product size (bp)</th>
<th>Positive control strain</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>( tcdA ) F:</td>
<td>CCCAATAGAAGATTTCAATA</td>
<td>251</td>
<td>SSCC</td>
<td>Kato <em>et al.</em>, 1991</td>
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<tr>
<td>( tcdA ) R:</td>
<td>TTAAGCTT</td>
<td></td>
<td>28297 (RT 027)</td>
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<tr>
<td>( tcdA1 ) F:</td>
<td>CAGTCACTGGATGGAGAAT</td>
<td>20</td>
<td>SSCC</td>
<td>E. Brion, 2016</td>
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Aspects of *Clostridium difficile* infection in pigs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Type</th>
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<th>Length</th>
<th>Reference</th>
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<tr>
<td>tcdA-2</td>
<td>R:</td>
<td>AAGGCAATAGCGGTATCAG</td>
<td>19</td>
<td>E. Brion, 2016 (RT 027)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>28297</td>
<td></td>
</tr>
<tr>
<td>tcdB</td>
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<td>GTGTAGCAATGAAAGTCCA</td>
<td>203</td>
<td>Kato <em>et al.</em>, 1999</td>
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<tr>
<td></td>
<td>R:</td>
<td>CACTTAGCTCTTGTATTGCT</td>
<td>203</td>
<td>Kato <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>cdtA</td>
<td>F:</td>
<td>TGAACCTGGAAAAGGTGAT</td>
<td>375</td>
<td>Stubbs <em>et al.</em>, 2000 (RT 027)</td>
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<td></td>
<td>G</td>
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<table>
<thead>
<tr>
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110
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<tr>
<th></th>
<th>R:</th>
<th>375</th>
<th>SSCC</th>
<th>Stubbs <em>et al.</em>, 2000</th>
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<tbody>
<tr>
<td>CD16S</td>
<td>CTGGGGTGAAGTCGTAACA AGG</td>
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<td></td>
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<td></td>
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<tr>
<td></td>
<td>AGGATTATTTACTGGACCA</td>
<td>28297</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>TTTG</td>
<td></td>
<td></td>
<td>(RT 027)</td>
</tr>
<tr>
<td><em>cdtB</em></td>
<td>F:</td>
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<td></td>
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<tr>
<td></td>
<td>CTTAATGCAAGTAAATACT GAG</td>
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<tr>
<td></td>
<td>R:</td>
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<tr>
<td></td>
<td>AACGGATCTCTTGCTTCAG TC</td>
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Aspects of *Clostridium difficile* infection in pigs

<table>
<thead>
<tr>
<th>CD23S</th>
<th>GCGCCCTTTGTAGCTTGAC</th>
<th></th>
<th></th>
<th></th>
<th>Stubbs et al., 1999</th>
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</table>

Positive control strain SSCC 28297 (RT 027) sourced from (Lefebvre et al., 2006).
### Table 3.2 Reagents and solvents used in the experiments

<table>
<thead>
<tr>
<th>Chemical/Reagents</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AmpliTaq Gold®</td>
<td>Applied Biosystems, Foster City, USA</td>
</tr>
<tr>
<td>Anti-Pig-IgG (whole molecule)</td>
<td>Sigma-Aldrich, Castle Hill, NSW, Australia</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>Sigma-Aldrich, St Louis, MO, USA</td>
</tr>
<tr>
<td>Chelex-100</td>
<td>Sigma-Aldrich, St Louis, MO, USA</td>
</tr>
<tr>
<td>Diethyl pyrocarbonate (DepC) (0.1% v/v)</td>
<td>Sigma-Aldrich, St Louis, MO, USA</td>
</tr>
<tr>
<td>Dulbecco’s modified Eagle’s medium</td>
<td>Sigma-Aldrich, Castle Hill, NSW, Australia</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>Fisher Biotech, Wembley, WA, Australia</td>
</tr>
<tr>
<td>Fetal bovine Serum</td>
<td>Sigma-Aldrich, St Louis, MO, USA</td>
</tr>
<tr>
<td>16% Formaldehyde (w/v)</td>
<td>Life Technologies, Vic, Australia</td>
</tr>
<tr>
<td>Formic acid</td>
<td>Sigma-Aldrich, St Louis, MO, USA</td>
</tr>
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</table>
Aspects of *Clostridium difficile* infection in pigs

<table>
<thead>
<tr>
<th>Chemical/Reagents</th>
<th>Manufacturer</th>
</tr>
</thead>
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<tr>
<td>IMJECT Alum</td>
<td>Life Technologies, Vic, Australia</td>
</tr>
<tr>
<td>L-proline aminopeptidase</td>
<td>Remel Inc, KS, USA</td>
</tr>
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<td>Magnesium chloride</td>
<td>Applied Biosystems, Foster City, USA</td>
</tr>
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<td>(MgCl$_2$)</td>
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<tr>
<td>N alpha-Tosyl-L-Lysine chloromethyl ketone hydrochloride</td>
<td>Sigma-Aldrich, Castle Hill, NSW, Australia</td>
</tr>
<tr>
<td>Phosphate buffered solution (PBS)</td>
<td>PathWest Media, Mt Claremont, WA, Australia</td>
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<tr>
<td>Potassium chloride (KCl)</td>
<td>Applied Biosystems, Foster City, USA</td>
</tr>
<tr>
<td>Protease inhibitor cocktail</td>
<td>Sigma-Aldrich, Castle Hill, NSW, Australia</td>
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<td>QIAxcel Alignment marker</td>
<td>QIAGEN, Germantown, MD, USA</td>
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<tr>
<td>QIAxcel size marker$^{a/b}$</td>
<td>QIAGEN, Germantown, MD, USA</td>
</tr>
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<td>QIAxcel DNA dilution buffer</td>
<td>QIAGEN, Germantown, MD, USA</td>
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<tr>
<td>QIAxcel separation buffer</td>
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### Chemical/Reagents

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<tr>
<td>QIAxcel wash buffer</td>
<td>QIAGEN, Germantown, MD, USA</td>
</tr>
<tr>
<td>QIAxcel mineral oil</td>
<td>QIAGEN, Germantown, MD, USA</td>
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<td>QIAxcel intensity calibration</td>
<td>QIAGEN, Germantown, MD, USA</td>
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<td>marker</td>
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<tr>
<td>Reaction buffer II</td>
<td>Applied Biosystems, Foster City, CA, USA</td>
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<tr>
<td>Sulfuric acid solution</td>
<td>Sigma-Aldrich, Castle Hill, NSW, Australia</td>
</tr>
<tr>
<td>Taurocholic acid sodium salt hydrate</td>
<td>Sigma-Aldrich, St Louis, MO, USA</td>
</tr>
<tr>
<td>3,3',5,5' Tetramethylbenzidine (TMB)</td>
<td>Sigma-Aldrich, Castle Hill, NSW, Australia</td>
</tr>
<tr>
<td>Tris</td>
<td>Invitrogen, Life Technologies, Vic, Australia</td>
</tr>
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<td>Tris Hydrochloric acid (Tris-HCl)</td>
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</tr>
<tr>
<td>Triton X-100</td>
<td>Sigma-Aldrich, Castle</td>
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Aspects of *Clostridium difficile* infection in pigs

<table>
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<tr>
<th>Chemical/Reagents</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hill, NSW, Australia</td>
</tr>
<tr>
<td>Tween-20</td>
<td>Sigma-Aldrich, Castle Hill, NSW, Australia</td>
</tr>
<tr>
<td>100 bp DNA ladder</td>
<td>Invitrogen, Life Technologies, Vic, Australia</td>
</tr>
<tr>
<td>0.1% peptone solution</td>
<td>PathWest Media, Mt Claremont, WA, Australia</td>
</tr>
</tbody>
</table>

*a* DNA size marker for ribotyping was 50-800 bp  
*b* DNA size marker for toxin profiling was 100bp-2.5 kb
3.4.6 Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) for 14 antimicrobials (amox-clavulanate, ceftriaxone, clindamycin, erythromycin, gentamicin, meropenem, metronidazole, moxifloxacin, piperacillin/tazobactam, spectinomycin, tobramycin, trimethoprim and vancomycin) were determined for a selection of isolates using the agar incorporation method as described by the Clinical and Laboratory Standards Institute (CLSI, M11-A7). A combination of CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints was used if available (CLSI, 2013; EUCAST, 2014). The quality control strains used were *Bacteroides fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29741, *C. difficile* ATCC 700057 and *Eubacterium lentum* ATCC 43055.
3.4.7 Inoculum preparations for microdilution tests

The test organisms (C. difficile) were revived on blood agar incubated anaerobically for 48 h. Then five colonies of similar morphology were suspended in sterile 0.85% normal saline in a Bijoux bottle (turbidity = McFarland 0.5 standard). This suspension contained approximately 1 to 2 x 10^8 CFU/mL for B. fragilis ATCC® 25285 and B. thetaiotaomicron ATCC® 29741, but 1 to 4 x 10^7 for C. difficile ATCC® 700057 (CLSI, M11-A7). All antimicrobial plates were commercially prepared by PathWest Media.

3.4.8 Inoculation of agar dilution plates

The inoculum as described above was inoculated onto antimicrobial-containing agar plates using a 48-pin inoculum replicating apparatus. The replicator pins deposited approximately 2 µL onto the agar surface. The final inoculum on the agar was approximately 10^5 CFU
per spot for *B. fragilis* and *B. thetaiotaomicron*. All manipulations were performed in air at room temperature. At the beginning of inoculation, two control plates with no antibiotics one labelled pre-oxygen (to check for contamination) and another pre-anaerobe (for the beginning anaerobic growth control).

The antimicrobial agar plates were inoculated starting with the lowest concentration to the highest. Thereafter, between each series of plates, one control plate without antimicrobials was inoculated as check for possible contamination that might occur as plates were being inoculated. At the end of the final series, two plates labelled post aerobic and post anaerobic to verify the final organism viability and purity (CLSI, M11-A7).

**3.4.9 Incubation of agar dilution plates**

Approximately 10 min after the agar plates had been inoculated, all the plates were placed in the anaerobic
incubation chamber at 35°C for 48 h. Control plates for aerobic incubation (to verify inability to grow in the presence of atmospheric oxygen) were placed in the incubator at 35°C for 48 h.

3.4.10 Reading agar dilution plates

The control plates were examined first to ensure that no aerobic contamination was present. After examining the control plates, the minimum inhibitory concentration (MIC) endpoints on test plates were determined. The MIC was considered as the concentration at which a marked reduction occurred in the appearance of growth on a test plate as compared to growth on the anaerobic control plates (CLSI, M11-A7).

The Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints were used if available (EUCAST, 2014; CLSI, 2013).
3.4.11 Inoculum preparations for epsilometer test (E-tests)

A *C. difficile* isolate was inoculated on BA and incubated anaerobically at 35°C for 48 h. A sterile swab was used to emulsify growth from the BA plate into 1mL of sterile 0.85% normal saline into a Bijoux bottle (McFarland standard 1.0 or 3.0 ×10⁸ cells). A sterile swab was used to lawn inoculate the entire surface of a BA plate. Excess moisture was allowed to be absorbed in the agar for 15-20 min before applying the E-test strips to the agar using sterile forceps. The strips were positioned in such a way that the MIC scale was facing upwards with the concentration maximum (marked E) nearest the rim of the plate. The air pockets were removed by gently pressing the strips using the forceps. The plates were incubated anaerobically for 48 h. The MIC values were read from where the edge of the inhibition ellipse intersected the strip.
Ten isolates were tested for susceptibility to azithromycin, clindamycin, metronidazole and moxifloxacin susceptibility using E-test strips (bioMérieux, Australia). The MIC breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines were adopted (CLSI, 2013).

3.4.12 Clostridium difficile toxoid vaccine development

3.4.12.1 C. difficile strains and toxin filtrate

*C. difficile* RTs 237 (A–B+C DT+) (WAP089) and 014/020 (A+B+C DT-) (ESP0119) were obtained from the Riley *C. difficile* research laboratory culture collection stored at -80°C. RT 237 (A–B+C DT+) and RT 014 (A+B+C DT-) were clinical isolates from diarrhoeic piglets in Western Australia (chapter 4) and Eastern Australia (Knight *et al.*, 2015). *C. difficile* strains were cultivated on BA plates in an anaerobic environment for 48 hr at 35°C to allow sporulation. A 48 h culture was inoculated
into brain heart infusion broth with L-cysteine -BHIB consisting of cycloserine and cefoxitin in a sterile DNase-free 50 mL centrifuge tube (Sigma-Aldrich, St Louis, MO, USA). The spore concentration was determined by dilution plating onto blood agar plates and stock solutions of $4 \times 10^7$ spores/mL. Briefly, after incubation at 35°C for 5 days on a shaker in an anaerobic environment, cells were removed by centrifugation at $16000 \times g$ for 20 minutes. The supernatant was filter sterilised using 0.2 µm syringe filter (Pall-Life Sciences, Cheltenham, VIC, Australia,) and stored at 4°C until use.

3.4.12.2 Toxin purity assessment

Toxin purity was tested for by Gram staining the filtrate, and culture of the BHIB and toxin filtrate under anaerobic and aerobic conditions. No growth of microorganisms from the filtrate under anaerobic and aerobic conditions signified that the filtrate was free from contamination. Toxin expression was confirmed by rapid
membrane enzyme immunoassay for simultaneous detection of *C. difficile* glutamate dehydrogenase antigen and toxins A and B (TechLab Blacksburg, VA, USA; C. DIFF QUIK CHEK COMPLETE). The detection limit for enzyme immunoassay for TcdA was $\geq 0.63$ ng/mL, for TcdB $\geq 0.16$ ng/mL, and for glutamate dehydrogenase at $\geq 0.8$ ng/mL. Toxin expression was measured in parallel with *C. difficile* ATCC 43255 ($A^+B^+CDT^-$) as a positive control, using a HiMarkTM standard protein, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

### 3.4.12.3 SDS-PAGE

Visualisation of a single band of protein was performed by SDS-PAGE to confirm protein identity. Briefly, SDS-PAGE was performed to separate proteins of *C. difficile* in the BHIB filtrate harvested as described in section 3.4.12.1. The filtrate was concentrated by centrifugation at $16000 \times g$ for 20 min and the final
concentration of the protein was $\geq 0.2$- $0.5$ optical density (OD) read at 600 nm. A 12% separating gel was prepared in a 15mL Falcon tube with 10% ammonium persulphate (APS) and tetramethylethlenediamine (TEMED). The plates were loaded with 12% separating gel using a pipette to a volume of $\sim 1$ cm below the comb depth. Then isopropanol was added on top of 12% separating gel and the remaining separating gel in the Falcon tube was used as an indicator of polymerisation. The isopropanol was decanted after the gel had set and then a 4% stacking gel was prepared in a 15-mL Falcon tube with 10% APS and TEMED added before loading. After the gels were set, the combs were removed and the gels assembled in the tank as per the manufacturer’s instruction. The tank was filled with 1× running buffer and samples were added to the wells (25 $\mu$L of samples, 25 $\mu$L of HiMark$^{TM}$ protein standard [ladder] (Thermofisher, Victoria, Australia). The empty wells were further filled with 1× sample buffer. The
SDS-PAGE was run at 200 V for 45 min at 4°C. After the SDS-PAGE finished running, it was equilibrated for 30 min in transfer buffer.

### 3.4.12.4 Coomassie Blue staining of SDS-PAGE gel

The gels were stained with Coomassie Brilliant Blue G-250 Dye (ThermoFisher Scientific, Pierce, Rockford, IL, USA) for 1-2 h with gentle agitation on a shaker and, thereafter, gels were destained overnight by gentle agitation in destaining solution.

### 3.4.12.5 Bradford assay for determining protein concentration

Determination of protein concentration in the toxin filtrates was performed using the Bradford method (Bradford, 1976). Briefly, Coomassie Blue reagent was vacuum filtered and stored in a bottle until use. Then 10 mg of bovine serum albumin (BSA) was dissolved in 10 mL of phosphate buffer. A varying amount of BSA in the range 5-100 µg was pipetted in triplicates for eight
different protein standards. Then varying amounts of PBS were pipetted into each of the different standard proteins to make up 100 µL for all standards. A blank containing 100 µL of BHIB was created and then 1000 µL of Coomassie Brilliant Blue G 250 was pipetted into each tube and vortexed for 5 s. Using the blank (100 µL BHIB) as the reference zero, the triplicate absorbance readings at 595 nm after 2 min of inoculation were averaged to plot an absorbance standard curve. For each RT 237 and RT 014/020 filtrate, 1 mL of concentrated protein was dissolved in 100 mL of deionised water. Then 100 µL of each sample was mixed with 1000 µL of Coomassie Brilliant Blue G 250 and vortexed for 5 s. The total protein concentration per mL of filtrate was estimated by extrapolating from the standard curve of serum.
3.4.13 Protein characterisation of filtrates using SDS-PAGE

The apparent molecular mass (MW) estimates of proteins secreted in BHIB were determined by comparing protein migration to the standard protein. A protein with apparent MW ~307 kDa and 270 kDa which corresponds to TcdA and TcdB were not visible on the SDS-PAGE gel. A protein with 48 kDa was present in RT 237, ATCC 43255, and in RT 014/020 (faint).

The apparent molecular masses of putative cell wall proteins (CWP) detected using SDS-PAGE were determined by estimating the protein migration in relation to the molecular mass of the standard protein as described by Fagan and others (2011). CWP25 (~33 kDa), CWP9, CWP14, CWP24 and CWP28 (~52.5 kDa), CWP20 (~111 kDa) and CWPV (~166.7 kDa) were detected RT 237 and ATCC 43255. CWP9, CWP14, CWP24 and CWP28
(~52.5 kDa) had less distinct bands. This is typical of intrinsic membrane proteins which show hydrophobic side chains that may or may not have denatured completely during sample processing. The surface layer proteins (SLP) for *C. difficile* are classified as low-molecular-weight (LMW-SLPs) and the high molecular weight proteins (HMW-SLPs) based on molecular weight. The first group of SLP molecular weights are in the range 32 kDa to 45-47 kDa, and the other group are in a range of 38 kDa to 42 kDa MW (Ferreira *et al.*, 2017; Takeoka *et al.*, 1991). The LMW-SLP is more immunogenic than HMW-SLPs (Ryan *et al.*, 2011).

**3.4.13.1 Determination of protein concentration in filtrates using the Bradford assay**

The total protein concentration in the vaccine was estimated as 21 mg/mL for RT 237 (OD 0.21) and 22 mg/mL for RT 014/020 (OD 0.22).
3.4.13.2 *Clostridium difficile* toxoid vaccine development

3.4.13.2.1 *C. difficile* strains and toxin filtrate

A *C. difficile* culture toxin filtrate consisting of a native TcdB and CDT elaborated from RT 237 or TcdA and TcdB from RT 014/020 was added to an equal volume of (1% v/v) formaldehyde with 4.5 mg of N\(\alpha\)-Tosyl-L-lysine chloromethyl ketone hydrochloride (Sigma-Aldrich, NSW, Australia) and incubated at 4°C for 120 h. Aluminium hydroxide (40 mg/mL) and magnesium hydroxide (40 mg/mL) plus inactive stabilisers (Imject Alum®) [Thermo Fisher Scientific, Rockford, IL, USA] was added to the toxoid according to the manufacturer’s instructions. Alum was added to the toxoid vaccine at the ratio of 2:3. We did not precipitate the toxins using ammonium sulphate because we wanted to harvest *C. difficile* cell wall proteins in the filtrate which have been described to enhance colonisation resistance against *C. difficile*.
Clostridium difficile (Huang et al., 2015; Senoh et al., 2015a; Willing et al., 2015; Fagan et al., 2011). For the control treatment, the filtrate of the BHIB was inoculated with alum in the ratio 2:3.

3.5 Animal selection

3.5.1 Serum and colostrum samples

Six pregnant gilts (treatment group) and six controls were selected for this study (Table 3.3). Both cohorts were sampled for blood and faeces 3-6 week’s prepartum and vaccinated thereafter. Pigs received two injections pre-farrowing. The vaccine was given at 3-weeks interval (priming inoculation plus one booster injection). The controls received adjuvant mixed with BHIB solution. At farrowing, both cohorts were sampled again for blood, colostrum and faeces to determine if there was any difference in samples collected before vaccination.
3.5.2 Blood recovery

Blood samples were collected by ear venipuncture using a 10-mL syringe fitted with a 20-gauge needle. Approximately 5 mL of blood was collected from each pig at each time point. Blood was allowed to clot and then serum was separated by centrifugation at 10000 × g for 10 min. Serum was stored at -80 °C until use.

Table 3.3 Vaccination protocol for C. difficile toxoid vaccine in gilts

<table>
<thead>
<tr>
<th>Group</th>
<th>TcdA</th>
<th>TcdB</th>
<th>CDTa/b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Absent</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Group 2</td>
<td>✓</td>
<td>✓</td>
<td>Absent</td>
</tr>
<tr>
<td>Group 3</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
</tbody>
</table>

✓ Antigen present in the toxoid vaccine and alum adjuvant: Group 1 toxoid vaccine made from ribotype 237 and Group 2 from ribotype 014/020.
± Alum adjuvant given to control group 3.

3.5.3 Determination of pig antibody titres by indirect ELISA

The analysis of humoral immune responses of sows’ serum and colostrum was collected as shown in Table 3.3 and Figure 8.1. Sample extractions were made at a one-fifth (wt/v) dilution in extraction buffer (2% foetal calf serum [FCS], Dulbecco’s modified Eagle’s medium [DMEM] plus protease inhibitor cocktails, trypsin [0.1 mg/mL], leupeptin [1 µg/mL], benzamide [1 µg/mL], aprotinin [10 µg/mL], phenylmethylsulfonyl fluoride [1 mM], and EDTA [0.05 mg/mL]). Samples were gently shaken for 30 min at 4°C to disrupt solid material and then centrifuged (16000 × g for 20 min). Supernatants were filter sterilised using sterile syringe filters with a 0.45-µm pore size (Pall-Life Sciences, Cheltenham, VIC, Australia) before analysis. Antibodies from sera and colostrum were
determined by indirect enzyme-linked immunosorbent assay (ELISA) as described previously with modifications (Permpoonpattana et al., 2011).

Nunc 96-well plates (Thermo Fisher Scientific, NSW, Australia) were coated with purified toxin filtrate TcdA and TcdB, or TcdB in bicarbonate buffer overnight at ambient temperature (AT). Thereafter, plates were blocked for 1 h at 30°C with 2% bovine serum albumin (BSA), 2-fold serially diluted samples were added, starting with an undiluted sample followed by a 1/2 dilution in diluent buffer (0.01 M PBS [pH 7.4], 0.5% [wt/v] bovine serum albumin [BSA], 5% [v/v] foetal bovine serum [FBS], 0.1% [v/v] Triton X-100, 0.5% [v/v] Tween 20). Replicate samples were examined together with a negative control (pre-immune serum). Plates were incubated for 2 h at AT before addition of appropriate horseradish peroxidase-conjugated anti-pig IgG antibodies in conjugate buffer (5% FBS [v/v], 1% BSA [wt/v], 0.05% Tween 20 in 0.01
M PBS). Peroxidase conjugated anti-porcine IgG was used as a secondary antibody in a 1:20000 dilution in TSB-Tween (0.2%). Plates were incubated for 1 h at AT and then developed using tetramethylbenzidine (TMB) substrate (0.1 mg/mL 3.3’, 5.5’-tetramethylbenzidine in 0.1 M sodium acetate buffer [pH 5.5] in distilled water). Reactions were stopped using 1 M H$_2$SO$_4$, and optical densities (ODs) were read at 450 nm with a reference filter of 540 nm using a micro-ELISA plate reader (xMark™ spectrophotometer - BIO-RAD 10193). Antibody levels were reported as mean OD of duplicate samples. Dilution curves were created for each sample, and endpoint titres for each specific antibody were estimated at the maximum dilution of serum giving an absorbance reading of 0.01 U over the ODs of naïve samples. Cross-protection assays were performed for both serum and colostrum in which RT 014/020 or RT 237 vaccinated pig serum were exposed to either of the two antigens. We determined the
ELISA cut-off as described by others (Ridge and Vizard, 1993).

The faecal samples from piglets were collected and processed as described in section 3.2 in this chapter. All positive cultures were characterised by standard molecular methods as described in this chapter.

3.5.4 Ethical Considerations

Animal ethics committee approval was obtained from The University of Western Australia Animal Ethics Committee (reference number RA/3/500/75) for the observational studies in chapter 3, and the Ethics approval for the vaccine study was obtained from The University of Western Australia Animal Ethics Committee (reference number RA/3/100/1481).

The ethical concern for this study was to keep safe identifier of the farm and individual animals in the study. The names of the farmers and animals were not used as
identifiers in our study. The dataset was de-identified therefore, the name of the farmer nor the owners were not identified in all our publications.
CHAPTER 4: PERSISTENCE OF CLOSTRIDIUM DIFFICILE RT 237 INFECTION IN A WESTERN AUSTRALIAN PIGGERY

4.1 INTRODUCTION

In 2009, a farrow to finish commercial piggery in Western Australian was experiencing idiopathic diarrhoea in up to 80% of neonatal pigs with mortality in the range 11-14%. The affected piglets had an early-onset of diarrhoea which was yellow, non-haemorrhagic, and pasty to watery. Untreated piglets had ill-thrift, became anorexic and dehydrated, and some died. Apparently healthy piglets (1-3 days old) were prophylactically treated with amoxicillin or penicillin. A cross-sectional study in the piggery found a C. difficile prevalence of 62% (114/185) in 5-7-day-old piglets (Squire et al., 2013). In that study, molecular typing revealed
all isolates of *C. difficile* recovered were an unusual RT 237, toxinotype XXXI (*tcdA*−, *tcdB*+), binary toxin positive (*cdtA/B*+) strain. Few studies have described the epidemiology of infections in livestock with RTs of *C. difficile* other than RT 078 (Knight *et al.*, 2015; Norén *et al.*, 2014; Squire *et al.*, 2013; Avberšek *et al.*, 2009).

This chapter describes two observational studies, a cross-sectional and a prospective cohort study, that further investigated CDI in the same piggery in Western Australia. The relationship between *C. difficile* colonisation/shedding and disease in piglets farrowed from a range of parity and litter sizes was examined. The prospective cohort study examined the relationship between age and *C. difficile* shedding over time. Finally, this chapter examines potential risk factors associated with *C. difficile* shedding in piglets.


4.2 Aims

The aims of this chapter were:

i) To determine if *C. difficile* RT 237 had persisted in the same piggery or whether there had been a temporal change in *C. difficile* diversity.

ii) To determine whether there were temporal changes in *C. difficile* strains circulating in piggeries to identify potential transmission pathways.

iii) To investigate the epidemiology of *C. difficile* acquisition in the piggery.

iv) To determine the production stage in the piggery that had the highest risk of dispersing *C. difficile* in the environment.

v) To determine factors which were significantly associated with *C. difficile* transmission within a piggery, such as age, litter size, mortality and diarrhoea.
4.2.1 Setting, study design and sampling

The study designs used to address the aims were single cross-sectional and prospective cohort studies, with sampling conducted from October to December 2014. The cross-sectional study was the appropriate study design in determining the prevalence of *C. difficile* in piglets because it is a robust epidemiological tool to determine a one-time point prevalence of *C. difficile* in piglets. The piggery was located across two sites. The farrow-to-wean site had two holdings separated by a fence, with approximately 5000 sows; holding “A” consisted of older breeding sows (parity > 1) and holding “B” consisted of gilts. Holding “C” was the finishing site some 20 km away. The sample size for the cross-sectional study was determined using Fleiss methods with a continued correction factor (Fleiss *et al.*, 2013). We estimated that 47.4% of non-diarrhoeic piglets were
Aspects of Clostridium difficile infection in pigs

shedding C. difficile and 92.8% of diarrhoeic piglets were exposed. The ratio of non-exposed piglets to exposed piglets was assumed to be 0.5, and with an odds ratio of 14, and a power of 80% to detect the difference if it existed, a sample size of 43 piglets was selected. Fresh faecal samples were collected via rectal swabs from 4 or 5 piglets randomly selected from each of 9 litters aged 1-10 days.

For the cohort study, we estimated a difference of 27% prevalence of C. difficile shedding between 1 day-old (77%) and 42-day-old piglets (50%) based on earlier studies (Squire et al., 2013). Using a two tail Z-test for logistic regression, with α of 0.05% and power of 80%, we determined that a total sample of 88 piglets was required. To account for possible loss to follow-up of 12%, 12 piglets were added to the sample to make a total of 100. Faecal samples (n=20) were randomly obtained from 5 piglets from each of 4 litters.
Aspects of *Clostridium difficile* infection in pigs

as described above on days 1, 7, 13 and 20, at the farrow-to-wean holding and on day 42 at the finishing site. One day before weaning, 20 piglets were ear tagged to allow follow-up at the finishing site. Among the four litters studied, two had 10 piglets each and the others had 14 piglets each. All swabs were transported in Amies transport medium with charcoal (Thermo Fisher Scientific, Waltham, MA, USA) in a cooler box at 4°C to The University of Western Australia, School of Pathology and Laboratory Medicine, for processing within 24 h.

This piggery had a two stage in-series anaerobic pond system for treatment of effluent. The primary aerobic pond has an inlet design to facilitate easier desludging of the pond. After moving through the primary pond, effluent moved to a secondary pond which allowed reuse and storage. No chemical disinfection was applied to the water. Therefore, an
additional four 30 mL specimen jars (Techno-Plas Pty Ltd, St Marys, Australia) of treated water held for under-pen flushing in storage tanks located adjacent to the farrowing shed, four 30 mL effluent samples from a drainage channel leading to the treatment pond, and six shed floor swabs transported in Amies transport medium with charcoal were obtained from holding “A”. The six-floor swabs were obtained by directly swabbing the wet floor from six pens.

Additional data were collected such as the health status of the piglets, age, litter size, mortality, parity of sow and farrowing date. A piglet was considered diarrhoeic at the sampling time using the following criteria: i) had yellow, non-hemorrhagic, and pasty to watery faeces and ii) any piglet painted red at the dorsum by personnel based on diarrhoea being observed, and that had a perineum soiled
Aspects of *Clostridium difficile* infection in pigs

with watery faeces. A litter was classified as diarrhoeic if one or more piglets had diarrhoea at the time of sampling.

### 4.2.4 Statistical analysis

The Chi-squared test was used to evaluate the association between isolation of *C. difficile* and diarrhoea in the cross-sectional study. *C. difficile* shedding over time was evaluated by the generalised estimating equations (GEEs) for longitudinal data collected in clusters that are repeated measures. The outcome variable was considered as binary (presence or absence of *C. difficile* per sample) and fixed effects models were employed in GEEs to adjust for the response variable from within clusters (litters) as well as over time (6 weeks). In fitting the data to the model, the independent working correlation structure was used as this implies that the within-litter correlation between all sampling was equal to zero. GEEs have been shown to be robust even
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when there is an error in specifying the working correlation structure (Zeger and Liang, 1986). All associations with a $p$-value < 0.05 were considered significant. All analyses were performed using Epi-Info™ 7.1.4.0 statistical software from the Centers for Disease Control and Prevention (CDC) and R version 3.2.2.

**Animal ethics committee approval.** This study was approved by The University of Western Australia Animal Ethics Committee (reference number RA/3/500/75).

**4.3 RESULTS**

**4.3.1 *C. difficile* carriage in piglets**

*C. difficile* was isolated from 19/43 (44.2%, 95% CI 29.3%-59.1%) faecal swabs by direct culture and 29/43 (67.4%, 95% CI 53.39-81.41) with enrichment media from holding “A”. Enrichment culture was significantly more sensitive than direct culture ($p=0.0002$, $\chi^2$). Of the diarrhoeic
Aspects of Clostridium difficile infection in pigs

piglets, 20 of 24 (83.3%) were C. difficile culture positive compared to 9 of 19 (47.4%) non-diarrhoeic piglets ($p = 0.0124$, $\chi^2$). C. difficile was isolated from piglets in 7 out of 9 pens (77.9%).

A total of 13/106 (12.3%) piglets died across the nine litters sampled in the cross-sectional study. However, the association between C. difficile positive status and mortality was not significant ($p = 0.74$). There were seven litters with and two without diarrhoea and a total of 24 out of 43 diarrhoeic piglets. The comparison between parity and C. difficile positive status of piglets was made between parity 3 (referent) and combined piglets from sows with parity 4, 5 and 6 because of sparse data. C. difficile distribution in piglets by parity of sow was parity 3 (13/19; 68.4%), parity 4 (7/10; 70%), parity 5 (5/9; 55.6%), and parity 6 (4/5; 80%). All C. difficile isolates from piglets were RT 237.
4.3.2 The prospective cohort study

*C. difficile* was isolated from 8/20 fecal samples (40%) on day 1, 10/20 (50%) on day 7, 4/20 (20%) on day 13, 0/20 (0%) on day 20, and 0/20 (0%) on day 42 (Table 4.1). The multivariate model evaluated the following variables: age of piglets, litter size, mortality and diarrhoea (Table 3.1). There was no significant difference between *C. difficile* shedding on day 1 versus day 7 (*p* = 0.10), nor day 1 versus day 13 (*p* = 0.10). However, there was a significant difference in *C. difficile* shedding between 1-day-old piglets and piglets at 20 and 42 days of age (*p* < 0.000). The regression coefficients were positively associated with *C. difficile* shedding on day 7 but were strongest and negatively (inversely) associated with shedding on day 13 to day 42 (Table 4.1). The risk of shedding *C. difficile* in the faeces by piglets significantly declined from day 13 onwards, as the regression coefficients
were negative [inverse] (Table 4.1). The overall prevalence of *C. difficile* was 22% (22/100). There were a total of 48 piglets from the four litters studied. This means that 42% of piglets were sampled at each time point indicating that each piglet had 42% chance of being sampled every week. *C. difficile* was isolated at least once from all study litters 100% (4/4).

*C. difficile* was not isolated from piglets aged 20 days and 42 days (n=20) (Table 3.1). There was a total of 36/100 cases (36%) of diarrhoea among the sampled piglets. The cases of diarrhoea in piglets per sampling time were as follows: day 1 (8/20; 40%), day 7 (6/20; 30%), day 13 (11/20; 55%), day 20 (9/20; 45%) and day 42 (2/20; 10%). However, the association between *C. difficile* positive status and diarrhoea for all cases was not significant (*p* = 0.67).
Nine piglets from four litters died in this study, giving a 9% mortality rate. Seven of the piglets were from diarrhoeic pens where \textit{C. difficile} was identified, while two were from non-diseased but \textit{C. difficile} positive pens. The regression coefficient for mortality was positively associated with \textit{C. difficile} shedding ($p = 0.001$, Table 4.1).

The toxin B gene (\textit{tcdB}) but not \textit{tcdA} was detected by PCR in all \textit{C. difficile} isolates from the 22 infected piglets, including both diarrhoeic and non-diarrhoeic animals. Binary toxin genes (\textit{cdtA} and \textit{cdtB}) also were detected in all isolates and all were RT 237.

\textbf{4.3.3 Environmental samples}

The effluent samples ($n = 4$) obtained from a drainage channel before the two-stage treatment ponds were all positive for \textit{C. difficile} by enrichment culture. Additionally, two of the four samples of treated water collected from the
farrowing sheds were positive. Furthermore, four of the six-floor swab samples collected from some of the pens of diarrhoeic and non-diarrhoeic litters were positive (67%). All environmental isolates were RT 237.
Table 4.1 Diarrhoea and *C. difficile* shedding over time by piglets in relation to their age

<table>
<thead>
<tr>
<th>Variable</th>
<th>C. difficile positive</th>
<th>C. difficile negative</th>
<th>GEEs Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a D+ c D- d Total</td>
<td>b D+ c D- d Total</td>
<td>Coefficients</td>
</tr>
<tr>
<td>Intercept</td>
<td>2 6 8</td>
<td>6 6 12</td>
<td>1.9218</td>
</tr>
<tr>
<td>Day 1</td>
<td>3 7 10</td>
<td>3 7 10</td>
<td>Referent</td>
</tr>
<tr>
<td>Day 7</td>
<td>4 0 4</td>
<td>7 9 16</td>
<td>-1.1701</td>
</tr>
<tr>
<td>Day 20</td>
<td>0 0 0</td>
<td>9 11 20</td>
<td>-43.15</td>
</tr>
<tr>
<td>Day 42</td>
<td>0 0 0</td>
<td>2 18 20</td>
<td>-43.15</td>
</tr>
</tbody>
</table>
Aspects of *Clostridium difficile* infection in pigs

<table>
<thead>
<tr>
<th>Litter size</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>-0.28</th>
<th>0.17</th>
<th>0.05</th>
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</thead>
<tbody>
<tr>
<td>Mortality</td>
<td>7</td>
<td>2</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.48</td>
<td>0.17</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

Note. *a* *C. difficile* test, *b*D⁺ diarrhoeic, *c*D⁻ non-diarrhoeic, *d>Total
4.3.4 Antimicrobial susceptibility profiles

MICs for 14 antimicrobials were determined for 29 isolates sourced from the cross-sectional study (Table 4.2). Despite the probability that these isolates were clonal, there were some small variations in susceptibility; however, all were susceptible to the antimicrobials for which breakpoints were available. There are no CLSI and EUCAST breakpoints available for the following antimicrobials; gentamicin had MIC range (32-64 mg/L), spectinomycin (128 mg/L), tobramycin (32-128 mg/L), and trimethoprim (32-64 mg/L).
Table 4.2 Minimal inhibitory concentration (MIC) range and proportion susceptible, intermediate, and resistant for RT 237 isolates ($n=29$) against a panel of 14 antimicrobial agents

<table>
<thead>
<tr>
<th>Agent</th>
<th>MIC range [mg/L]</th>
<th>Clinical breakpoints</th>
<th>Proportion susceptible, intermediate and resistant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>I</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1</td>
<td>≤ 2</td>
<td>NR</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>0.25 - 1</td>
<td>≤ 8</td>
<td>16</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.25 - 4</td>
<td>≤ 2</td>
<td>4</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.25 - 0.5</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Amox-clavulanate</td>
<td>0.12 - 0.25</td>
<td>≤ 4</td>
<td>8</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>8</td>
<td>≤ 16</td>
<td>32</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>1</td>
<td>≤ 2</td>
<td>4</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>Susceptible (S)</td>
<td>Intermediate (I)</td>
<td>Resistance (R)</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----------------</td>
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</tr>
<tr>
<td>Meropenem</td>
<td>0.25 - 2</td>
<td>≤ 4</td>
<td>8</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.12</td>
<td>≤ 4</td>
<td>8</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>2 - 4</td>
<td>≤ 32</td>
<td>64</td>
</tr>
</tbody>
</table>

Note. The susceptible (S), intermediate (I), and resistance (R) interpretive values when available were obtained from CLSI or EUCAST (vancomycin only). If breakpoints were not available from CLSI and EUCAST then a no range (NR) was recorded.
4.4 DISCUSSION

This study aimed to determine if *C. difficile* RT 237 had persisted in the Western Australian piggery that was investigated in 2009 (Squire *et al.*, 2013). *C. difficile* RT 237 was found again and the prevalence in the cross-sectional study (67.4%) was similar to the earlier study (62%) (Squire *et al.*, 2013), and the same as a national prevalence study conducted recently in 21 Australian piggeries (67%) (Knight *et al.*, 2015). In the Australian national survey RT 014/020, a strain commonly reported in human hospital settings (Foster *et al.*, 2014; Barbut *et al.*, 2007), was the most prevalent RT found (36/154; 23%). Overall these prevalence results are consistent with findings in studies from Europe (Norén *et al.*, 2014) and North America (Keel *et al.*, 2007). However, the reasons for continuing predominance of RT 237 in this piggery are unclear. One possible explanation is that the piggery
generates its own replacement breeding stock and this could have prevented the introduction of new *C. difficile* strains from other piggeries. Our findings suggest that new strains of *C. difficile* are not commonly introduced from other sources such as rodents or birds on this piggery. An important factor could be the geographical location of the piggery both within the State of Western Australia, and within Australia generally where there is a large expanse of desert and great distances separating eastern and western Australia.

The prevalence in the cross-sectional study on holding “A” was 67.4% in piglets aged 1-10 days, and the overall prevalence of *C. difficile* from the cohort study was 22% (22/100). There was a gradual decline in *C. difficile* shedding in faeces with increasing age of piglets in the cohort study on holdings “B” and “C”. These findings are in agreement with similar studies from elsewhere (Álvarez-Perez *et al.*, 2013; Weese *et al.*, 2010a) and with
other cross-sectional studies (Knight et al., 2015; Norén et al., 2014; Squire et al., 2013; Álvarez-Perez et al., 2009; Norman et al., 2009) which reported a lower prevalence of *C. difficile* in older (>14 days) piglets than in younger piglets. Álvarez-Pérez et al. (2009) reported a 26% prevalence of *C. difficile* in piglets aged 1-7 days in Spain but zero prevalence in pigs aged 1 to 2 months, while a study conducted in an integrated swine production system in the USA found that faecal shedding of *C. difficile* was 50% in suckling piglets, 6.5% in weaner pigs (3-10 weeks old) and 3.9% in both fattening pigs (up to 22 weeks) and adult breeding boars and sows (Norman et al., 2009). Another longitudinal study undertaken in Canada found a *C. difficile* prevalence of 74% (day 2), 55% (day 7), 40% (day 30), 23% (day 44) and 3.7% (day 62) (Weese et al., 2010a). These findings support the hypothesis that *C. difficile* colonisation declines with increasing age, possibly due to interference from developing components of the
normal intestinal microbiota in a phenomenon referred to as “colonisation resistance” (Kim et al., 2017: Buffie et al., 2015).

A high prevalence of *C. difficile* in slaughter age pigs could pose a risk of foodborne infection to humans through consumption of contaminated meat. The current study did not examine slaughter age pigs, but the overall prevalence found in younger pigs was 22% (22/100), lower than that reported in Canada (96%) (Weese et al., 2010a) and in the Netherlands (100%) (Hopman et al., 2011), but similar to that reported in Spain (25.6%) (Álvarez -Perez et al., 2013), although the RTs detected were different. Álvarez -Perez et al. (2013) found a peak prevalence on day 15 (85%) compared to day 7 (50%; 10/20), but they sampled from the same piglets over time up to day 50 as opposed to sampling a subset of the same litters over time. The decline in *C. difficile* shedding to
zero by day 20 was similar to (Grzeskowiak et al., 2016) but earlier than reported in other studies (Álvarez-Perez et al., 2013; Weese et al., 2010a) where C. difficile shedding continued up to day 50. Weese and colleagues (2011) reported a farm level C. difficile prevalence of 6.5% (30/346) in slaughter age pigs in Canada. In that study, various strains of C. difficile were detected, but RT 078 was the predominant strain on farms, with a prevalence of 67% (Weese et al., 2011). Many other studies have documented the presence of C. difficile in meat products such as retail beef, pork and turkey (Rodriguez-Palacios et al., 2013; Al Saif and Brazier, 1996). The fact that C. difficile was not isolated in older pigs (6 weeks old) in the present study suggests that slaughter age pigs at this piggery are unlikely to pose a risk for human infection. However, there is a need to carry out further studies at local piggeries with different circulating RTs and in
abattoir environments to be able to exclude local meat products as a source of *C. difficile*.

The contaminated farm environment may provide a source of *C. difficile* for human infection. *C. difficile* can be dispersed by wildlife (Álvarez-Perez *et al.*, 2014), vermin (mice and flies on a piggery) (Burt *et al.*, 2012), wind (Keessen *et al.*, 2013), and manure (Al Saif and Brazier, 1996). RT 078, a well-established animal pathogen, has increasingly been isolated from humans, particularly those living near pig farms in Europe (Knetsch *et al.*, 2014; Keessen *et al.*, 2013). Knetsch *et al.* (2014) reported indistinguishable strains of *C. difficile* RT 078 in pig farmers and pigs by whole genome sequence techniques. In the present study, RT 237 was detected from the floor, treated water, and also from the effluent from a drainage channel before the two–stage treatment pond at the piggery. Similarly, Squire and colleagues
(2011) isolated *C. difficile* RT 237 from treated pig effluent planned for use in cleaning the pig sheds. However, RT 237 has been detected rarely in clinical specimens obtained from human patients in Western Australia (Foster *et al.*, 2014; Elliott *et al.*, 2011), suggesting, perhaps, that it does not adapt well to a human host.

At the study piggery, a sporicidal disinfectant (sodium hypochlorite) has been used in pig sheds for the last few years. An explanation for detection of *C. difficile* from pen floor and waste-water is not obvious although the suboptimal concentration of the disinfectant used cannot be ruled out. *C. difficile* spores can persist in the environment for a long time, therefore additional control measures such as providing education to all working staff at the farm could further reduce the incidence of CDI. Overall, these findings suggest that sporicidal disinfectants
in pig sheds analogous to hospital environments may reduce piglet infections (Weese et al., 2003).

All the *C. difficile* isolates sourced from the cross-sectional study had similar susceptibilities to a panel of antimicrobials, with no resistance detected (Table 4.2). This finding was expected because all isolates were most likely clonal. In an earlier smaller study of RT 237 isolates from the same piggery no resistance was detected (Hammer and Riley, 2015). In contrast, Peláez et al. (2013) reported a 9% prevalence of metronidazole resistance (MIC > 256 mg/mL) and nearly 50% multi-drug resistance in *C. difficile* in swine herds in Spain. In general, there is a paucity of information on the antimicrobial susceptibility of *C. difficile* in livestock.

This study has some limitations. The number of sows enrolled for the longitudinal study was small to investigate the role of maternal age and *C. difficile* colonisation in
Aspects of Clostridium difficile infection in pigs

their progenies. Although the farm has a high biosecurity system, future studies should investigate the presence of pests as they could play a role in C. difficile transmission. (Pest control at pig farms) is important because it has potential to prevent pathogen transmission within and between farms (Andrés-Lasheras et al., 2016; Andres and Davies, 2015).

4.4.1 CONCLUSIONS

RT 237 has persisted for at least 5 years and remains the predominant strain of C. difficile in piglets on a piggery in Western Australia. This unusual RT has been detected in human patients in Australia but not in high numbers. The decline of C. difficile shedding to zero by day 20 suggests that slaughter age pigs are unlikely to be greatly contaminated with C. difficile in this piggery. Further research is warranted to determine the sources of the persisting RT 237 on the piggery, and to reduce
contamination levels in the piggery environments to limit piglet and potentially human exposure.
CHAPTER 5: OUTBREAK OF CLOSTRIDIUM DIFFICILE INFECTION IN SUCKLING PIGLETS IN EASTERN AUSTRALIA

5.1 INTRODUCTION

In the previous chapter, the epidemiology of CDI in a piggery in Western Australia was described. Diarrhoeic piglets were more likely to be shedding C. difficile than the subclinical colonised piglets. The two studies showed that CDI and/or colonisation with C. difficile were highly correlated with the age of piglets. This chapter describes a CDI outbreak in a piggery from an eastern State of Australia, Victoria. In this chapter, the possibility that CDI outbreaks could be occurring in some piggeries in Australia is explored along with the possibility that some CDI outbreaks could be missed. The use of molecular typing techniques such as ribotyping for surveillance and for characterising C. difficile outbreaks in livestock is
examined. Furthermore, this chapter provides recommendations for prevention and control of transmission of *C. difficile* in a piggery during an outbreak.

CDI is associated with nosocomial outbreaks in medical settings and is an emerging pathogen in livestock such as pigs (Squire *et al.*, 2013; Songer, 2004), horses (Båverud *et al.*, 1998) and calves (Magistrali *et al.*, 2015). CDI outbreaks have been reported in piggeries (Songer, 2004; Nagy and Bilkei, 2003; Waters *et al.*, 1998), stud farms (Båverud *et al.*, 2004) and veterinary hospitals (Weese and Armstrong, 2003; Weese *et al.*, 2000) outside Australia but none has been recorded in Australia. *C. difficile* is ubiquitous in the environment. Environmental sources common to animals and humans could play an important role in *C. difficile* transmission (Hensgens *et al.*, 2012).
C. difficile is an opportunistic pathogen that colonises the gastrointestinal tract of animals/humans whose microbiota has been perturbed (Båverud et al., 1998; Van der Waaij et al., 1971). Neonatal pigs are born without an established microbiota in the gastrointestinal tract and are therefore at risk of C. difficile colonisation or CDI. Older piglets (> 7 days) are less susceptible because they have an established microbiota that outcompetes C. difficile (chapter 4). The risk factors for CDI in piggeries include change of diet, stress, poor shed hygiene (Bilkei et al., 1995), and use of disinfectants that are not sporicidal.

Since the year 2000, there have been reports of C. difficile outbreaks in medical hospitals in Europe (Eyre et al., 2012) and the USA (Muto et al., 2005) but very few outbreaks reported in livestock (McElroy et al., 2016; Songer, 2004; Nagy and Bilkei, 2003; Weese and Armstrong, 2003; Waters et al., 1998). The underlying
discrepancy in reporting outbreaks in livestock could be attributable to CDI diagnostic challenges in the veterinary industry compared to medical settings. This could lead to most of the outbreaks in livestock being missed.

Recently, a piggery in Victoria, Australia, experienced an increased number of diarrhoea cases in piglets aged 1-12 days (median age 4 days). This chapter describes the investigation of these cases and the characteristics of *C. difficile* isolates associated with the outbreak.

5.2 Aims

The aims of this study were first to characterise a *C. difficile* outbreak occurring in neonatal pigs in Eastern Australia. The second aim was to identify drivers for *C. difficile* outbreaks and to provide preventative measures to minimise the possible future occurrence of CDI outbreaks in piggeries across Australia.
5.2.1 Setting and historical perspective

In October 2015, a pig veterinarian reported an unusually high number of piglets aged 1-12 days (median age 4 days) with diarrhoea at a piggery in the state of Victoria in Australia. The piggery was part of an 1800 sow farrow-to-finish facility with a 5-year history of idiopathic diarrhoea in piglets aged 1-7 days. Affected litters had 80-100% of piglets affected, with clinical signs of pasty yellow diarrhoea progressing to watery yellow diarrhoea. Diarrhoeic piglets were severely dehydrated and were provided with electrolytes as soon as diarrhoea commenced. Treatment with trimethoprim sulphate was given to all piglets in the litter for 3 days although this did not stop diarrhoea. Pre-weaning mortality was 16-18% which was an increase above the baseline for this site. There was a longer tail of lower weight piglets at weaning from affected litters.
Routine treatment of each piglet and processes that occurred after birth included the following; on the first day, piglets received 1 mL of apple cider vinegar orally, followed by an anticoccidial, Baycox® (toltrazuril), and an iron injection and prophylactic penicillin was given to all piglets after tail docking on day 2. Occasionally, sows were treated with penicillin in the farrowing crate, but no other antibiotics were administered.

5.2.2 Sow environment

In the affected piggery, there were 300 farrowing crates in five sheds. Two sheds had automated temperature control installations while the others were naturally ventilated. When a sow weaned her litter, she was removed from the farrowing crate. The crate was pressure washed and disinfected with Microtech® (benzalkonium chloride and poly-hexamethylene biguanide-hydrochloride) then given 12-24 h to dry. The sows were
brought into farrowing crates approximately 3-4 days prior to farrowing. The original crates had a concrete floor, which was undergoing replacement with plastic slatted flooring. Heating lamps were temperature controlled and piglet behaviour in the crates indicated that they were at a comfortable temperature on the day of a visit by a veterinarian.

5.2.3 Sampling

Thirty rectal swabs and 12 fresh colon content samples (dead piglets) were obtained from clinically diarrhoeic litters, the latter during necropsy. This was done by identifying the affected litters, choosing three piglets with diarrhoea per litter and taking a rectal swab. A piglet was considered diarrhoeic at the sampling time using the following criteria: i) the piglet had yellow, non-haemorrhagic, and pasty to watery faeces and ii) any piglet painted red at the dorsum by personnel based on diarrhoea
being observed, and that had a perineum soiled with watery faeces. A litter was classified as diarrhoeic if one or more piglets had diarrhoea at the time of sampling. There were three separate sample submissions made to our laboratory in October 2015 and April 2016, with a total of 75 samples. For the first two submissions, piglets were aged 1-12 days (median age 4 days) \([n = 42]\). In the third submission, 33 piglets aged 1-21 days were randomly sampled (1 day old, \(n = 14\), 2 days old, \(n = 8\), 3-7 days old, \(n = 5\), and 14-21 days old, \(n = 5\)) 6 months after the CDI outbreak. All swabs were transported to Western Australia for processing in Amies transport medium without charcoal (Thermo Fisher Scientific, Waltham, MA, USA) in a cool box at 4ºC. Porcine reproductive and respiratory syndrome (PRRS), porcine epidemic diarrhoea (PED), and transmissible gastroenteritis (TGE) were not tested for as they are considered exotic (no outbreaks reported in the last two decades) in Australia.
5.2.4 Necropsy

A subset of piglets in this study was humanely euthanised and submitted for necropsy and histopathology. The pathologist was blinded to the *C. difficile* status of piglets.

5.2.4.1 Histopathology

The following tissues were processed using standard histopathology methods; lung, brain, heart, kidney, liver, spleen, stomach, small intestines and the spiral colon (Table 5.2).

5.2.4.2 Antimicrobial susceptibility tests using the E-test

Ten isolates were tested for azithromycin, clindamycin, metronidazole and moxifloxacin susceptibility using E-test strips (bioMérieux, Australia) on BA). MICs were recorded after 48 h of anaerobic incubation. The MIC breakpoints recommended by the
Aspects of *Clostridium difficile* infection in pigs

CLSI were used (CLSI, 2013). Antimicrobials that are frequently used in piggeries in Australia were selected.

5.2.5 Statistical analysis

The Chi-squared test was used to evaluate if there was a difference in *C. difficile* shedding from piglets during and post-outbreak. Statistical analysis was performed in Epi-infor™ version 7.5.1.

5.3 RESULTS

5.3.1 Prevalence of *C. difficile*

The prevalence of *C. difficile* for all outbreak samples was 64% (27/42) by direct culture and no additional samples were positive by enrichment culture. All isolates were toxigenic having both *tcdA* and *tcdB* (27/27) (Table 5.1). Additionally, all isolates possessed the binary toxin gene *cdt*. Only one RT, QX 450 (A+B+CDT+), was isolated at the piggery. Distribution of *C. difficile* by age was 50% (2/4) in 1 day old piglets, 55% (5/9) in 2 day olds, 67%
(6/9) in 4 day olds, and 76.5% (13/17) in 5-6 day olds.

There was no *C. difficile* detected in three samples from piglets aged 9-12 days.

Samples obtained from piglets (n = 33) 6 months after the outbreak were also analysed. *C. difficile* prevalence was 15.1% (5/33) by direct culture and 21.2% (7/33) by enrichment. The distribution of *C. difficile* prevalence by age was 7% (1/14) in 1 day olds, 25% (2/8) in 1-3 day olds, and 50% (3/6) in 3-7 day olds. None of the five samples from piglets aged 14-21 days tested positive for *C. difficile*. There was a three-fold reduction in *C. difficile* prevalence in this piggery 6 months post-outbreak and this was significantly less than that reported during the outbreak ($p < 0.0001$, $\chi^2$, Table 5.1).
Table 5.1 Distribution of *C. difficile* during and 6 months post-outbreak in a piggery in Victoria, Australia

<table>
<thead>
<tr>
<th>PCR ribotype</th>
<th>Toxin profile</th>
<th>Toxigenic culture</th>
<th>C. difficile status</th>
<th>Chi-square</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>QX 450</td>
<td>A+B+C</td>
<td>Positive</td>
<td>27</td>
<td>0.0001**</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>DT+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Negative</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>42</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

***Significant at 5% probability level.

5.3.2 Clinical signs

The diarrhoeic piglets were emaciated and presented with mild to severe dehydration depending on the time of onset of infection. They all had yellow pasty to watery diarrhoea. Two piglets with severe diarrhoea had
haematochesia. The five piglets submitted for necropsy experienced seizures and were in lateral recumbency.

5.3.3 Necropsy and histopathology

Gross necropsy of five piglets revealed consolidation of the cardiac lung lobe while the liver had non-specific lesions (orange tinged colouration). Three piglets had stomachs nearly full of milk curd and colons filled with a greenish fluid. All the five necropsied piglets had an oedematous spiral colon (Figure 5.1 and Table 5.2). Furthermore, piglets had mesocolonic oedema with neutrophilic infiltration.

The histopathological lesions were consistent with CDI in piglets as there was multi-systemic organ dysfunction (Figure 5.2 and Table 5.2). The lung, kidney, liver, spleen, small intestines and the colon tissues showed extensive microscopic lesions but not the brain, heart or stomach tissues (Table 5.2). The colon had
pseudomembranous exudate similar to the small intestines. The colonic mucosa was eroded, with severity ranging from mild epithelial cell necrosis through to patchy luminal exudate. Piglets with severe colonic erosions had large numbers of bacilli adherent to the mucosal surface (Figure 5.2).

Figure 5.1 Severe oedema of the mesocolon (≥ 3 mm between loops) observed in piglets infected with *C. difficile* (Squire, 2015).
Figure 5.2 Gross and histopathologic lesions in piglets with CDI. [A], Normal spiral colon of a piglet. [B], Moderate mesocolonic oedema and congestion of the spiral colon of a piglet. [C], Mild inflammation and minimal luminal debris in the colon shown in [A]. [D], intact mucosa and mild neutrophilic infiltrate of the colonic epithelium shown in [C]. [E], Moderate to severe neutrophilic inflammation and multifocal ulceration of the colon shown in [B]. Asterisk denotes copious mucus, cellular debris, and bacteria forming diphtheritic membrane in colonic lumen. [F], Erosion of colonic mucosa and abundant neutrophils in lumen of colon shown in [E] (Sponseller et al., 2015, J Infect Dis, 211, 1334-1341).
Table 5.2 Histopathology of tissues from 2 days old suckling piglets naturally infected with *C. difficile* ribotype QX 450

<table>
<thead>
<tr>
<th>Tissue examined</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>Focal lobular, moderately severe, acute interstitial pneumonia characterised by thickened alveolar walls and alveolar spaces filled with exudate. The exudate primarily consisted of highly degenerate and necrotic neutrophils with streaming nuclear material and fewer macrophages. Affected portions comprised 10%-30% of the section.</td>
</tr>
<tr>
<td>Brain and heart</td>
<td>No significant lesions.</td>
</tr>
<tr>
<td>Kidney</td>
<td>Multifocal, mild to moderate, acute, tubular epithelial necrosis with cellular cast formation.</td>
</tr>
<tr>
<td>Liver</td>
<td>Increased number of</td>
</tr>
</tbody>
</table>
### Aspects of Clostridium difficile infection in pigs

<table>
<thead>
<tr>
<th>Tissue examined</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>pigments (probably iron) laden macrophages.</td>
<td></td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td>Mild to moderate lymphoid depletion.</td>
</tr>
<tr>
<td><strong>Stomach</strong></td>
<td>No lesions.</td>
</tr>
<tr>
<td><strong>Small intestine</strong></td>
<td>The luminal surfaces covered by a pseudomembrane composed of degenerate and necrotic desquamated epithelial cells, cells debris, and inflammatory cells predominantly neutrophils, and a variable number of bacteria (mostly bacilli). In addition, moderate to severe villous atrophy were present in most sections. Mild to moderate reduction in length of the villi accompanied by occasional foci of suppuration within the</td>
</tr>
</tbody>
</table>
Aspects of *Clostridium difficile* infection in pigs

<table>
<thead>
<tr>
<th>Tissue examined</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon</td>
<td>A pseudomembranous exudate similar to that in small intestines was present. Mild epithelial cell necrosis of the mucosal surface with a patchy luminal exudate, sometimes adherent to the mucosa, and often containing moderate to large numbers of bacilli.</td>
</tr>
</tbody>
</table>

### 5.3.4 Antimicrobial susceptibility tests using E-test

All 10 isolates were susceptible to azithromycin (MIC 1 to 1.5 µg/mL) with the clinical break points defined as ≥ 8 µg/mL (resistance), clindamycin (MIC 1.5 to 2 µg/mL) with the clinical break points defined as ≤ 2 µg/mL (susceptible), 4 µg/mL (intermediate), and ≥ 8
Aspects of *Clostridium difficile* infection in pigs

µg/mL (resistance), metronidazole (MIC 0.016 to 0.047 µg/mL) with the clinical break points defined as ≤ 8 µg/mL (susceptible), 16 µg/mL (intermediate), ≥ 32 µg/mL (resistance) and moxifloxacin (MIC 0.75 µg/mL) clinical breakpoints defined as ≤ 2 µg/mL (susceptible), 4 µg/mL (intermediate), and ≥ 8 µg/mL (resistance).

5.4 DISCUSSION

The main objective of this study was to characterise a CDI outbreak occurring in suckling piglets in eastern Australia. In this piggery, the prevalence of *C. difficile* in submitted samples during the outbreak was 64% (27/42), declining to 21.2% (7/33) post outbreak. There was clonal clustering of an unusual RT, RT QX 450 (A+B+CDT+), in this piggery. This clonal clustering of RTs within a piggery is characteristic of a common source CDI outbreak occurring (Knight *et al.*, 2017; Squire, 2015).
Aspects of *Clostridium difficile* infection in pigs

*C. difficile* strains that produce TcdA and TcdB, or TcdB alone, are known to cause diarrhoea (Steele *et al.*, 2013; Steele *et al.*, 2012; Waters *et al.*, 1998). A survey of *C. difficile* in piglets aged 2-7 days old found that *C. difficile* was common and toxins were detected in 29% of individual suckling piglets submitted to Iowa State Veterinary Hospital (Yaeger *et al.*, 2002). Yaeger *et al.* (2007) showed that there was a correlation between piglets with toxins in the colon and enterocolitis. In the present study, histopathological examinations from five piglets revealed the presence of pseudomembranous colitis, and mild epithelial cell necrosis of the mucosa, with luminal exudate containing large numbers of bacilli in the colon (Figure 5.2 and Table 5.2). The detection of lesions in the small intestines was unexpected (Table 5.2) because CDI in piglets is mostly associated with colonic lesions (Keel and Songer, 2006). The observed lesions in piglets in this
study could be explained by bacterial characteristics (early germination resulting in more toxin production) and/or the density of TcdA receptors in the duodenum, jejunum, caecae and colon (Keel and Songer, 2007). This finding is similar to a report of a human patient with CDI who had pseudomembranous enteritis in which both the small intestine and the colon had lesions (Elinav et al., 2004). Similarly, foals often present with severe necrotic mucosa, pseudomembrane, haemorrhage and vascular thrombosis in the small intestine and colon (Uzal et al., 2012; Weese et al., 2001). The involvement of other pathogens in the infected piglets should not be overlooked as has been shown in other disease outbreaks in piglets in Europe (Larsson et al., 2015). However, the histopathological lesions detected in this study were consistent with CDI toxin-mediated lesions detected in the colon and other organs of piglets that are naturally infected (Yaeger et al.,
2007; Waters et al., 1998) and those experimentally infected (Sponseller et al., 2015; Arruda et al., 2013; Lizer et al., 2013; Steel et al., 2010).

In a follow-up study, there was a reduction in the C. difficile prevalence by three-fold (64% versus 21.2%) 6 months after the CDI outbreak in the studied piggery (Table 5.1). The most remarkable finding in this study was that no piglet older than 14 days was culture positive for C. difficile emphasising how common CDI is in piglets aged 1-7 days. In hospitals, the use of chlorine-based disinfectants, improved cleaning, and the use of routine molecular typing have prevented CDI outbreaks (Mutters et al., 2017; Barbut, 2015; Eyre et al., 2012; Weese and Armstrong, 2003; Wilcox et al., 2003). Although a disinfectant (Microtech® - benzalkonium chloride and poly-hexamethylene biguanide-hydrochloride) was regularly used in pig sheds in this piggery, it did not
prevent the CDI outbreak. This is probably because Microtech® disinfectant is not sporicidal in nature. Therefore, it does not matter how frequently this product was used in the piggery, the spore load would have remained the same or even higher. In this study, the outbreak was brought under control after using sodium hypochlorite (bleach) disinfectant similar to a study by Weese and Armstrong (2003). Researchers have evaluated the efficacy of disinfectants in medical settings (Vohra and Poxton, 2011; Maillard, 2011; Wilcox et al., 2003), but not in animal sheds where the organic debris load is higher than in medical settings. The greater amounts of organic debris present in the piggery environment may require a high dosage of disinfectant or higher frequency of disinfection and longer exposure time to eliminate C. difficile spores. There is a need to evaluate the efficacy of
the widely used disinfectants in piggeries against spore
forming bacteria including *C. difficile*.

CDI in piglets was first described in gnotobiotic pigs infected with *Brachyspira hyodysenteriae* and accidentally infected with *C. difficile* (Lysons et al., 1980). The piglets excreted mucoid faeces tinged with blood and were dehydrated. Three years later, Jones and Hunter (1983) recovered *C. difficile* in association with *Balantidium coli* and *Salmonella enterica* serovar Typhimurium from colonic contents of 8-week-old piglets. In that study, piglets exhibited pseudomembranous exudate in the colonic mucosa. In 1998, Waters *et al.* reported a CDI outbreak in 5-day old piglets from a herd of 600 gilts. In the study conducted by Waters *et al.* (1998), piglets had dyspnoea, hydrothorax, abdominal distension, ascites and emaciation, oedema of the mesocolon, scrotal oedema and diarrhoea. The findings in the present study are consistent
with previous reports describing the clinical signs of CDI in piglets (Yaeger et al., 2007; Songer, 2004; Waters et al., 1998; Yaeger et al., 2002). Waters and colleagues (1998) detected *C. difficile* and toxins from colonic contents, concluding that the toxins were responsible for the enteropathy. However, they did not type the *C. difficile* isolates. In this study, a heavy growth of *C. difficile* was observed from both colon contents and rectal swabs on primary culture on *C. diff* ChromID™ and this was later shown to be an unusual RT of *C. difficile*, RT QX 450.

In Australia, the national prevalence of *C. difficile* carriage in piglets aged 1-7 days was reported as 67% (154/229) (Knight et al., 2015), similar to Europe 71% (143/201) (Schneeberg et al., 2013) and the USA 47.6% (~97%) (Songer, 2004). The prevalence of *C. difficile* in individual piglets within a farrowing shed was as high as 67% in the Western Australian study reported in chapter 4.
Aspects of Clostridium difficile infection in pigs

and 35% in the USA (Songer, 2004). All these figures are in agreement with the findings of the current study where prevalence was 45%.

The predisposing factors for CDI in piglets under field conditions are not obvious (Keessen et al., 2011b). The risk factors that led to this outbreak are not clear either, but hygiene cannot be ruled out. In this piggery, piglets were raised on a concrete slab floor which is difficult for removing spore contamination from cracks and crevices. This suggests that piglets raised on the concrete floor may have been frequently in contact with faeces and C. difficile spores. This notion is supported by a study that found an association between poor hygiene and CDI in piglets (Nagy and Bilkei, 2003). Airborne transmission of C. difficile in piggeries has been described also under field conditions (Keessen et al., 2011a).
However, other host factors such as litter size have not been associated with CDI in piglets (chapter 4).

In this study, antimicrobial (penicillin) administration to piglets post tail docking could have predisposed piglets to CDI by slowing down microbiota establishment. Although still controversial, antibiotic use has been associated with CDI outbreaks in sows in east Europe (Kiss and Bilkei, 2005). However, an experimental study did not find any association between antibiotic therapy and CDI in a day old piglets (Arruda et al., 2013). In the study conducted by Arruda and colleagues (2013), it is not surprising that they did not find an association between antibiotic therapy and CDI in piglets because the neonatal pigs were only a day old and not expected to have fully established functional microbiota. Given this background, it is possible that antimicrobials could delay the
establishment of microbiota and therefore predispose piglets to colonisation by *C. difficile*.

All 10 *C. difficile* isolates tested in this study were susceptible to azithromycin, clindamycin, metronidazole, and moxifloxacin. This uniformity in susceptibility pattern was expected because each of the 10 isolates tested belonged to the same RT QX 450. However, the overall finding of susceptibility is consistent with previous reports in Australian piggeries where *C. difficile* isolates have not shown resistance to antimicrobials (chapter 3; Knight *et al.*, 2015). Nonetheless, there is a great need to restrict/reduce the widespread use of antibiotics in food producing animals (McEwen and Fedorka-Cray, 2002).

Livestock or pets have been suggested as reservoirs for CA-CDI in humans. Livestock could contribute to CA-CDI through meat and meat by-products (Weese *et al.*, 2010b; Songer *et al.*, 2009), and effluent (Squire *et al.*, 2010).
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2011; Norman *et al.*, 2009). A recent study found a human patient infected with a unique RT 237 which is predominantly associated with pigs in Western Australia (McGovern *et al.*, 2016). This report of *C. difficile* RT 237 infection of a human patient suggests potential zoonosis or common source acquisition. In addition, surveillance studies have shown that the incidence of CA-CDI has increased in the last decade in Australia (Slimings and Riley, 2014) and abroad (Hensgens *et al.*, 2012). These findings imply that the environment could be an important source of novel *C. difficile* clones circulating in the community due to contamination from animal manure or biosolids.

This study has some limitations. For example, only samples from diarrhoeic piglets were processed during the outbreak, and therefore the prevalence of subclinical shedders was not determined. Piglets without diarrhoea
but still shedding *C. difficile* may be important for maintaining the infectious cycles. In the follow-up study the prevalence was lower (21%) than during the outbreak corroborating the hypothesis that symptomatic piglets shed more spores in the environment than the asymptomatic ones. On the other hand, clustering of RT QX 450 within the farm may not necessarily mean that transmission is occurring as this may just point to the fact RT QX 450 was endemic in the studied piggery. Therefore, routine typing of pathogens during increased incidence of diarrhoea in pigs could aid early detection and implementation of infection control to prevent *C. difficile* outbreaks.

**5.4.1 CONCLUSION**

In this study, a CDI outbreak in a piggery was characterised using molecular and phenotypic methods. *C. difficile* RT QX 450 isolates clustered together both in space and time suggesting that an outbreak was occurring
in the piggery. There is a need to recognise CDI as an important pre-weaning cause of neonatal morbidity and mortality in piglets. Furthermore, there is even a greater need to increase awareness among veterinarians, laboratory pathologists, and farmers if early detection and control of this pathogen are going to succeed in preventing further CDI outbreaks.

The use of sporicidal disinfectants in piggeries during an outbreak appears to be an appropriate short-term intervention in preventing *Clostridium difficile* transmission. The major challenges with sporicidal disinfectants include the cracks and crevices on the floor and the amount of organic debris. This implies that the frequency of application of disinfectant and the concentrations of the solvent may determine success or failure. Therefore, improved hygiene in the piggery could further reduce spore contamination.
CHAPTER 6: DETECTION OF CLOSTRIDIUM DIFFICILE FROM THE ENVIRONMENT EXPOSED TO PIG MANURE, EFFLUENT AND COMPOST IN AUSTRALIA

6.1 INTRODUCTION

In the previous chapter, an outbreak of CDI in a piggery was described. A unique clonal cluster of toxigenic C. difficile ribotype QX 450 (A^+B^+CDT^+) was detected. It was concluded that the source of the outbreak was the environmental contamination of the pig shed. This chapter was conceived particularly to examine the extent to which animal pathogens such as C. difficile were dispersed in the agricultural settings. Although the use of animal manure as a fertiliser for crops and other plants has occurred for a long time, there have been concerns about potential transmission of the pathogenic organism through contamination of food produce for many years (Casey et
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*al., 2013; Carrington et al., 1988*. Although data conclusively supporting the zoonotic transmission of *C. difficile* through food has not yet emerged, it is possible that application of contaminated manure for landscaping could be driving CA-CDI. This chapter describes the prevalence of *C. difficile* in the pig farm environment, land amended with pig manure, retail compost and human biosolids. The safety of animal or human biosolids used as a fertiliser in crops has long been a source of concern to public health workers because of the many foodborne disease outbreaks that have been attributed to manure contamination (Fry *et al.*, 2014; Pell, 1997). For over a century, manure has been used as a fertiliser for crops (Wood, 2012). Currently, animal production systems have become more intensified in many countries (O’Donoghue *et al.*, 2011). Intensive animal farming results in more manure being produced per farm that can be used in crops.
The same applies to humans because the population has generally increased in many countries resulting in excess biosolids produced (Suslow et al., 2003). The major challenge is how to utilise animal or human biosolids without impacting on public health (Fry et al., 2014; Létourneau et al., 2010; Carrington et al., 1988). This chapter reviews risk factors associated with the use of treated or raw animal or human biosolids on crops or plants in general. Soil application of pig manure or human biosolids treated by mesophilic methods could lead to dissemination of \textit{C. difficile} in the environment.

6.2 Aims and objectives

The first aim of this chapter was to describe, characterise and quantitate \textit{C. difficile} spore contamination in pig environments, and farmland amended with pig manure. The second aim was to determine the prevalence of \textit{C. difficile} in animal and human biosolids from garden retail
shops. The last aim was to determine the efficacy of mesophilic treatment of effluent in reducing *C. difficile* contamination by comparing *C. difficile* prevalence in treated effluent to influent water at pig farms.

### 6.3 Study settings

**6.3.1 Sample description**

Soil samples were classified as background soil if they were not exposed to pig or other animal effluents (*n* = 6), two randomly obtained effluent soil (1 and 2), soil exposed to pig effluent (*n* = 11), raw animal waste or influent (*n* = 7) and effluent as treated raw animal waste (*n* = 7) obtained from treatment ponds. There were three (*n* = 3) manure samples assumed to have been obtained from retail shops whose location was indeterminate. There was a total of six (*n* = 6) floor swabs obtained from one piggery. Nearly all the seven farms contributed each of the stated sample categories. There was a total of 37 samples
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obtained from seven pig farms and three composts from unknown shops, and 85 gardening products (garden mixes, human biosolids, mulch, organic fertiliser, and soil conditioner) obtained from retail shops in Western Australia.

6.3.2 Study location and samples

Soil and effluent samples were collected from seven farms in four states of Australia; Queensland, South Australia, Victoria and Western Australia.

A total of 85 samples of gardening products (human biosolids, organic liquid fertilisers, soil conditioners, mulches, garden mixes, and organic fertiliser) were obtained from nine wholesalers, two manufacturing plants, two online suppliers of manure (free) and retail shops in Western Australia. The retail shops stocked a range of gardening products some of which had origins in the eastern states of Australia. There were human biosolids (n
Aspects of *Clostridium difficile* infection in pigs

= 4), organic liquid fertiliser (n = 6), soil conditioner (n = 37), mulches (n = 12), garden mixes (n = 22) and organic fertiliser (n = 1).

Lastly, there were six floor swabs obtained from one piggery (Western Australia) plus three compost samples from unknown shops.

Each soil sample weighed ~50 g and each liquid effluent sample was ~30 mL transported at ambient temperature in specimen jars (Techno-Plas Pty Ltd, St Marys, Australia) to The University of Western Australia. Nearly all the seven farms contributed samples in each category.

6.4 RESULTS

6.4.1 Prevalence of *C. difficile* in farm environmental samples

The overall prevalence of *C. difficile* from 40 environmental samples from all the seven farms and plus
Aspects of *Clostridium difficile* infection in pigs

compost was 72.5%. All the seven farms were *C. difficile* culture positive in at least one sample (100%) (Table 6.1). The farm prevalence of *C. difficile* in background soil was 33% (2/6) and in influent and effluent 100% (7/7) (Table 6.1). The prevalence of *C. difficile* in soil (effluent irrigated) was 90.9% (10/11).

Toxin profile distribution among the toxigenic RTs was 24.1% (7/29) A* B+CDT−, 13.8% (4/29) A−B−CDT+, 13.8% (4/29) A−B+CDT+, and 17.2% (5/29) A+B+CTD+ and 13.0% (9/29) A−B−CDT− (Table 6.2).

Six of the seven farms had piglets experiencing diarrhoea at the time of sampling (Table 6.2). Many RTs identified had both toxin genes *tcdA* and *tcdB*, or *tcdB* alone (55.2%; 16/29). However, farm “E” did not report any diarrhoea in piglets, although RT 014/020 was recovered from the influent samples from pig sheds (Table 6.2).
A total of 13 ribotypes was detected including two novel non-toxigenic (A\(^{-}\)B\(^{-}\)CDT\(^{-}\)) strains, type A and type B (Table 6.2). The proportion distribution of RTs was 014/020 (20.6.2%; 6/29), 033 (13.8%; 4/29), 237 (10.3%; 3/29), 051 (10.3%; 3/29), 281 (3.4%; 1/29), QX 521 (10.3%; 3/29), QX 412 (3.4%; 1/29), QX 450 (10.3%; 3/29), QX 451 (3.4%; 1/29), QX 167 (3.4%; 1/29), novel type A (6.9%; 2/29) and novel type B (3.4%; 1/29).

Many RTs in the influent samples were identical to the RTs in the effluent samples (Table 6.2). RTs in effluent liquid and soil samples obtained from locations exposed to pig effluent were indistinguishable in five farms 71.4% (5/7) (Farms C, D, E, F, and G). Three out of seven farms (42.8%) had single clonal RTs across all samples processed and the other four farms had multiple RTs.
Table 6.1 *C. difficile* distribution by sample type

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Farm prevalence (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background soil</td>
<td>33.3% (2/6)</td>
</tr>
<tr>
<td>Effluent soil</td>
<td>90.9% (10/11)</td>
</tr>
<tr>
<td>Influent</td>
<td>100% (7/7)</td>
</tr>
<tr>
<td>Effluent</td>
<td>100% (7/7)</td>
</tr>
<tr>
<td>Floor swab</td>
<td>100% (4/6)</td>
</tr>
<tr>
<td>Compost (from pig manure)</td>
<td>66.7% (2/3)</td>
</tr>
</tbody>
</table>
### Table 6.2 C. difficile ribotype distribution by farm and sample type

<table>
<thead>
<tr>
<th>Farm ID</th>
<th>Ribotype</th>
<th>Toxin profile</th>
<th>Sample type</th>
<th>*Clinical status</th>
<th>State</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>QX450</td>
<td>A⁺B⁺CDT⁺</td>
<td>Effluent soil 1</td>
<td>-</td>
<td>Diarrhoeic, Victoria</td>
</tr>
<tr>
<td>A</td>
<td>QX451</td>
<td>A⁺B⁺CDT⁺</td>
<td>Effluent soil 2</td>
<td>-</td>
<td>Diarrhoeic, Victoria</td>
</tr>
<tr>
<td>A</td>
<td>UK 051</td>
<td>A⁻B⁻CDT⁻</td>
<td>Effluent (liquid)</td>
<td>-</td>
<td>Diarrhoeic, Victoria</td>
</tr>
<tr>
<td>A</td>
<td>UK 051</td>
<td>A⁻B⁻CDT⁻</td>
<td>Influent (liquid)</td>
<td>-</td>
<td>Diarrhoeic, Victoria</td>
</tr>
<tr>
<td>B</td>
<td>UK 014/020</td>
<td>A⁺B⁺CDT⁻</td>
<td>Effluent soil 2</td>
<td>-</td>
<td>Diarrhoeic, South Australia</td>
</tr>
<tr>
<td>B</td>
<td>Novel type A</td>
<td>A⁻B⁻CDT⁻</td>
<td>Effluent after (liquid)</td>
<td>-</td>
<td>Diarrhoeic, South Australia</td>
</tr>
<tr>
<td>B</td>
<td>Novel type A</td>
<td>A⁻B⁻CDT⁻</td>
<td>Influent (liquid)</td>
<td>-</td>
<td>Diarrhoeic, South Australia</td>
</tr>
<tr>
<td>C</td>
<td>QX 450</td>
<td>A⁺B⁺CDT⁺</td>
<td>Effluent soil 1</td>
<td>-</td>
<td>Diarrhoeic, Victoria</td>
</tr>
</tbody>
</table>
## Aspects of *Clostridium difficile* infection in pigs

<table>
<thead>
<tr>
<th>Farm ID</th>
<th>Ribotype</th>
<th>Toxin profile</th>
<th>Sample type</th>
<th>*Clinical status</th>
<th>State</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>QX 450</td>
<td>$A^+B^+CDT^+$</td>
<td>Effluent soil 2</td>
<td>Diarrhoeic</td>
<td>Victoria</td>
</tr>
<tr>
<td>C</td>
<td>QX 167</td>
<td>$A^+B^+CDT^+$</td>
<td>Effluent (liquid)</td>
<td>Diarrhoeic</td>
<td>Victoria</td>
</tr>
<tr>
<td>C</td>
<td>UK 051</td>
<td>$A^-B^-CDT^-$</td>
<td>Influent (liquid)</td>
<td>Diarrhoeic</td>
<td>Victoria</td>
</tr>
<tr>
<td>D</td>
<td>UK 033</td>
<td>$A^-B^-CDT^+$</td>
<td>Effluent soil 1</td>
<td>Diarrhoeic</td>
<td>South Australia</td>
</tr>
<tr>
<td>D</td>
<td>UK 033</td>
<td>$A^-B^-CDT^+$</td>
<td>Effluent soil 2</td>
<td>Diarrhoeic</td>
<td>South Australia</td>
</tr>
<tr>
<td>D</td>
<td>UK 033</td>
<td>$A^-B^-CDT^+$</td>
<td>Effluent (liquid)</td>
<td>Diarrhoeic</td>
<td>South Australia</td>
</tr>
<tr>
<td>D</td>
<td>UK 033</td>
<td>$A^-B^-CDT^+$</td>
<td>Influent (liquid)</td>
<td>Diarrhoeic</td>
<td>South Australia</td>
</tr>
<tr>
<td>E</td>
<td>QX 521</td>
<td>$A^-B^-CDT^-$</td>
<td>Background soil</td>
<td>Non-diarrhoeic</td>
<td>Queensland</td>
</tr>
</tbody>
</table>
### Aspects of *Clostridium difficile* infection in pigs

<table>
<thead>
<tr>
<th>Farm ID</th>
<th>Ribotype</th>
<th>Toxin profile</th>
<th>Sample type</th>
<th>*Clinical status</th>
<th>State</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>QX 521</td>
<td>A⁻B⁻CDT⁻</td>
<td>Effluent soil 1</td>
<td>Non-diarrhoeic</td>
<td>Queensland</td>
</tr>
<tr>
<td>E</td>
<td>QX 521</td>
<td>A⁻B⁻CDT⁻</td>
<td>Effluent soil 2</td>
<td>Non-diarrhoeic</td>
<td>Queensland</td>
</tr>
<tr>
<td>E</td>
<td>QX 412</td>
<td>A⁺B⁺CDT⁻</td>
<td>Effluent (liquid)</td>
<td>Non-diarrhoeic</td>
<td>Queensland</td>
</tr>
<tr>
<td>E</td>
<td>UK 014/020</td>
<td>A⁺B⁺CDT⁻</td>
<td>Influent (liquid)</td>
<td>Non-diarrhoeic</td>
<td>Queensland</td>
</tr>
<tr>
<td>F</td>
<td>UK 014/020</td>
<td>A⁺B⁺CDT⁻</td>
<td>Background soil</td>
<td>Diarrhoeic</td>
<td>Queensland</td>
</tr>
<tr>
<td>F</td>
<td>UK 014/020</td>
<td>A⁺B⁺CDT⁻</td>
<td>Effluent soil 1</td>
<td>Diarrhoeic</td>
<td>Queensland</td>
</tr>
<tr>
<td>F</td>
<td>UK 014/020</td>
<td>A⁺B⁺CDT⁻</td>
<td>Effluent (liquid)</td>
<td>Diarrhoeic</td>
<td>Queensland</td>
</tr>
</tbody>
</table>
### Aspects of *Clostridium difficile* infection in pigs

<table>
<thead>
<tr>
<th>Farm ID</th>
<th>Ribotype</th>
<th>Toxin profile</th>
<th>Sample type</th>
<th>*Clinical status</th>
<th>State</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>UK 014/020</td>
<td>A⁺B⁺CDT⁻</td>
<td>Influent (liquid)</td>
<td></td>
<td>Diarrhoeic, Queensland</td>
</tr>
<tr>
<td>Shop A</td>
<td>Novel type B</td>
<td>A⁻B⁻CDT⁻</td>
<td>Raw manure</td>
<td>aN/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Shop B</td>
<td>UK 281</td>
<td>A⁻B⁺CDT⁺</td>
<td>Raw manure</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>G</td>
<td>UK 237</td>
<td>A⁻B⁺CDT⁺</td>
<td>Treated water</td>
<td></td>
<td>Diarrhoeic, Western Australia</td>
</tr>
<tr>
<td>G</td>
<td>UK 237</td>
<td>A⁻B⁺CDT⁺</td>
<td>Floor swabs</td>
<td></td>
<td>Diarrhoeic, Western Australia</td>
</tr>
<tr>
<td>G</td>
<td>UK 237</td>
<td>A⁻B⁺CDT⁺</td>
<td>Water collected in channels before digesting ponds</td>
<td></td>
<td>Diarrhoeic, Western Australia</td>
</tr>
</tbody>
</table>

*Clinical status represents samples obtained from pig farms with a history of idiopathic neonatal diarrhoea.

1 First soil sample randomly obtained from the field applied with pig effluent.
Aspects of *Clostridium difficile* infection in pigs

² Second soil sample randomly obtained from the field applied with pig effluent.
³N/A-means not applicable or no available information.
6.4.2 Prevalence of *C. difficile* in retail compost, manure, and human bio-solids

The overall prevalence of *C. difficile* in gardening products was 26.8% (22/82) (Table 6.3). The prevalence of *C. difficile* varied from 0% to 75% in gardening products. Three of the four samples from human biosolids were culture positive to *C. difficile*.

There were five toxigenic RTs (005, 012, 014/20, 017, 056) in soil conditioners, followed by organic fertiliser with two toxigenic RT (237, 080) [Table 6.3] and (Figure 6.1).
Table 6.3 Prevalence of *C. difficile* and toxin profiles in gardening products

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Prevalence (n) %</th>
<th>Number of isolates (%)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A⁻B⁻CDT⁻</td>
<td>A⁺B⁺CDT⁻</td>
<td>A⁻B⁺CDT⁺</td>
<td>A⁻B⁺CDT⁻</td>
<td></td>
</tr>
<tr>
<td>Human biosolids</td>
<td>(3/4) 75.0</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Organic liquid fertiliser</td>
<td>(3/6) 50.0</td>
<td>1</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Soil conditioner</td>
<td>(11/37) 29.7</td>
<td>8</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Mulches</td>
<td>(2/12) 16.7</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Garden mixes</td>
<td>(3/22) 13.6</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Organic fertiliser</td>
<td>(0/1) 0.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Overall prevalence</td>
<td>(22/82) 26.8</td>
<td>20</td>
<td>10 (27.8)</td>
<td>5 (13.9)</td>
<td>1 (2.8)</td>
<td></td>
</tr>
</tbody>
</table>
6.4.3 Viable colony counts

There were 17/35 samples (48.6%) that yielded *C. difficile* colonies in the enumeration assay (Table 6.4). The range of viable counts per sample type was as follows; effluent samples 7.6-14.8 log CFU/mL (57.1%; 4/7), influents samples 3 - 14.8 log CFU/mL (71.4%; 5/7), soils exposed to pig effluent 8.0-11.9 log CFU/mL (54.5%; 6/11) and compost manure 8.6 log CFU/g (33.3%; 1/3). The highest concentration of spores was in samples
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obtained from Victoria and South Australia (14.8 log CFU/mL), while the lowest concentration (6.9 log CFU/mL) was for samples obtained from Queensland (Table 6.4). The detection limit for the assay was >1 CFU/mL or >1 CFU/g (Table 6.4). The positive samples in the viable spore counts came from three states; Queensland (n = 7), Victoria (n = 5) and South Australia (n = 4).
Table 6.4 *C. difficile* enumeration (CFU) in influent, effluent and compost samples

<table>
<thead>
<tr>
<th>Farm ID</th>
<th>Location</th>
<th>Sample type</th>
<th>Viable count (CFU/)</th>
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</tr>
<tr>
<td>A</td>
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</tr>
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</tr>
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<td>A</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
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</tr>
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<tr>
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<tr>
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<tr>
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<td>Effluent soil (S)</td>
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Aspects of *Clostridium difficile* infection in pigs

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<td>Influent (L)</td>
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<td>E</td>
<td>Queensland</td>
<td>Influent (L)</td>
<td>1.0 $\times 10^3$</td>
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<td>Shop A</td>
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<td>Compost (S)</td>
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</tbody>
</table>

Note: Sample type designated “L” was liquid therefore spore concentration units were CFU/mL and that designated “S” were solid hence the spore concentration...
units were CFU/g. NA is for samples whose source location was indeterminate.
6.5 DISCUSSION

In this chapter, the aim was to characterise and quantitate *Clostridium difficile* spore contamination in pig environment and farm soils exposed to pig manure. The major challenge associated with the use of manure in crop farming is that there are a substantial number of pathogenic microorganisms that could be viable and have potential to cause foodborne infection. In addition to foodborne infection, some pathogens could be acquired from manure through recreational reservoirs such as lawns, and contaminated waterways. Another aim was to establish possible transmission pathways for *Clostridium difficile* and determine how these might relate to the high rates of CA-CDI in Australia.

In the USA, there are many reports of outbreaks of pathogenic *Escherichia coli* associated with farm produce contamination with manure (Gould *et al.*, 2013; Painter *et
Aspects of *Clostridium difficile* infection in pigs


In Australia, pig effluent treatment involves removal of solid wastes and then holding liquid effluent in anaerobic ponds under mesophilic anaerobiosis (Kruger *et al.,* 1995).

The treatment method for human raw effluent in Switzerland involved grid separation, primary sedimentation and secondary biological treatment (activated sludge process). Although there have been no foodborne outbreaks reported for CDI, reports of CA-CDI have increased over recent years (Slimings *et al.,* 2014; Kutty *et al.,* 2011). CA-CDI has been reported in younger people and even those without health care facility contact (Khanna *et al.,* 2012; Goorhuis *et al.,* 2008). In this regard, compost should be screened for pathogenic microorganisms such as *C. difficile* before use. Highly contaminated manure should not be used or processed into compost.
The detection of *Clostridium difficile* in influent, effluent and soil amended with manure has important public health implications. The concentration of spores in the animal effluent was considerably higher than in raw influent $2.8 \times 10^6$ CFU/mL vs. $6.0 \times 10^2$ CFU/mL in effluent in this study (Table 6.4). This suggests that *C. difficile* spores may have been amplified in the anaerobic ponds. The concentration of spores was slightly higher than reported on calf carcases and in the faeces of veal calves [$2.0 \times 10^3$ to $2.3 \times 10^6$ CFU/mL] (Knight *et al.*, 2016). This finding suggests that food such as root vegetables that come into contact with effluent could pose a greater risk for acquiring CDI than meat. The fact that in the quantitation assays, all *C. difficile* was recovered by direct culture suggests that the contamination in the soil was likely significant. Our findings are in agreement with others who have detected *C. difficile* in human biosolids that are
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mesophilically treated (Xu *et al.*, 2015; Romano *et al.*, 2012; Viau and Peccia, 2009). McCarthy *et al.* (2013) suggests that the use of manure from farms that have a lower prevalence of pathogenic microorganism for composting could reduce *Salmonella spp.* and *E. coli* in the final composted product. This strategy could further be enhanced by separating manure of young animals from older ones as this has potential to reduce the amount of pathogenic organism including *C. difficile* in compost.

The most likely at-risk group for exposure to *C. difficile* are industry workers who could directly be exposed to manure during processing compost either by aerial transmission or orally if there is poor hand hygiene. In addition, if disease does not directly affect people in the industry, they might be transient shedders who could indirectly become a source of infection within their households. For example, recent studies have reported
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household transmission of *C. difficile* from CDI patients or asymptomatic people and animals in North America (Loo *et al.*, 2016; Shaughnessy *et al.*, 2016). Similarly, other studies have found that residential proximity to swine farms or contact with livestock was the major risk factor of community associated (CA)-MRSA (Deiters *et al.*, 2015; Carrel *et al.*, 2014; Casey *et al.*, 2013). The findings in this study suggest that the transmission pattern described in piggeries is likely occurring in human populations using contaminated manure with *C. difficile* spores.

RT 014/20 was the most frequently detected strain (20.7%) in the pig environment in this study. Other studies have reported RT 014/20 as being among the most frequently detected strains in human medical settings in Australia (Cheng *et al.*, 2016; Foster *et al.*, 2014), England (Fawley *et al.*, 2016) and the USA (Tickler *et al.*, 2014).
In continental Europe, RT 014/20 has been reported as the third highest most commonly detected strain (Davies et al., 2016). The prevalence of RT 014/20 was comparable to that reported previously in pigs in Australia (23.4%) (Knight et al., 2015). Tickler et al. (2014) described *C. difficile* RT014/020 as an emerging strain in certain regions of the USA. Factors that drive the emergence of some ribotypes like RT 014/020 are unknown, but common source or zoonotic transmission is possible. RT 014/020 belongs to clade 1 [toxinotype 0, North American Pulsotype (NAP) 4] which is a highly successful lineage of *C. difficile* consistently among the most common RTs causing CDI in medical settings (Collins et al., 2016; Schwartz et al., 2014) and piggeries in Australia (Knight et al., 2015). A study has found a high genetic relatedness of clinical isolates (RT014) obtained from CDI patients and porcine using WGS in Australia (Knight et al., 2017).
The findings in this study suggest that animal manure could be an important reservoir for *C. difficile*.

The overall prevalence of *C. difficile* in retail gardening products was 26.8% (Table 6.3). The detection of RT 010 (A⁻B⁺CDT⁻) and 014/020 (A⁺B⁺CDT⁻) (11.1%) and 237 (Figure 6.1) was not surprising because they are commonly isolated from the animals and the environment in Western Australia. However, the detection of RTs 005, 012 and 017 was unexpected as these RTs are associated with CDI in patients in Western Australia but they are not common (Collins *et al.*, 2017). *C. difficile* RT 017 in particular is often found in Asia (Collins *et al.*, 2013) and among the emerging ribotypes in Europe (Davies *et al.*, 2016).

Interestingly, *C. difficile* RT 237 which has been exclusively associated with one piggery in Western Australia (chapter 4; Knight *et al.*, 2015; Squire *et al.*, 2015).
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2013) was detected from both pig environment and retail gardening products. The detection of RT 237 in piglet faeces, pig effluent and organic liquid fertiliser, suggests that manure could be the dispersal vehicle for *C. difficile*. This is supported by the recent report of a human case of CDI with RT 237 in Western Australia (McGovern *et al.*, 2016).

In this study, *C. difficile* RT 033 (A-B-CDT+) was the second most frequently detected strain (13.8%) in the pig environment. Generally, non-toxigenic strains are not frequently reported in patients (Eckert *et al.*, 2015; Barbut *et al.*, 2007). However, some studies have found an association between colonisation with RT 033 and clinical diarrhoea (Lyon *et al.*, 2016; Androga *et al.*, 2015; Eckert *et al.*, 2015; Gerding *et al.*, 2014). Most animal strains of *C. difficile* produce a binary toxin (Jhung *et al.*, 2008). Although still controversial, binary toxin has been shown
Aspects of *Clostridium difficile* infection in pigs

to cause a cytopathic effect in *in vitro* studies but does not cause death in a hamster infection model (Geric *et al*., 2006). However, a recent study evaluating the toxicity of the binary toxins found that binary toxin caused death in hamsters and mice like toxin A and B (Wang *et al*., 2016). In the present study, RT 033 was recovered from a pig farm with a history of idiopathic diarrhoeic piglets. Longitudinal studies investigating the association of RT 033 in pig farms with clinical diarrhoea are needed to evaluate the relevance of RT 033 in CDI. These findings suggest that RT 033 could be of clinical relevance in livestock and human CDI.

The prevalence of *C. difficile* in the background soil in this study was 33.3% (2/6) which is comparable to that reported in soils in Zimbabwe (37%) (Simango, 2006), South Wales (21.4%) (Al Saif and Brazier, 1996) and the USA (6.5%) (Higazi *et al*., 2011). Although this was a
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small study, it demonstrated that mesophilic anaerobiosis treated pig effluent at pig farms should not be directly amended into the soil on vegetable farms as *C. difficile* spores remain viable after treatment. These findings are in agreement with others who detected *C. difficile* in treated animal manure (Norman *et al.*, 2011) and human biosolids (Xu *et al.*, 2014; Romano *et al.*, 2012; Viau and Peccia, 2009). The fact that there was a wide variation of *C. difficile* prevalence by farm shows that the risk of *C. difficile* contamination of produce might be low for some farms and high for others. This finding reinforces the idea that farm manure must be screened for important pathogens before applying it to the soils as a fertiliser.

This study has some limitations. The number of farms surveyed was relatively small (n = 7), therefore possibly making the results difficult to generalise in Australia and abroad. However, although a limited number
of samples and farms were studied, there was a wide spatial coverage consisting of four states in Australia.

While most of the samples studied was pig manure. Additionally, we did not enumerate spore concentration in all manure, compost and biosolids samples obtained from retail shops. Therefore, it is difficult to generalise the risk of CDI from retail shop manure, compost, and biosolids. The samples came from four states in Australia; however, future studies should target individual states because of variations in farming practices within and between states might mean that the risk factors could be unique in each state.

6.5.1 CONCLUSIONS

The widespread prevalence of *C. difficile* in pig manure in this study was not surprising. The detection of indistinguishable ribotypes from pig influent, effluent, and farm soil amended with manure and retail manure is of
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public health importance. The detection of RT 237 in effluent and retail manure is important because a case of CDI with RT 237 has been reported recently in a human patient (McGovern *et al.*, 2016). The fact that RT 237 is exclusively associated with one piggery suggests there is a potential that it could be zoonotically transmitted.

Application of manure or compost to land used for crop production could lead to *C. difficile* contaminating farm produce. This is supported by the fact that *C. difficile* was detected in both raw and retail packaged manure or compost, although the contamination level may depend on several factors, such as the source of manure, mode of action of disinfectant, and concentration at individual farm. In the case of landscaping, it is likely that the spores could be higher than detected in farm produce because there are less likely mitigating factors such as washing of vegetables. Since the prevalence of CA-CDI is increasing
worldwide, it is reasonable to suggest that the environment is an important source of infection. Manure and its by-products are known to harbour gastrointestinal tract infectious organisms including *C. difficile*. There are no studies that have demonstrated zoonotic transmission of *C. difficile* infection, although manure is a plausible mode by which *C. difficile* could be transmitted. Based on this study and others, discharging animal effluent on farmland, water bodies, and landscaping is likely to increase human and animal exposure to *C. difficile*. 
CHAPTER 7: DETECTION OF CLOSTRIDIUM DIFFICILE FROM LAWNS IN WESTERN AUSTRALIA

7.1 INTRODUCTION

Work in the previous chapter showed that *C. difficile* was present in both compost and manure. Compost or manure sold as agricultural or landscape fertiliser contained a high prevalence of *C. difficile*. While *C. difficile* is a well-established hospital pathogen, recently it has increasingly been detected in patients without hospital contact. Given this rise in community-associated infections with *C. difficile*, it was hypothesised that the environment could play an important role in the transmission of spores. At a meeting with pig farmers in Western Australia it was revealed that much of the pig manure from their farms was sold to turf farms in Perth to produce “roll-out” lawn. The concept that lawn could be a
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reservoir for *C. difficile* had not been considered previously. In a small (n = 1) pilot study of a new lawn at the Queen Elizabeth II Medical Centre a heavy growth of *C. difficile* was obtained on direct and enrichment culture. On this basis, a larger study was planned.

7.2 **Aim and objectives**

The main aim of this study was to determine the prevalence of *C. difficile* in newly established (NL) and older (OL) lawns in public spaces in Perth and to characterise any *C. difficile* isolates by phenotypic and genotypic techniques. A second aim was to investigate factors such as the location and size of the lawn to see if they could predict *C. difficile* status. The last aim was to quantitate the *C. difficile* in the lawn samples by undertaking viable colony counts.
7.2.1 Study location and samples

NL (defined as ≤ 4 months old) and OL (defined as > 4 months old) samples were collected from 20 public spaces within 11 postcodes in Perth and surrounding areas between February and June 2016. Lawns in Australia are considered young up to 4 months of age by the turf producing industry (Scott Garden Team, 2016); therefore, this seemed a logical cut-off. Lawns in autumn through winter tend to lose colour and become dormant, so during autumn most lawns are rehabilitated, and those not rehabilitated are visible by the characteristic changes associated with dormancy. Therefore, the study was initiated during this period when it was relatively easy to identify lawns that were new versus those that were old. Lawn in Western Australia can either be raised as seeding lawn or instant turf (Scott Garden Team, 2016). Seeded lawn has a lower cost to plant compared to the instant turf;
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however, it takes long to mature and is labour intensive compared to turf rolls. Turf rolls are fast and easy to lay and can be used within a week; hence the high demand in Western Australia. Soft-leaved buffalo grass is a widely used variety in Western Australia because it can withstand droughts. In Western Australia, most lawns are established in early autumn or late summer to avoid extreme temperatures in summer (Scott Garden Team, 2016). Lawn maintenance is achieved by applying fertiliser around autumn, spring, and early summer. A NL was identified by the suture line patterns adjoining two or more patches of laid down lawn which remained visible for up to 4 months. Lawns that did not have these signs were assumed to be old (Scott Garden Team, 2016). When information of the age of lawns in some areas was unavailable, photographic identification was employed. This involved comparing photographs of the test lawn
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with a series of photographs taken of lawns of known age (NL appeared bright green, luxuriant and with sharp edges). Public space lawns without this information were in the minority. Although it is difficult to age lawns using photographic evidence, this technique has been widely used in the USA (Giner *et al.*, 2013). Lawns were sampled within 5 - 30 km north and 5 - 80 km south of Perth city. To be included in the study, all public spaces were had to have a commercially available variety of lawn which was either new or old. Public spaces were categorised by size (small [0.5-1 km$^2$], medium [1.1-2 km$^2$], large [2.1-2.9 km$^2$], and extra-large [$\geq 3$ km$^2$]) based on area data from local council authority websites in each study area or, where no information was available, the area of the space was estimated in relation to other known areas. All the public spaces that met the size cut off and had grass variety which is commercially available were included in
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the sampling frame. To prevent over sampling of one size of public space, a balanced assignment of public spaces across postcodes was employed. It was estimated that 150 samples were appropriate to detect an 18% difference in C. difficile prevalence between NL and OL ($\alpha = 0.05$, power 80%, 95 CI). To account for clustering, the 150 samples obtained were multiplied by simple random effect by 2 design effect. A total of 311 NL or OL samples were collected in sterile 200 mL specimen jars. Each public space was divided into four quadrants with the centre generally being a children’s play facility. Four samples were obtained per quadrant starting from the centre and moving outwards. A lawn sample constituted grass and its root system attached to soil, and was obtained using a new set of examination gloves and sterile tongue depressor which was used to dig out grass and its root system. Each
lawn sample weighed approx. 50 g and these were transported at ambient temperature to the laboratory.

7.2.2 Isolation of \textit{C. difficile} from lawn samples

Briefly, 90 mL BHIB-S was pre-reduced for 4 h in the A35 anaerobic chamber. The lawn samples were aseptically cut in \(~5 \text{ cm}^2\) pieces weighing approximately 5 g, put into the broth and gently shaken, and then incubated in the anaerobic chamber for 5 days with loose lids. A 5 mL aliquot of culture broth was mixed with 5 mL anhydrous ethanol (96\%) and incubated for 1 h before being centrifuged at \(3000 \times g\) for 10 min. The pellet was plated onto \textit{C. diff} ChromID™ agar (bioMérieux) and plates incubated anaerobically for 48 h. \textit{C. difficile} was identified based on its characteristic chartreuse fluorescence detected with UV light (~360 nm wavelength), morphological characteristic (ground glass appearance) and odour (horse dung) (Knight \textit{et al.}, 2013).
Identification of uncertain isolates was achieved by Gram staining and detection of L-proline aminopeptidase (Remel Inc., Lenexa, KS, USA).

**7.2.3 Quantitation of *C. difficile* in lawn samples**

A viable count of *C. difficile* was performed on a subset of lawn samples using previously described methods with some modifications (Knight *et al.*, 2016; Housman *et al.*, 2015; USEPA, 2001). Briefly, approx. 5 g of soil was aseptically obtained from an approx. 10g lawn sample by shaking the root system and placed in a Stomacher bag (Colworth, London, UK) containing 25 mL of phosphate buffered saline at pH 7.4 supplemented with 0.1% Tween 20 (PBST) or peptone saline also containing 0.1% Tween 20 (PST) and shaken for 60 s. A 100 µL aliquot was removed and directly inoculated onto *C. diff* ChromID agar, spread using a sterile hockey stick (InterPath Services Pty Ltd, West Victoria, Australia) and
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incubated anaerobically. The remaining volume was centrifuged at 3000 g for 10 min, the supernatant discarded, the pellet resuspended in 1000 µL of either PBST or PST and 100 µL directly inoculated on C. diff ChromID. Viable colony counts were performed on 48 h old culture. A negative control (either PBST or PST) was used in each assay to monitor for potential contamination. Molecular typing was performed on isolates from all positive samples using toxin gene PCR and PCR ribotyping. The detection limit for the viable count was > 1 CFU per g of soil.

**7.2.4 Statistical analyses**

Univariable random effect logistic regression analysis was used to describe the relationship between *C. difficile* culture status (response variable), and independent variables: age of lawn (new vs. old), sampling site, location (north vs south), postcode, size of playground
Aspects of *Clostridium difficile* infection in pigs (small, medium, large, and extra-large) and season (autumn: March, April, May and winter: June, July August). Two random effects, postcode and sampling site, were included in the model to account for clustering or autocorrelation among samples from the same sampling site and postcode. Univariable models were constructed including one main effect and two random effects per model, then a backwards stepwise multivariable model including the interactions terms, main effects and random effects. A cut off of 10% was used for variable to proceed to the multivariable analysis. In the multivariable model, all variables that were not significantly improving the model were excluded with the assumption that they were not confounders. Variables were retained in the model if they were significant or part of their interaction terms was significant or they were considered as confounders. Random effects were excluded from the model if they
explained very little from the models based on the change in Akaike’s Information Criterion (AIC), Bayesian Information Criterion and deviance $\chi^2$ (Dohoo et al., 2009). Model selection was also evaluated by the likelihood ratios using the generalized linear model (GLM) framework using R version 3.3.1 (R Core Team 2015).

7.3 RESULTS

7.3.1 Prevalence of \textit{C. difficile}

The overall prevalence of \textit{C. difficile} in lawn samples was 59% (182/311) varying from 0% to 70% by postcode. The prevalence of \textit{C. difficile} was significantly higher in NLs 65% (129/198) compared to 47% (53/113) in OLs (Table 7.1). In the unadjusted univariable model, \textit{C. difficile} was more likely to be detected in NL than OL (OR= 2.11, 95% CI: 1.32-3.4, $p = 0.001$) compared to the adjusted model (OR=2.30, 95% CI: 1.16-4.57, $p=0.015$)
Aspects of Clostridium difficile infection in pigs (Table 7.1). In the multivariable analysis, the age of lawn was also the only variable that was significantly associated with detection of C. difficile (OR= 1.92, 95% CI: 1.15-3.21, \( p=0.013 \)). The size and location of the lawns were not associated with detection of C. difficile and the study period was too short to observe any effect of season on recovery.
Table 7.1 The relationship between the prevalence of *C. difficile* in lawn and the age of the lawn, its size, sampling site, location, postcode, and season in Perth

<table>
<thead>
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<th>Variable</th>
<th>Variable categories</th>
<th><em>C. difficile</em> prevalence (%)</th>
<th>Univariable models</th>
<th>Covariate Odds ratios (95% C.I.)&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>Odds ratios (95% C.I.)&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
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Aspects of *Clostridium difficile* infection in pigs

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<td>98 (60.9)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Season</th>
<th>Autumn (n=224)</th>
<th>Referent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>135 (60.3)</td>
<td></td>
</tr>
</tbody>
</table>
Aspects of *Clostridium difficile* infection in pigs

<table>
<thead>
<tr>
<th></th>
<th>Winter (n= 87)</th>
<th>47 (54)</th>
<th>0.77 (0.47- 1.28)</th>
<th>0.67 (0.28- 1.62)</th>
</tr>
</thead>
</table>

\( ^a \) CI; The 95% confidence interval of the odds ratio for the covariate estimates.

\( ^b \) CI; The 95% confidence interval of the odds ratio for the univariable estimates.

\( ^c \) Age of lawn; New lawn approximately ≤ 4 months, Old lawn > 4-8 months.

\( ^d \) PS; Public space.

\( ^e \) P values are based on likelihood ratios and \( P < 0.05 \) is considered significant**.

A univariable logistic regression model with random effect (site of sampling and postcode). The random effect term for postcode was not included in all the models because its addition or removal did not change the model estimates significantly.
7.3.2 Quantitation of *C. difficile* in lawn

Viable counts were performed on 10 lawn samples taken from one positive area by direct culture on *C. diff* ChromID agar (Table 7.2). Samples were also concentrated by centrifugation. Using PBST or PST diluent, the highest number of *C. difficile* positive cultures was 8 out of 10 after centrifugation. *C. difficile* recovery was higher in PST than PBST by both direct culture (50% vs 40%) and after centrifugation (80% vs 60%). The highest viable count of *C. difficile* detected was 1200 CFU/g of soil and the lowest 50 CFU/g of soil (Table 7.2).

7.3.3 Molecular characterisation

There were 35 unique RTs detected in this study. Of the toxigenic RTs detected (*A*\(^+\) *B*\(^+\) *CDT*\(^-\)), 11 isolates harboured *tcdA* and *tcdB* genes but no binary toxin genes (Table 7.3). The toxigenic RTs accounted for 47% (86/182) of the total and included internationally
recognised RTs that are associated with hospital infections. The 24 non-toxigenic RTs (A–B–CDT–) accounted for 53% (96/182). RT 014/020 was predominant (39.01%), followed by RT 010, QX 189, RT 393, 142, and QX 601 (Table 7.3). Toxigenic RTs isolated from NL accounted for 33.52% (61/182) of all isolates compared to 14.28% (26/182) in OL. In the NL among toxigenic strains only, RT 014 was over-represented 78.69% (48/61), followed by RT 054 (3.28%), 056 (3.28%), 002, 018, QX 610, and QX 611). In the OL among toxigenic strains only, RT 014/020 was also over represented 88.46% (23/26), followed by RTs 002, 106 and QX 409.
Table 7.2 Viable counts of *Clostridium difficile* in soil from lawn samples using either phosphate buffer solution (pH 7.4) (PBST) or peptone saline (PST), both containing 0.1% Tween 20, as a diluent

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Sample (viable count cfu/g of soil)</th>
<th>No. samples positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>PBS + 0.1% Tween 20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PS + 0.1% Tween 20</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

‡PBS; Phosphate buffer solution (pH 7.4).
Table 7.3 The frequency of *C. difficile* ribotypes in lawns in Perth

<table>
<thead>
<tr>
<th>Ribotype</th>
<th>Toxin genes</th>
<th>Number, (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tcdA</td>
<td>tcdB</td>
</tr>
<tr>
<td>014/020</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>010</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>QX 077</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>QX 189</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>039</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>QX 601</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>393</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>QX 142</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>002</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Others*</td>
<td>Various</td>
<td>Various</td>
</tr>
</tbody>
</table>

*Presence of toxin gene.

*Absence of toxin gene.

*Others: QX 518, QX 611, QX 608, QX 610, RT 056, RT 054, QX 607, QX 606, QX 605, QX 603, QX 602, QX 550, QX 449, QX 409, QX 393, QX 210, RT 125, QX 121, RT 106, QX 072, QX 067, QX 054, RT 018, RT 012, RT 009, and RT 043.
7.4 DISCUSSION

The aim of this study was to investigate the prevalence of *C. difficile* in public space lawns in Perth, Western Australia. The reasons for undertaking the project were three-fold. First, there has been an increase in the incidence of CA-CDI in Australia (Slimings *et al.*, 2014). Second, although many production animals carry *C. difficile* in their gut, particularly young animals, it is unlikely that meat contamination plays a major role in CA-CDI (Limbago *et al.*, 2012). Last, anecdotally we learned that manure from pig farms was being used by turf farms for the production of lawn.

The overall prevalence of *C. difficile* in lawn samples was 59%, varying from 0% to 70% by postcode. In the adjusted univariable model, *C. difficile* was more likely to be detected in NL than OL (OR = 2.30, 95% CI: 1.16–4.57, \( p = 0.015 \)) (Table 1). Other studies have reported a high prevalence (30–68%) of *C. difficile* in the household.
environment and biosolid treatment plants (Alam et al., 2014: Romano et al., 2012), similar to this study. The reason for the lower prevalence of *C. difficile* in OL (> 4 months) is not clear, although an effect of natural exposure of germinated spores to oxygen or ultraviolet light cannot be excluded. It is unlikely that *C. difficile* can multiply to any great extent in the lawn and the counts detected were not very high. A recent study has shown that ultraviolet light devices can be used as sanitizers in hospitals to reduce pathogenic organisms including *C. difficile* (Weber et al., 2016). Another study found that *C. difficile* spores could survive up to ~5 months in the environment (Kramer et al., 2006). Therefore, the difference in prevalence between NL and OL could be explained by environmental factors such rain, wind, and ultraviolet light over time, and contact with people and animals which could help disperse the initial spore load, particularly on the surface of the lawn. The majority of the NL in this study was located
within recently built suburbs in Perth. The relationship between new suburb buildings and lawn expansion is well studied (Robbins and Birkenholtz, 2003). Our findings suggest that new suburbs and consequent expansion of NL could be contributing to dispersal of *C. difficile* in the environment and variables other than NL were not significantly associated with isolation of *C. difficile*.

Nearly 25% of global disease burden has been attributed to environmental risk factors (Prüss-Üstün and Corvalán, 2007). Animal manure is widely used in both agriculture and landscaping (USEPA, 2012; NEBRA, 2007; Iranpour *et al.*, 2004; Suslow *et al.*, 2003), and anecdotal evidence suggests this practice is widespread in Australia. In the USA, manure that has been composted is exempted from federal or state rule regarding pathogen levels if it is going for land application (Suslow *et al.*, 2003). The major problem which is often overlooked in
applying animal manure or biosolids to land is consideration of the duration of persistence of pathogens on plants (Suslow et al., 2003). Animal effluent treatment involves removal of solid wastes and then holding liquid effluent in anaerobic ponds under mesophilic anaerobiosis. The treatment method for human raw effluent in Switzerland involved grid separation, primary sedimentation, and secondary biological treatment (activated sludge process) (Romano et al., 2012). The major problem in animal manure treatment is a lack of separation of faeces from young animals versus older animals. Young animals are associated with shedding high levels of pathogenic organisms including, and in particular, *C. difficile* (Knight et al., 2013; Houser et al., 2012; Suslow et al., 2003). Currently, there is pressure on the turf industry to meet the demand for lawns in newly built suburbs both private and public lawns. This has led the industry to develop the lawn farming system of roll-
out lawns which can easily be transported to clients on demand. The lawn, like other plants, is grown on manure or biosolids for rapid growth and early maturation. The fact that even composted manure or treated animal effluent does not inactivate *C. difficile* spores implies that lawns could be heavily contaminated with *C. difficile* and our data suggest this is the case. Even compost, manure or biosolids that have been produced with specific standards (Iranpour *et al.*, 2004) have caused disease outbreaks in the USA (Gould *et al.*, 2013; Painter *et al.*, 2013; Doyle *et al.*, 2008; Gerba *et al.*, 2005). In this regard, manure, compost and biosolids should be screened for pathogenic microorganisms such as *C. difficile* before being applied to land for recreational or agricultural use.

Although the infectious dose for *C. difficile* is unknown, there are suggestions that it could be low (100-1000 spores) (Warriner *et al.*, 2016) and infection could be
driven mainly by host factors. In this study, we found that the highest *C. difficile* spore concentration in the lawn samples was 1200 CFU/g of soil (Table 7.2). The concentration of *C. difficile* in faeces of cattle has been reported as $2.5 \times 10^4$ CFU/mL (Knight *et al.*, 2016) and in human faeces as $6.66 + 0.62 \log_{10} \text{CFU/mL}$ (Housman *et al.*, 2015). It is unclear how much exposure to lawns is required for a human or animal to be contaminated with sufficient spores that could lead to infection with *C. difficile*. The fact that ingestion of *C. difficile* from the environment does not quickly result in infection is due to the complex nature of the pathogenesis of disease that requires a gut insult, usually in the form of antibiotic exposure (Kuntz *et al.*, 2011). Although we did not find any seasonal fluctuations of *C. difficile* in lawn samples in this study, it is likely that recreational exposure to *C. difficile* could be higher in some seasons when people regularly use public spaces and lower in low activity
periods. Therefore, studies that quantitate spore load on fomites such as shoes are needed because they may explain the rise of CA-CDI.

There were 35 unique RTs detected in this study, including the clinically important RTs 014/020, 056, 054, 002, 018, 012 and 043 (Table 7.3). The detection of RT 014/020 in this study was expected because RT 014/020 is the most predominant RT in laboratory samples from diarrheic patients (Barbanti and Spigaglia, 2016; Cheng et al., 2016; Collins et al., 2016; Eyre et al., 2015) and livestock in Australia (Knight et al., 2015). Other researchers have reported detection of RT 014/020 in river water (Zidaric et al., 2010) and treated sludge (Romano et al., 2012).

The detection of RTs 012, 002, and 018 was unexpected because they are not routinely detected from the environment (Romano et al., 2012; Zidaric et al.,
2010), although they are increasingly associated with CA-CDI (Dauby et al., 2017; Cheng et al., 2016; Collins et al., 2016). Recently, RTs 056, 010, 078, 213, 009 and 020 were detected in dogs (Álvarez-Perez et al., 2015). RT 056 was also recently detected in gastrointestinal tracts of veal calves at slaughter (Knight et al., 2013). Several of the other RTs detected in our study (056, 054, 018 and 002) have been associated with human CDI in Europe (Dauby et al., 2017; Fawley et al., 2016), Japan (Senoh et al., 2015b) and Australia (Eyre et al., 2015). These findings suggest that all lawns may be getting contaminated with animal strains of C. difficile and that this could be contributing to an increase in CA-CDI.

Interestingly, no binary toxin-producing C. difficile isolates were cultured in this study although such strains have been recovered previously in high numbers from production animals in Australia (Knight et al., 2015; Knight et al., 2014; Knight et al., 2013). The reasons for
Aspects of Clostridium difficile infection in pigs

This is unclear. Our earlier animal studies have suggested that different strains of C. difficile tend to infect/colonise herds of animals in particular geographic areas (Knight et al., 2015; Knight et al., 2013) and these are not always binary toxin producers, such as RT014/020 in pigs (Knight et al., 2015). The lack of binary toxin producers in the current study may just reflect a relatively small sample size and the fact this study was undertaken in Western Australia only.

The lawns sampled were located within the metropolitan area of Perth, suggesting that people and dogs walking on lawns could transfer C. difficile into the household. A high prevalence (32.3%) of C. difficile in household environs has been reported, particularly on the soles of shoes (Alam et al., 2014). It is also possible that shoes could be vehicles by which C. difficile could be introduced in health care facilities. The detection of C.
Clostridium difficile in lawn could help explain the substantial proportion of cases (45%) of CDI originating from unknown reservoirs as recently described by others (Kubota et al., 2016; Eyre et al., 2013).

This study has some limitations. First, the study was undertaken only in Western Australia which may impact its generalisability to other regions of Australia and the world. We only sampled a relatively small number of public spaces (n = 20), however, all lawn/turf producing companies have access to the same sources of manure in Western Australia, suggesting that the problem could be similar throughout the metropolitan area of Perth. The age of some of the lawns was estimated with a series of photos of lawns with a known age and this could have resulted in misclassification bias. However, this would not have affected the overall outcomes of the study as both OL and NL had relatively high prevalence of C. difficile. We
investigated management practices of commercial lawn farms; however, the frequency of maintenance of the lawns was not established.

7.4.1 CONCLUSION

In conclusion, the high prevalence of *C. difficile* in lawn is potentially an important finding; however, it is difficult to estimate the risk of CDI through exposure to *C. difficile* in lawns. There is a need for further detailed and structured studies to examine how modern lawns are grown, and to investigate the role of any manure and biosolids applied as fertiliser as a source of CA-CDI. The relevance of these findings should be further confirmed by studies comparing the relatedness of isolates in this study to those CA-CDI cases using whole genome sequencing.

Future research should be directed towards residential and public lawn care practices (Giner *et al.*, 2013; Law *et al.*, 2004). Studies investigating lawn management
practices and any correlations with CA-CDI are needed. Furthermore, studies investigating the source of manure, quantity and the frequency of manure used in lawns are needed.
CHAPTER 8: DEVELOPMENT OF A

CLOSTRIDIUM DIFFICILE TOXOID VACCINE TO
PREVENT C. DIFFICILE COLONISATION AND
INFECTION IN PIGLETS UNDER FIELD
CONDITIONS AND NATURAL CHALLENGE

8.1 INTRODUCTION

This chapter examines the potential of passive immunotherapy using a toxoid vaccine for CDI in gravid gilts. In chapter 6, the environmental sources of C. difficile at pig farms were discussed, including detection of C. difficile in compost, manure and soils exposed to pig effluent. In the previous chapter, detection of C. difficile on lawn grown using pig manure was described. This finding is not surprising given that the prevalence of C. difficile in piglets is high (> 50%). A plausible cost-effective control of CDI in animals, and by extension in humans, could be by employing a vaccination strategy. In
the present chapter, development of a *C. difficile* toxoid vaccine that has potential to reduce *C. difficile* shedding in piglets is described. It was hypothesised that vaccinating pregnant gilts in addition to increased use of sporicidal disinfectants in a piggery could possibly break the infectious cycle of *C. difficile*. In the second part of this chapter, an anamnestic response of gilts to the *C. difficile* toxoid vaccine compared to the controls is examined.

### 8.2 Aims and objectives

The aim of this study was to develop a *C. difficile* toxoid vaccine consisting of TcdA and TcdB or TcdB and CDT, and cell wall proteins using RT014/020 (A+B+CDT−) and RT 237 (A−B+CDT+). A second aim was to investigate whether passive immunity could protect neonatal piglets born from vaccinated gilts against natural CDI and colonisation by stimulating sufficient protective humoral immunity in gilts.
Ethics statement

All experiments were conducted in accordance with The University of Western Australia Animal Ethics Committee (AEC) guidelines (approved protocol number RA/3/100/1481).

8.2.1 Experimental settings

8.2.1.1 Serum, colostrum and faecal sample collection

Three gravid gilts received either a toxoid vaccine formulated with *C. difficile* RT014/020 (A+B+CDT-) or RT237 (A-B+CDT+). Each treatment group was assigned to three control adjuvant injected gravid gilts. Rectal swabs (n = 188) were obtained from 12 litters after farrowing. Half of the faecal samples (n = 94) were collected on the farrowing day (n = 44, treatment group) and a second sampling (n = 94) on days 5 (2 litters) and 6 (Figure 8.1). Baseline serum was collected from all gilts before vaccine administration and at farrowing, together
with colostrum. The gilts received two doses of intramuscular injections (~42 mg of proteins each), the first vaccination at 6 weeks pre-farrowing followed by a booster vaccination 3 weeks pre-farrowing (Figure 8.1).

![Image of vaccination and sampling time frame for serum and colostrum from gilts, and faeces from progeny.](image)

Figure 8.1 Vaccination and sampling time frame for serum and colostrum from gilts, and faeces from progeny.

The optimal route for vaccine administration was adapted from the Committee for Medicinal Products for Veterinary Use (CVMP) assessment report for Porcilis ColiClos (EMEA/V/C/002011) based on *C. perfringens* and *E. coli* vaccine standards by The European Medicine Agencies, document number EMA/260393/2012, Veterinary Medicines and Product Data Management 2012 (CVMP,
Porcilis ColiClos is a vaccine containing *E. coli* (F4ab, F4ac, F5, F6, and LT) and *C. perfringens* type C antigens. We reasoned that since *C. perfringens* belongs to the same family of clostridia as *C. difficile* by extension, the intramuscular route of vaccine administration in this study was deemed appropriate.

### 8.2.2 Statistical analysis

Unconditional univariate logistic regression analysis was used to describe the relationship between *C. difficile* culture status (response variable), and independent variables. For a variable to enter the multivariate model, a conservative cut off was set at 10% in the univariate model. Multivariable analysis was performed using the generalised estimating equations (GEE) model to assess the impact of passive immunity on *C. difficile* shedding in piglets born from vaccinated gilts. Pen identity number was included in all multi-variate analysis in all GEE models to account for within pen/litter clustering as previous described (chapter 4). For a variable to remain in
the final model, it either had to be a priori or had a Chi-square \( p \)-value < 0.05 using the Wald test as described previously (Halekoh et al., 2006). Wilcoxon signed-rank test was performed to determine differences between immunised and control sera and colostral antibody titres using the ELISA units obtained by optical density at 450 nm. Wilcoxon signed-rank test a non-parametric test was used because the sample did not satisfy the normal distribution assumption. A two-sided \( p \)-value < 0.05 was considered significant for all associations. All statistics were performed in R version 3.3.3 (R Core Team, 2017).

8.3 RESULTS

8.3.1 Faecal culture \textit{C. difficile} prevalence

The overall culture prevalence of \textit{C. difficile} in faecal samples of piglet from the vaccine study was 25\% (47/188) by enrichment and 21.3\% (40/188) by direct culture (Table 8.1). The prevalence of \textit{C. difficile} in the
piglets born to vaccinated sows was 15.9% compared 33% in the control piglets (unadjusted $p = 0.008$, Table 8.2). In the multivariable analysis, piglets born to vaccinated sows were less likely to shed *C. difficile* compared to piglets from control sows ($p = 0.042$, GEE, Table 8.3).

### 8.3.2 *C. difficile* molecular characterisation

One *C. difficile* isolate possessed ($tcdA^+\), $tcdB^+\), $cdtA^-\) and all the other isolates had $tcdB$ and binary toxin ($cdtA^+/B^+$) genes but not $tcdA$. RT 014/020 ($A^+B^+CDT^-$) and RT 237 ($A^-B^+CDT^+$) were detected.

### 8.3.3 Passive immunisation

In the cross-protection assay, gilts vaccinated with RT 014/020 ($A^+B^+CDT^-$) had higher antibody ELISA titres (IgG) than the baseline serum from control gilts ($p = 0.019$) (Table 8.4). Serum IgG antibodies from gilts vaccinated with RT 014/020 was also greater than the baseline sera from control gilts ($p = 0.0119$) when
challenged with antigens of the same RT used in the vaccine. However, in the cross-protection assay, gilts vaccinated with RT 237 did not produce greater antibody ELISA titres (IgG) than the baseline sera of control gilts ($p = 0.272$) (Table 8.4).

The colostral ELISA titre (IgG) antibodies were generally higher for vaccinated gilts than control gilts, but not significantly different ($p > 0.05$) (Table 8.5).
Table 8.1 Enrichment culture prevalence of *Clostridium difficile* in passively immunised piglets from six gilts vaccinated using extracts from *C. difficile* ribotype 014/020/or 237 and six controls

<table>
<thead>
<tr>
<th></th>
<th><em>C. difficile</em> culture +ve</th>
<th><em>C. difficile</em> culture -ve</th>
<th>No. samples</th>
<th>Prevalence %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinated</td>
<td>14</td>
<td>74</td>
<td>88</td>
<td>15.9</td>
</tr>
<tr>
<td>Control</td>
<td>33</td>
<td>67</td>
<td>100</td>
<td>33.0</td>
</tr>
<tr>
<td>Totals</td>
<td>47</td>
<td>141</td>
<td>188</td>
<td>25</td>
</tr>
</tbody>
</table>
Table 8.2 Univariate logistic regression models describing the relationship between an independent variable and the outcome variable “Clostridium difficile shedding” of passively immunised piglets compared to piglets born from control gilts

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient Estimates (OR)</th>
<th>Std. Error</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccination status</td>
<td>-0.957</td>
<td>0.360</td>
<td>0.008**</td>
</tr>
<tr>
<td>Referent (Control group)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RT014/020 vaccine</td>
<td>-1.444</td>
<td>0.518</td>
<td>0.005**</td>
</tr>
<tr>
<td>RT237 vaccine</td>
<td>-0.528</td>
<td>0.434</td>
<td>0.223</td>
</tr>
<tr>
<td>Litter size</td>
<td>0.184</td>
<td>0.100</td>
<td>0.065</td>
</tr>
<tr>
<td>Referent (1-day old)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 5</td>
<td>2.054</td>
<td>0.413</td>
<td>&lt; 0.001**</td>
</tr>
<tr>
<td>Day 6</td>
<td>-15.320</td>
<td>1.054</td>
<td>&lt; 0.001**</td>
</tr>
</tbody>
</table>
Aspects of Clostridium difficile infection in pigs

Note: Significance level was set at $P$ value < 0.05**

Table 8.3 Multivariable analysis using Generalised Estimating Equations model showing the impact of passive immunity on *C. difficile* shedding in piglets born to sows vaccinated with a *C. difficile* toxoid

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient Estimates</th>
<th>Std. Error</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Referent (Day 1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 5</td>
<td>2.059</td>
<td>0.590</td>
<td>0.00048**</td>
</tr>
<tr>
<td>Day 6</td>
<td>-39.817</td>
<td>1.147</td>
<td>&lt; 0.001**</td>
</tr>
<tr>
<td>Vaccination status</td>
<td>-0.822</td>
<td>0.406</td>
<td>0.042**</td>
</tr>
</tbody>
</table>

Note: Significance level was set at $P$ value < 0.05**
Table 8.4 Antibody titres of pooled serum samples obtained from vaccinated gilts at farrowing following two injections of a toxoid vaccine formulated from RT 014/020 or 237 extracts at 3-weeks intervals and non-vaccinated naïve control gilts processed by indirect ELISA

| Sample | Ribotype used in vaccine formulation | Antigen on ELISA plate | Antibody ELISA Titre (IgG) | Wilcoxon rank sum test on ELISA units | P-value  
Mean pooled sera samples for 3 sows by vaccine type | Mean non-vaccinated pooled sera samples |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sera₁</td>
<td>014/020</td>
<td>237</td>
<td>4096</td>
<td>128</td>
<td>0.019**</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean pool sera samples</td>
<td>Mean non-vaccinated pool sera samples</td>
<td></td>
</tr>
<tr>
<td>Sera₂</td>
<td>237</td>
<td>237</td>
<td>4096</td>
<td>0</td>
<td>3.602e-05**</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean pool sera samples</td>
<td>Mean non-vaccinated pool sera samples</td>
<td></td>
</tr>
<tr>
<td>Sera₃</td>
<td>014/020</td>
<td>014/020</td>
<td>4096</td>
<td>0</td>
<td>0.0119**</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean pool sera samples</td>
<td>Mean non-vaccinated pool sera samples</td>
<td></td>
</tr>
</tbody>
</table>
Aspects of *Clostridium difficile* infection in pigs

---

Data used to calculate Wilcoxon rank sum test in Table 8.4

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Antigen</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>S&lt;sub&gt;1&lt;/sub&gt;</td>
<td>237</td>
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<tr>
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<td>0.233</td>
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<td>0.16</td>
<td>0.14</td>
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<td>0.16</td>
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<td>0.12</td>
<td>0.111</td>
<td>0.11</td>
</tr>
<tr>
<td>S&lt;sub&gt;3&lt;/sub&gt;</td>
<td>014/020</td>
<td>0.202</td>
<td>0.20</td>
<td>0.168</td>
<td>0.157</td>
<td>0.13</td>
<td>0.14</td>
<td>0.121</td>
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<td>0.12</td>
<td>0.116</td>
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</tr>
<tr>
<td>S&lt;sub&gt;4&lt;/sub&gt;</td>
<td>014/020</td>
<td>0.208</td>
<td>0.11</td>
<td>0.101</td>
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<td>0.1</td>
<td>0.108</td>
<td>0.10</td>
<td>0.116</td>
<td>0.10</td>
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</tbody>
</table>
Note: S₁ and S₂ are vaccinated serum mean optical density values (450 nm) used to determine titres in sera₁ and sera₂ whereas S₃ and S₄ are sera₃ and sera₄. C₀ is the mean control sera used to compare with S₁ and S₂, while C₁ is the mean control sera used to compare with S₃ and S₄.

Note: Significance level was set at $P$ value < 0.05**
### Table 8.5 Antibody titres of pooled colostrum samples obtained from vaccinated gilts using a vaccine formulated from ribotype 014/020 or 237 extracts or non-vaccinated controls using either RT 014/020 or 237 extracts as coating antigens on indirect ELISA

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Ribotype used in vaccine formulation</th>
<th>Antigen on ELISA plate</th>
<th>Antibody ELISA Titre (IgG)</th>
<th>Wilcoxon rank sum test on ELISA units</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean pooled colostrum samples of 3 sows by vaccine type</td>
<td>Mean pooled non-vaccinated colostrum samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colostrum(_1)</td>
<td>014/020</td>
<td>237</td>
<td>512</td>
<td>128</td>
<td>[ ] [ ]</td>
</tr>
<tr>
<td>Colostrum(_2)</td>
<td>237</td>
<td>237</td>
<td>512</td>
<td>1024</td>
<td>[ ] [ ]</td>
</tr>
<tr>
<td>Colostrum(_3)</td>
<td>014/020</td>
<td>014/020</td>
<td>512</td>
<td>512</td>
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</tr>
</tbody>
</table>
Data used to calculate Wilcoxon rank sum test in Table 8.5

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<th>3</th>
<th>4</th>
<th>5</th>
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</thead>
<tbody>
<tr>
<td>C1</td>
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<td>0.25</td>
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<td>0.20</td>
<td>0.13</td>
<td>0.101</td>
<td>0.08</td>
<td>0.079</td>
<td>0.07</td>
<td>0.086</td>
<td>0.084</td>
<td>0.165</td>
<td>0.117</td>
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<td>6</td>
<td>6</td>
<td>3</td>
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<tr>
<td>C2</td>
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<td>0.20</td>
<td>0.113</td>
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<td>0.099</td>
<td>0.07</td>
<td>0.085</td>
<td>0.074</td>
<td>0.164</td>
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</tr>
<tr>
<td>Cα</td>
<td>237</td>
<td>0.29</td>
<td>0.26</td>
<td>0.20</td>
<td>0.13</td>
<td>0.108</td>
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<tr>
<td>C3</td>
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<td>7</td>
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<td>4</td>
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<td>3</td>
</tr>
<tr>
<td>Cb</td>
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<td>0.12</td>
<td>0.13</td>
<td>0.11</td>
<td>0.115</td>
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<tr>
<td></td>
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<td>0</td>
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<td>5</td>
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<td>5</td>
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</tr>
</tbody>
</table>
Note: C₁ and C₂ is vaccinated colostrum mean optical density values (450 nm) used to determine titres for colostrum₁ and colostrum₂ in Table 8.5 whereas C₃ and C₄ are colostrum₃ and colostrum₄. Cₐ is the mean control colostrum used to compare with C₁ and C₂, while Cₐ is the mean control colostrum used to compare with C₃ and C₄.

Note: Significance level was set at $P$ value < 0.05**
8.4 DISCUSSION

The overarching aim for developing a \textit{C. difficile} vaccine for pigs was to complement efforts towards reducing diarrhoea associated with CDI in piglets. This is because piglets with CDI take longer to wean and are underweight at weaning. The vaccine intervention could reduce faecal \textit{C. difficile} shedding by piglets through passive immuno-transfer, consequently preventing environmental contamination.

In this study, \textit{C. difficile} prevalence was lower in progeny of vaccinated gilts, 15.9\% versus 33\% in controls (adjusted $p = 0.042$, GEE, Table 8.3). The overall \textit{C. difficile} prevalence in this study was lower (25\%) than reported in our previous studies (62\%-67\%) in the same piggery (chapter 4; Squire \textit{et al}., 2013). There are likely many factors that led to a significant drop in \textit{C. difficile} prevalence in this study overtime. The fact that there has
been increased awareness of *C. difficile* among the pig
farm staff and better infection control attempts, such as the
intermittent use of sporicidal disinfectants (> 8 years),
could partly explain the findings in the present study.
Recently, a study showed that infection control strategies
other than vaccination led to a decline in the *C. difficile*
incidence in humans by ~80% in England (Dingle *et al.*, 2017). However, a simulation study using a 30-bed ward
in a hospital has found that vaccinating patients could
prevent up to 43% new cases of CDI (van Kleef *et al.*, 2016). Similarly, *in vivo* studies have reported low faecal
centre of *C. difficile* spores in vaccinated mice
(Bruxelle *et al.*, 2016; Péchiné *et al.*, 2016; Huang *et al.*, 2015) and hamsters (Bruxelle *et al.*, 2016). More recently,
Hong *et al.* (2017) showed that a vaccine using *Bacillus
subtilis* spores expressing carboxyl-terminal of TcdA
prevented *C. difficile* colonisation and infection in
hamsters. A mouse infection model has shown that
hyperimmune bovine colostrum from cows receiving a vaccine formulated from TcdB, spore and/ or vegetative cell wall using *B. subtilis* spores expressing C-terminal of TcdB of *C. difficile* prevented CDI recurrence by 67% (Hutton *et al.*, 2017). Therefore, vaccination and disinfection of piggeries as described in this chapter could explain the reduction in faecal shedding of *C. difficile* by piglets.

In the present study, the vaccine was formulated as a combination of *C. difficile* toxins and SLPs. Although *C. difficile* TcdA and TcdB are well described virulence factors, other factors have been reported (Pechine *et al.*, 2016; Dingle *et al.*, 2013). For example, *C. difficile* uses the SLPs as a defensive mechanism to evade host immunity (Dingle *et al.*, 2013). Drudy and colleagues (2004) showed that CDI patients who experienced only a single episode of diarrhoea had significantly higher IgM
antibodies to SLPs compared to those who had recurrences. Many in-vitro (Senoh et al., 2015a; Willing et al., 2015) and in-vivo (Huang et al., 2015) studies have shown that SLP-based vaccines could prevent bacterial colonisation including C. difficile (Pechine et al., 2016). Senoh et al. (2015a) showed that serum and intestinal fluid isolated from mice immunised with nontoxigenic C. difficile cell membrane fraction prevented adhesion of C. difficile to human Caco-2 intestinal cell explants. For this work, it was hypothesised that a combination of protein antigens could synergistically elicit protective immunogenic antibodies with the ability to prevent infection and colonisation. Although an ELISA to detect specific antibodies against SLPs was not done, it is likely that SLP could have played a role in enhancing colonisation resistance.
A high serum IgG against toxin A in medical settings has been associated with asymptomatic carriage of *C. difficile* (Kyne *et al.*, 2000). Additionally, patients with a high serum IgG that had experienced a single episode of diarrhoea were less likely to develop CDI recurrence (Kyne *et al.*, 2001). In the present study, serum ELISA titres for gilts that were vaccinated either with RT 014/020 or RT 237 formulations had higher IgG titres than baseline serum from controls (Table 8.4). Additionally, serum IgG titres from gilts vaccinated with RT014/020 (A+B+CDT-) formulated vaccine were consistently higher than baseline controls when exposed to RT014/020 or RT237 toxin filtrate antigens on ELISA. However, serum IgG titres from gilts vaccinated with the RT 237 formulated vaccine were not greater than the baseline control serum when exposed to RT 014/020 toxin filtrate antigen (Table 8.4). This supports the already established knowledge about the importance of both TcdA and TcdB in vaccine
formulations (Pechine et al., 2016). In agreement with this study, Steel and colleagues (2013) found that administering monoclonal or polyclonal antibodies against TcdA and TcdB or TcdB alone prevented severe diarrhoea in gnotobiotic piglets.

Squire and Riley (2013) suggest that maternal colostral antibodies determine whether a piglet became infected or remained without infection. In this study, the colostral antibodies from gilts vaccinated with RT 014/020 formulated vaccine or those vaccinated with RT 237 were generally higher than controls although not significantly different \((p > 0.05)\) (Table 8.5). The lack of statistical difference between vaccinees and control colostrum antibody titres could possibly be attributable to poor assay performance or unspecific antibody binding. In the cross-protection assay on ELISA, colostrum from gilts vaccinated with RT 014/020 performed better than those of RT 237 probably because of the high concentration of
toxin A IgG antibody (Table 8.5). Bauer and colleagues (2014), suggest that antibodies associated with mucosal immunity (sIgA) could be important in influencing the outcome of *C. difficile* associated disease. A pig CDI model showed that piglets that received hyperimmune bovine colostrum did not experience any severe diarrhoea compared to control piglets (Sponseller *et al.*, 2015). Although we only tested for the presence of colostral IgG and not IgA and IgM, it is evident that vaccinated gilts were generally producing relatively higher IgG than controls. Together, these data in this work suggest that the vaccine used was immunogenic in pigs and that passive immuno-transfer played a role in *C. difficile* colonisation resistance.

The association of CDT as a virulence factor was first described in patients experiencing pseudomembranous colitis in 1997 in France (Perelle *et al.*, 1997). Geric *et al.* (2006) showed that binary toxins caused enterotoxicity in
Aspects of *Clostridium difficile* infection in pigs

the rabbit ileal loop but not mortality in hamsters. More recently, Kuehne *et al.* (2014) showed that 33% mice infected with a *C. difficile* RT 027 (A-B-CDT+) double toxin mutant succumbed to CDI. Wang *et al.* (2016) demonstrated that CDT caused systemic disease and death in mice and hamsters. Similarly, RT 033 has been recovered from diarrhoeic piglets (Knight *et al.*, 2015) and calves (Schneeberg *et al.*, 2013). Recently, Secore and colleagues (2017) showed that binary toxin enhanced the efficacy of a vaccine in the *C. difficile* infection model using Syrian golden hamster. Together, these studies suggest that CDT could be an important antigen in the vaccine development as shown in this study when using RT 237 (A-B+CDT+).

**Limitations:**

In this study, only 12 gravid gilts and the progeny were studied in one piggery, therefore making it difficult
to extrapolate these data to older sows (more than one birth) and other piggeries that could be managed differently. Additional studies should be conducted to determine *C. difficile* spore concentration in faeces as this could show the relative impact of the vaccine on spore clearance in the GIT. Given that there was a low *C. difficile* prevalence in this study may suggest that spores were less concentrated in faeces. These findings in the present study are consistent with others who have investigated passive immuno-transfer in animal models (Hutton *et al.*, 2017) and human neonates (Tullus *et al.*, 1989; Cooperstock *et al.*, 1983; Cooperstock *et al.*, 1982). Future studies should evaluate specific important antigens in a toxoid vaccine in the field settings. The problem is that field studies examining the efficacy of *C. difficile* toxoid vaccines in farm animals are not available. The available vaccination data is that performed in laboratory animals. This becomes difficult to compare studies that
have used high doses of spores in CDI models in laboratory animals which may overestimate the infectious dose of *C. difficile*.

### 8.4.1 CONCLUSIONS

A two dose, *C. difficile* toxoid vaccine given to pregnant gilts at 6 and 3 weeks before farrowing reduced the prevalence of *C. difficile*. Protection was demonstrated against natural challenge by a high serum and colostral IgG titres in vaccinated gilts compared to baseline serum from control gilts. Although colostral IgG titres in vaccinated gilts were not significantly different from control gilts, they were generally higher in the vaccinated gilts than controls. This study forms the basis for further investigations into cost-effective way to manage the ubiquitous *C. difficile* pathogen in piggeries. Further studies evaluating vaccine efficacy, including specific antigens for *C. difficile* vaccine formulations are required.
and should be tailored to monitoring exposure and shedding of spores in the environment. Overall, this study suggests that a combined use of a toxoid vaccine and improved disinfection protocols could be effective in reducing colonisation and/or infection in piggeries.
CHAPTER 9: GENERAL DISCUSSION AND CONCLUSIONS

9.1 INTRODUCTION

*C. difficile* is an important pathogen of humans (Lessa *et al.*, 2015; Bartlett, 2008) and animals (Songer *et al.*, 2006). It is a Gram positive, anaerobic, spore forming bacterium, which is ubiquitous in the environment due to its ability to withstand harsh environmental conditions. *C. difficile* colonises the large intestine and the caecae when the endogenous microbiota has been disrupted (Lyra *et al.*, 2009; Jank and Aktories, 2008; Keel and Songer, 2007) and produces two toxins, A (TcdA) and B (TcdB), as its main virulence factors (Jank and Aktories, 2008). Some *C. difficile* strains produce a binary toxin.

Neonatal pigs with CDI may develop enteritis resulting in stunted growth, delay in weaning, and increased mortality (up to 16%) (Songer and Uzal, 2005).
Poor farrowing shed hygiene, high spore dosage (contamination) and age are risk factors for CDI in piglets (Arruda et al., 2013). The major risk factors for CDI in humans are exposure to *C. difficile* spores and antimicrobials which are known to disrupt the resident microbiota (Hurley and Nguyen, 2002). Conversely, in animals, the epidemiologic link between antibiotic exposure and CDI is not as clear.

Although *C. difficile* was confirmed as a pathogen in hospitalised patients receiving antibiotics such as clindamycin in the late 1970s (Bartlett, 2008), it was not recognised as a pathogen for food animals until in the late 1980s. The epidemiology of CDI has evolved significantly over the past decades. *C. difficile* has increasingly been detected in people without a history of hospital contact. For example, in the Netherlands, indistinguishable *C. difficile* RT 078 strains have been detected from pigs and
farmers (Keessen et al., 2013; Goorhuis et al., 2008). In some Western Australian piggeries, *C. difficile* RT 237 is endemic (Squire et al., 2013). Although RT 237 appears not to be well adapted to human hosts, recently, there have been human cases of CDI with this ribotype in Western Australia (McGovern et al., 2016; Foster et al., 2014). Furthermore, direct transmission of *C. difficile* from pigs to humans has been suggested in the Netherlands although not proven (Knetsch et al., 2014). Taken together, this recent evidence supports a hypothesis of an alternative mode of zoonotic transmission of *C. difficile*.

Recently, the rates of CA-CDI have increased worldwide (Slimings et al., 2014; Kutty et al., 2011). This has led to a search for reservoirs of *C. difficile* outside the hospital with various possibilities being suggested (Bauer and Kuijper, 2015). Multiple food sources have been investigated for the presence of *C. difficile* (Knight et al.,
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2016; Rahimi et al., 2014; Pasquale et al., 2012; Pasquale et al., 2011; Al Saif and Brazier, 1996). The prevalence of C. difficile in food animal products is low (Limbago et al., 2012). Furthermore, a study found a low prevalence of C. difficile in food given to patients in a hospital (Kwon et al., 2016). Although the prevalence of C. difficile in different animal food products is low, it is unclear what the concentration of spores in food actually is. The concentration of bacteria required to cause an infection is the infectious dose. For C. difficile this is unknown. However, the prevalence of C. difficile in vegetables in Australia (S.C. Lim et al., J Appl Microbiol accepted for publication) and Europe (Eckert et al., 2013; Bakri et al., 2009) has been found to be relatively high. Although foodborne transmission of CDI is plausible, it has never been proven (Kwon et al., 2016; Limbago et al., 2012). Further studies are required to investigate the
concentration of spores in foods as this could be a better predictor of CDI.

On the other hand, the environment in general may be an important source of CA-CDI (Bauer and Kuijper, 2015; Limbago et al., 2012). This is evident from many studies where various RTs of *C. difficile* have been detected from the environment including so-called hypervirulent strains (RTs 027, 045, 066, 078 and 126) (Alam et al., 2014; Romano et al., 2012). It is possible that environmental contamination with *C. difficile* spores may be a more important source for CA-CDI than food specifically (Kwon et al., 2016; Limbago et al., 2012). The fact that indistinguishable strains of *C. difficile* have been detected from humans, animals, and crops irrigated with manure suggests that *C. difficile* could be acquired from a common source or zoonotically transmitted (Knetsch et al., 2014;
Eckert et al., 2013; Keessen et al., 2013; Marsh et al., 2011).

Although there has been some research on this subject, the epidemiology of CDI in piggeries is still poorly understood. Although calves have been shown to be colonised by \textit{C. difficile}, no study has confirmed disease (Rodriguez-Palacios \textit{et al.}, 2007). A recent study showed that calves receiving antibiotics were twice as likely to shed \textit{C. difficile} as the control group (Magistrali \textit{et al.}, 2015). Some studies have also shown that piglets can be colonised with \textit{C. difficile} without clinical disease although in other piglets develop overt diarrhoea (chapter 4; Weese \textit{et al.}, 2010a). Squire and Riley (2013) suggested that maternal colostral antibodies determine whether a piglet becomes infected or remains as a subclinical shedder. Another study found that there was no association between antibiotic therapy and CDI in 1-day
old piglets (Arruda et al., 2013). This may be true for piglets as colonisation is reported to occur within an hour of farrowing and disease manifests in piglets less than 14 days of age (Keessen et al., 2011a). In captive or farmed wildlife, CDI outbreaks have been associated with antibiotic therapy (Álvarez-Pérez et al., 2014; Jardine et al., 2013; Silva et al., 2013; Thakur et al., 2011) but this remain controversial. The epidemiology of CDI in pigs and other food animals has been complicated by the practices of prophylaxis or metaphylaxis, particularly the latter when whole herds or farrowing sheds of animals are “treated” to prevent disease (McEwen and Fedorka-Cray, 2002). Therefore, increased surveillance of antimicrobial usage in animals is required to try and better understand what is required to prevent C. difficile amplification.

Current measures for preventing C. difficile colonisation and/or infection in pig pens are limited to
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routine infection control such as use of disinfectants.

Although sporicidal disinfectants are used in piggeries, C. difficile spores have been detected in disinfected pig pens (Squire et al., 2011). This is compounded by the fact that water which is contaminated is recycled in many piggeries (Squire et al., 2011). Despite the success of sporicidal disinfectants in stopping or controlling CDI outbreaks in hospitals (Weber et al., 2016; Wilcox et al., 2003) and veterinary clinics (Weese et al., 2003), spores are not always all eradicated even though hospital environments are cleaner than animal sheds (Maillard, 2011). The reason for the failure of sporicidal disinfectants on environmental surfaces has been attributed to insufficient exposure time and not necessarily the concentration (Maillard, 2011). To efficiently manage contamination of pig pens in relation to CDI, additional steps that could break the infection cycle are required. Sporicidal disinfectants capable of preventing sporadic infections or outbreaks of CDI are not
a long-term preventative measure by themselves for endemic infectious agents. It is reasonable to suggest that a combination of sporicidal disinfectants, together with other possible interventions such as with compounds to prevent spore germination, and immunogenic vaccines, could prevent CDI outbreaks.

There are other modalities that may also be worth exploring. The use of probiotics in both medical and veterinary settings is popular as a mode of treatment for gastrointestinal infection (Hill et al., 2014; Weese and Martin, 2011; Parkes et al., 2009). The Food and Agriculture Organisation of the UN and WHO have described probiotics as, “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). Because the normal gut microbiota has inhibitory effects against *C. difficile*, there has been an accelerated interest in the use of
probiotics to prevent CDI (O'Toole and Paoli, 2017; Parkes et al., 2009). In some studies, the use of probiotics in horses and other animals has not been beneficial against CDI (Schoster et al., 2015) and a study evaluating the benefit of a lactobacilli probiotic in piglets was inconclusive (Arruda et al., 2016). However, a multi-strain probiotic consisting of *Lactobacillus murinus*, *L. salivarius* subsp. salivarius, *L. pentosus* and *Pediococcus pentosaceus* reduced faecal shedding of *Salmonella enterica* Serovar Typhimurium in piglets (Casey et al., 2007). Another study using competitive exclusion culture reported a reduction of enterotoxigenic *E. coli* shedding and mortality in neonatal pigs (Genovese et al., 2000). Due to the contradictory therapeutic potential of probiotics, research focusing on understanding the functionality of various host microbiomes as a treatment option for many infectious diseases, including *C. difficile*, has increased (O'Toole and Paoli, 2017; Kim et al., 2017;
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Carlucci *et al.*, 2016; Buffie *et al.*, 2015; Harvey *et al.*, 2006; Parkes *et al.*, 2009; Pillai and Nelson, 2008). Hell *et al.* (2013) suggest that specific probiotics should be developed for a specific pathogen. Apart from preventing CDI, probiotics have been found to have other functions such as immune stimulation and suppression of colonisation, adhesion and invasion of pathogens (Fitzpatrick, 2013; Hell *et al.*, 2013; Parkes *et al.*, 2009).

*L. plantarum* NCIMB 8826 (human saliva isolate) probiotics temporarily colonise the gut in humans for approximately 7 days (Mercenier *et al.*, 2000). The duration of colonisation of various microorganisms used in many probiotics needs to be evaluated in various animal species including pigs. The fact that the half-life of colonisation by probiotic microorganisms in many animal species including pigs is unknown is significant. Another challenge associated with some commercially available
probiotics is that the concentration of viable microorganisms is often lower than recommended by scientific bodies (Hill et al., 2014; Weese and Martin, 2011). A major challenge is that probiotics can be marketed without scientific proof of their claims (Hoffmann et al., 2013). This and many other factors could potentially explain the varied response of probiotics to infectious pathogens including against CDI in various animal species. To try and understand these conflicting results associated with different animal models, it has been suggested that a pig model is used (Heinritz et al., 2013). This is because any success in a pig model with probiotics has the potential to benefit humans because of the similarities in physiology and nutrition between the two species (Heinritz et al., 2013). Future studies should explore the bioavailability of viable microorganisms in probiotics for piglets as this will inform optimal treatment dosage and frequency.
Giving non-toxigenic \textit{C. difficile} strains to piglets (Arruda \textit{et al.}, 2016; Songer \textit{et al.}, 2007) and hamsters (Merrigan \textit{et al.}, 2009) resulted in a reduction in \textit{C. difficile} shedding. The basis of this approach is that non-toxigenic strains of \textit{C. difficile} out-compete toxigenic strains for the same niche in the gastrointestinal tract. However, it is possible that non-toxigenic \textit{C. difficile} can acquire the PaLoc from toxigenic strains and thus become pathogenic (Brouwer \textit{et al.}, 2013). Therefore, non-toxigenic strains of \textit{C. difficile} may not be suitable for treatment or more correctly prevention of CDI. Future studies investigating potential immunotherapy against CDI in livestock are likely to provide a better way of CDI prevention.

\textbf{9.1.1 Significant scholarly contribution of this thesis}

This thesis focused on piggeries as potential reservoirs of \textit{C. difficile} that might be driving the
worldwide increase in CA-CDI and provides action plans to prevent transmission of *C. difficile* from the environment to people. In chapters 4, 5, 6, 7 and 8, the prevalence of *C. difficile* in piglets, the farm environment, retail compost, manure and human biosolids, and lawn was determined. RT 014/020 *C. difficile* was most commonly detected from effluent/soil (20.7%). In Australia, this is the same RT most frequently detected in piggeries (chapter 8, Knight *et al.*, 2015) and hospital settings (Collins *et al.*, 2016). In Western Australia, a piggery which has a well described endemic strain RT 237 (chapter 4) has recently seen the emergence of RT 014/020 (chapter 8). The factors leading to the appearance of RT 014/020 in this piggery are unclear at present although asymptomatic human carriage by farm workers cannot be excluded. Others have reported detecting indistinguishable *C. difficile* strains from pigs and farmers although the direction of transmission was not established
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(Knetsch *et al.*, 2014; Bakker *et al.*, 2010; Norman *et al.*, 2009; Goorhuis *et al.*, 2008). The fact that RTs 014/020 and 237 were recovered from effluent at pig farms (chapters 4, 6, 7 and 8) and from retail compost, manure and human biosolids suggests that transmission of *C. difficile* to pigs and possibly humans could happen through contact with manure. The data presented in chapters 4, 5, 6 and 7 supports the hypothesis that pigs and piggeries are playing a significant role in driving the increase in rates of CA-CDI through contaminated manure. Indeed, detection of clinically relevant toxigenic strains of *C. difficile* from pig faeces, commercially available compost and lawns farmed using contaminated pig manure suggest that contaminated animal manure is the main reason for *C. difficile* dissemination. This hypothesis for *C. difficile* dissemination is supported by a recent study in Australia which showed that *C. difficile* RT 014 from pigs and humans were indistinguishable by WGS (Knight *et al.*, 2014; Bakker *et al.*, 2010; Norman *et al.*, 2009; Goorhuis *et al.*, 2008).
2017). In addition, transmission was thought to have occurred over distances of thousands of kilometres implying other possible vehicles, such as food (Knight et al., 2017). Others have found that residential proximity to swine farms or contacts with livestock were the major risk factors of CA-MRSA (Deiters et al., 2015; Carrel et al., 2014; Casey et al., 2013). Similarly, a study found that *C. difficile* carriage in dogs visiting health care facilities was significantly higher than a control cohort (Lefebvre et al., 2009). The paucity of data on other potential reservoirs of *C. difficile* CA-CDI remains an issue.

Furthermore, a diverse group of RTs including clinically relevant ones was detected from effluent and commercially available manure, compost and lawn (chapters 4, 6 and 7). The detection of clinically relevant RTs and the diversity seen in this study suggests that CDI could be zoonotically acquired from multiple sources.
Without changes in the way animal manure is being used, it is likely that the rates of CA-CDI will keep rising. In Australia, studies investigating *C. difficile* colonisation and CDI in people working at pig farms, composting companies and turf farming industries are needed to determine the risk of disease following human exposure to *C. difficile*. Further research should be directed towards understanding the epidemiology of *C. difficile* in wildlife in Australia, particularly as Australian wildlife being predominantly marsupials may be affected differently to other mammals. This is likely to enhance our understanding of the other potential reservoirs of CA-CDI.

Molecular speciation of *C. difficile* strains in this thesis was performed using ribotyping and toxin gene PCR (chapter 3). Although ribotyping has a low discriminatory power to distinguish between two isolates of the same strain, it is a robust tool for surveillance (Knetsch *et al.*, 2010).
2013). Ribotyping can be used in combination with other highly discriminatory diagnostic tools such as the MLVA or WGS when tracking *C. difficile* outbreaks. Although performing PCR ribotyping is relatively easy, it requires specialised laboratory bench equipment. The development of tools that can accurately diagnose CDI in livestock will be crucial in improving our understanding of the evolving epidemiology of CDI and thus its control. Future studies investigating the circulating strains of *C. difficile* in livestock and indeed humans should use WGS to establish transmission links, and direction of *C. difficile* movement. Although WGS is desirable, it is expensive, has a relatively long turnaround time and is labour intensive, particularly the bioinformatics component, therefore currently limiting its application in routine laboratory surveillance. However, the effectiveness of routine molecular typing in reducing hospital infection
transmission is positively correlated with the frequency of its application (Mutters et al., 2017; Nakaie et al., 2016).

Although most reports about CDI in neonatal pigs outside Australia have been associated with hypervirulent strains of *C. difficile* such as RT 078 (Keessen et al., 2013; Goorhuis et al., 2008; Keel et al., 2007), in this thesis different strains of *C. difficile* that have potential to cause infection and sometimes outbreaks were described in chapters 4 and 5. Some novel toxigenic strains of *C. difficile* were detected including RT QX 450 which was associated with a CDI outbreak in a piggery. Furthermore, *C. difficile* shedding in piglets in Australia was found to be age dependent similar to findings observed outside Australia (Álvarez-Perez et al., 2013; Weese et al., 2010a; Songer, 2004). The most important findings from the cross-sectional study (chapter 4) were that *C. difficile* prevalence in piglets (1-10 days old) was relatively stable
(67.4%) compared to 62% in the earlier study at the same piggery (Squire et al., 2013) and that RT 237 (A-B+CDT+) was again the only strain detected. There was no association between age of sow and C. difficile shedding in their progeny, perhaps because of a small sample size of sows studied (n = 9). Diarrhoeic piglets were more likely to shed C. difficile than non-diarrhoeic and this is significant. Additional work should focus on identifying risk factors such as those associated with the pig production type as this is likely to enhance our understanding of epidemiology CDI in piglets.

The detection of only RT 237 C. difficile in chapter 4 of this thesis was not surprising. This is because other studies have shown that there was less C. difficile diversity in piggeries (Keessen et al., 2010; Avberšek et al., 2009; Keel et al., 2007), compared to horses (Avberšek et al., 2009; Keel et al., 2007) and poultry (Zidaric et al., 2008).
However, these are completely different “production” systems compared to high intensity pig rearing. The apparently stable population of *C. difficile* within piggeries is likely to be influenced by production type and this is often the case for commercial production enterprises. There is strict biosecurity enforcement in the Australian pig industry and livestock movement between enterprises is uncommon. In addition, in Australia, there are often vast distances between piggeries making movement of strains less likely. In contrast, the production of broiler chickens involves hatching of eggs that have been acquired from many different facilities allowing for a mixing of RTs (Leibler *et al.*, 2008). As an aside, however, broiler chickens in Australia are not colonised nor infected with *C. difficile* most likely due to the lack of antimicrobial use in the industry in Australia (Riley, unpublished data). This diversity in pigs may have significant implications for vaccine development. Currently how much cross-
reactivity there might be between strains of \textit{C. difficile} is largely unknown.

Finally, to prevent piglets from colonisation or infection with \textit{C. difficile}, a toxoid vaccine was developed and administered to gilts (chapter 8). This is one of the first studies to investigate the impact of passive immunotransfer from a toxoid vaccine on \textit{C. difficile} shedding in neonatal pigs in a commercial piggery setting. The findings from this study showed that piglets born from vaccinated gilts were less likely to shed \textit{C. difficile} compared to control progeny. Generally, there was also a decline in the overall prevalence reported in this study compared to the previous studies on this farm (Squire \textit{et al.}, 2013), suggesting that other interventions such as the use of sporicidal disinfectants and good hygiene in the pig sheds were significant. The use of sporicidal disinfectants and good hygiene in a piggery are important preventative
measures to end a CDI outbreak in a piggery as shown in chapter 5.

The toxoid vaccine formulated using *C. difficile* RTs 014/020 performed better than the one from RT 237. The difference observed could partly be attributable to RT 014/020 having TcdA which is more immunogenic than TcdB (Leuzzi *et al.* 2013; Permpoonpattana *et al.*, 2011). However, it is unclear if different strains of *C. difficile* could confer cross-protection. Further studies investigating the impact of specific antigens in the vaccine and studies investigating the dosage and frequency of vaccinations are required. Furthermore, studies investigating the mechanism by which specific antigens in a toxoid vaccine enhances colonisation resistance of pathogenic microorganisms will advance our understanding of vaccines. Future work should also examine vaccine
response in older pigs as this will help optimise vaccination strategy in piggeries.

Vaccination of livestock is a well-known control and prevention strategy against zoonotically transmitted infectious pathogens (Monath, 2013; Cybulski et al., 2009). For example, vaccinating livestock against *Bacillus anthracis* infection, another spore-forming bacterium, reduces morbidity and mortality in humans (Cybulski et al., 2009). The most plausible method of control of *C. difficile* in animals and the environment is by employing a vaccination strategy in animals. The findings in this thesis are in agreement with others investigating *C. difficile* vaccine therapy (Hutton et al., 2017).

9.2 FUTURE STUDIES

The reservoirs which sustain the *C. difficile* transmission cycle in the community are poorly understood. Risk based surveillance studies are needed to
establish the likely sources and direction of *C. difficile* transmission. This is because risk based surveillance is the most efficient way to identify a subpopulation of people that are likely subclinical or transient shedders of *C. difficile* in the community. The subpopulations which have not been studied in Australia include people employed by pig farms, abattoirs, the composting industry and turf farms as they are likely to come in contact with contaminated animal manure. Although the employees may not be directly at-risk of CDI if they are healthy, they may transiently shed *C. difficile* spores and potentially contaminate their households. Another subpopulation that needs surveying are households where people working in high risk industries reside, to determine if this could predict CA-CDI. Further, studies to establish the possible link between CA-CDI and lifestyle activities such as the use of manure in their home-grown vegetable gardens and lawns are needed. Finally, studies that target house pets
which visit public spaces in Australia may help determine potential transmission pathways of *C. difficile* from the environment. These studies are important as they will inform the direction of CA-CDI transmission pathways and this will help in the design of control measures.

### 9.3 CONCLUSION

*C. difficile* is an important pathogen for humans (Lessa *et al.*, 2015) and animals (Songer and Anderson, 2006). The epidemiology of CDI has greatly evolved in the past 40 years. The increase in CA-CDI worldwide is concerning but the reservoirs of CDI remains to be identified. Correspondingly, there has been an increase in clinically relevant strains of *C. difficile* detected from the environment. The findings in this thesis support the hypothesis that pig manure is responsible for much of the increasing rates of CA-CDI. Therefore, monitoring of *C.
difficile strains from animal sources which are clinically relevant in the hospital settings is urgently needed.

In this thesis, new strains of *C. difficile* capable of causing CDI outbreak were detected in piggeries. *C. difficile* strains detected from pig manure or human biosolids and the environment were indistinguishable by ribotyping. The transmission pathway of *C. difficile* from pig manure should be confirmed by WGS. Surveillance of animal populations for *C. difficile* is needed to clarify the relationship between livestock-associated CDI, contamination of food or the environment, and human CDI. Ultimately, the promotion of a dialogue between physicians, veterinarians, and food scientists in the development of a One Health approach will be essential to control CDI.

The major challenge in treating CDI patients with antibiotics is the high recurrence rate (up to 30%). The use
of a vaccine is unlikely to disturb gut microbiota, therefore, less likely to cause CDI recurrence. In livestock, there is no standard treatment for CDI. Reduced *C. difficile* colonisation in progeny of vaccinated gilts could result in reduced faecal shedding; therefore, less environmental contamination with *C. difficile* spores, thus possibly less CA-CDI. A combination of vaccination and sporicidal disinfection of piggeries is required if outbreaks of CDI are to be prevented. Some research should be directed towards identifying immunogenic antigens for CDI. Furthermore, research directed towards understanding the functionality of various host microbiomes as a treatment option for many infectious diseases, including CDI, is needed.
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APPENDICES

APPENDIX 1. PAPERS
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Review Article

*Clostridium difficile* Infection in Production Animals and Avian Species: A Review

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Abstract

*Clostridium difficile* is the leading cause of antibiotic-associated diarrhea and colitis in hospitalized humans. Recently, *C. difficile* infection (CDI) has been increasingly recognized as a cause of neonatal enteritis in food animals such as pigs, resulting in stunted growth, delays in weaning, and mortality, as well as colitis in large birds such as ostriches. *C. difficile* is a strictly anaerobe spore-forming bacterium, which produces two toxins A (TcdA) and B (TcdB) as its main virulence factors. The majority of strains isolated from animals produce an additional binary toxin (*C. difficile* transferase) that is associated with increased virulence. *C. difficile* is ubiquitous in the environment and has a wide host range. This review summarizes the epidemiology, clinical presentations, risk factors, and laboratory diagnosis of CDI in animals. Increased awareness by veterinarians and animal owners of the significance of clinical disease caused by *C. difficile* in livestock and avians is needed. Finally, this review provides an overview on methods for controlling environmental contamination and potential therapeutics available.

Keywords: *Clostridium difficile* infection, production animals, avian species, laboratory diagnostics, clinical impact, environmental control

Introduction

*C. difficile* is a strictly anaerobic Gram-positive bacillus that is the leading cause of antibiotic-associated diarrhea in humans, emerging as a significant cause of gastrointestinal infection in animals. An important property of *C. difficile* is its ability to form highly resistant spores that survive for a long time (~5 months) on contaminated surfaces (Kramer et al., 2006). *C. difficile* infection (CDI) is transmitted by the fecal–oral route through the ingestion of these spores. The clinical presentation of CDI in humans and livestock varies from asymptomatic/subclinical carriage to mild diarrhea, severe diarrhea, and sometimes, life-threatening pseudomembranous colitis in humans (Hurley and Nguyen, 2002; Keesies et al., 2011). Disease arises due to the activity of two exotoxins TcdA and TcdB that are expressed in the gut by toxigenic strains of *C. difficile*. The presence of toxin receptors is required for toxin uptake by colonocytes (Kee and Songer, 2006). Different animal species vary in the type of toxin receptors present in the gut (Kee and Songer, 2006), but this does not correlate with disease severity. In addition, some *C. difficile* strains produce a binary toxin (*C. difficile* transferase, CDT) that has been associated with enhanced virulence in human disease (Schwan et al., 2009). Some strains also differ in nucleic acid composition of tcdC, a toxin regulatory gene, which may result in an increased toxin production (Merrigan et al., 2010). However, the significance of these latter features (CDT and tcdC) remains to be debated (Carter et al., 2011; Goldenberg and French, 2011).

The incidence and severity of CDI in humans have increased over recent years. In Europe and North America, this change has been attributed to the emergence since the early 2000s of a "hypervirulent" strain of *C. difficile*, ribotype 027 (NAP1/BD) which is non-encapsulated resistant (Loo et al., 2003). In addition, RT 078, a similar strain predominantly responsible for human infection in Europe (Goebbels et al., 2008; Bauer et al., 2011). *C. difficile* RT 027 and RT 078 both produce all three toxins, TcdA, TcdB, and CDT (Merrigan et al., 2010; Bauer et al., 2011), and some strains have reduced susceptibility to various antimicrobics used for treatment such as metronidazole (Álvarez-Pérez et al., 2011).
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et al., 2013). Countries outside Europe and North America, including Australia, have seen a similar, but more recent rise in the incidence of CDI (Shimings et al., 2014). Furthermore, CDI has been reported in people who have not been exposed to traditional CDI risk factors such as antibiotics, hospitalization, and living in an aged care facility (Khamna et al., 2013).

Some strains of C. difficile recovered from different animal species and humans are indistinguishable by conventional molecular typing techniques, including polymerase chain reaction (PCR) ribotyping, multilocus sequence typing, and multilocus variable tandem repeat analysis (Goethais et al., 2008; Balcker et al., 2010; Marsh et al., 2011; Jancetic et al., 2014; Kaestch et al., 2014). More recently, this genetic overlap was further confirmed by whole genome sequencing and core genome single-nucleotide polymorphism typing, which showed that pigs and pig farmers were colonized by indistinguishable strains of C. difficile (Kaestch et al., 2014). These findings have raised concerns that cases of CDI could arise by zoonotic transmission. Transmission could occur by direct contact with live animals or their environment (Keesen et al., 2013) or during or after slaughter, since C. difficile has been isolated from animals at slaughterhouses (Knight et al., 2013) and also from retail meat (Honore et al., 2012). The possibility that meat and meat products could play a role in human CDI although zoonotic transmission has not yet been conclusively proven. In addition, contamination of predominantly root vegetables has been reported to a lesser extent, despite early evidence of these foods as a potential source of C. difficile (Ali Safi and Buzari, 1996).

Pathogenesis of and Host Susceptibility to CDI

C. difficile causes disease through expression of two main virulence factors, the toxins TcdA and TcdB (Mertens et al., 2010). The corresponding genes, tcdA and tcdB, are located on the chromosome alongside three accessory genes tcdC, tcdR, and tcdB that together form a 196-kb pathogenicity locus (PaLoc) (Britton and Young, 2014). Most toxigenic C. difficile isolates possess tcdA and tcdB, however, some variant isolates do not produce TcdA (Squire et al., 2013) and others are missing the PaLoc altogether, instead having a 115 bp insertion. TcdA has been described as an enteroxin because it causes exudative colitis. TcdB is cytotoxic and causes epithelial cell collapse, apoptosis, and cell death. TcdA and TcdB are both large molecular weight toxins (308 and 209 kDa, respectively) belonging to the large clostridial toxin family (Jund and Adolfs, 2008). Some strains of C. difficile, particularly those associated with livestock, produce binary toxin, the function of which remains speculative even though it has been associated with so-called “hypervirulence” (Ruehne et al., 2014). It is thought to enhance microtubule protrusion from gut epithelial cells, leading to formation of a network of mesh around the bacterial cells resulting in adhesion (Schwan et al., 2009).

As in humans, the intestinal microbiota is likely to play an important role in the susceptibility of animals to CDI. The intestinal microbiota prevents overgrowth of C. difficile and other enteric pathogens by competing for nutrition or acting as a mechanical blockade of enterocytes in a process that is often referred to as colonization resistance (Thienot and Young, 2015). Furthermore, the gut microbiome plays a role in the deconjugation of tauroursodeoxycholate to deoxycholate, a key component in inhibiting spore germination in the small intestine and ceca (Gil et al., 2010; Britton and Young, 2014), and the bioconversion of secondary bile salts such as deoxycholate, which in turn vegetative cell growth in the colon (Gil et al., 2010; Theriot and Young, 2015). When the commensal intestinal microbiota has been disrupted, there is an increased production of cholate from bile salts that promotes spore germination (Gil et al., 2010). This was recently demonstrated in a murine model and expanded to human studies. Mice treated with clindamycin developed an altered gut microbiota with a reduced ability to convert primary bile salts into secondary bile salts that correlated with the susceptibility to infection by C. difficile (Buttle et al., 2015).

Disruption of the normal gut microbiota by antibiotics is the best known predisposing mechanism leading to C. difficile colonization of the large intestine (Theriot and Young, 2015). In particular, later generation cephalosporins, penicillins, carbapenems, clindamycin, trimethoprim/sulfonamides, and fluoroquinolones (in the United States) have been associated with greater risk (Shimings and Riley, 2014). Recently, an epidemiological study in real calves found an association between antibiotic exposure and C. difficile shedding (Magistris et al., 2015), however, the association between antibiotic exposure and CDI has not been commonly reported in livestock as animals can develop diarrhea associated with antibiotic therapy that is unrelated to CDI. Animals that rely on fermentation in the hindgut, known as pseudomonaecostrophic animals (hamsters, horses, guinea pigs, and rabbits), are highly dependent upon commensal bacterial populations for digestion of fiber. Interestingly, most pseudomonaecostrophic animals are more susceptible to severe colitis and death associated with CDI compared with true monogastric animals (Keesel and Songer, 2006). The evidence so far suggests that antibiotics may play a significant role in precipitating colonization of livestock by C. difficile and antibiotics may escalate CDI in animals without clinical disease. It is possible that the impact of antibiotics on the normal gut microbiota for pseudomonaecostrophic animals such as hamsters is more sudden than for true monogastric animals.

Clinical Signs of CDI

Intestinal colonization with C. difficile, and disease, is common in neonatal piglets within 7 days of farrowing (Norman et al., 2009; Weese et al., 2010; Monno et al., 2016). The most common clinical sign for CDI in livestock is diarrhea, which may be acute or chronic; however, many neonatal animals remains without clinical disease probably due to acquired colostral immunity (Squire and Riley, 2013). CDI may be self-limiting, intermittent, or continuous in nature. Piglets infected by C. difficile may present with a yellow or watery, nonhemorrhagic diarrhea. Orabch chicks often experience anorexia, weight loss (Bhuvanagiri, 2003), acute diarrhea, and sudden death within 3 days (Frazier et al., 1993; Cooper et al., 2015). CDI should be considered in the differential diagnosis in poultry, as one of many diarrhoea-causing enteropathogens (Cooper et al., 2015). Obstruction and constipation, scrotal edema, and dyspnea occur uncommonly in piglets (Steele et al., 2010). In humans, CDI occurs in older people as noctones are thought not to have toxin
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receptors. Clinical presentation in humans is similar to ani-
mals and patients may present with malaise, abdominal pain,
nausea, anorexia, watery diarrhea, low-grade fever, and
peripheral leukocytosis (Shelley and Nguyen, 2002).

Laboratory Diagnosis of CDI

The diagnostic tests available for detection of C. difficile in
humans can broadly be classified into three categories
(Croebbe et al., 2016). First, there are tests such as toxigenic
culture to isolate toxins-producing C. difficile, second, tests
that detect C. difficile products such as glutamate dehydro-
genase (GDN) and toxins A and B; and last, tests that
detects C. difficile genes.

Although toxigenic culture for C. difficile is labor-intensive
with a long turnaround time, it is still regarded as one of the
gold standards for diagnosis of human CDI (Croebbe et al.,
2016). Toxigenic culture involves isolating C. difficile from
feces by using selective culture media and determining if the
isolate is toxin producing (Barnham and Carroll, 2013; Lund
and Peck, 2015). The methods for isolating C. difficile from
feces either by direct plating on selective media or selec-
tive enrichment broth, followed by plating on selective
media, have been extensively reported (Lund and Peck, 2015).
By direct culture, chromogenic agar (BioMérieux, Marcy
l’Etoile, France) gives a shorter turnaround time (21h) in
comparing to preenriched cycloserine-cefoxitin-fructose
agar with added sodium taurocholate (Caneon et al., 2013).
Pre-
sumptive C. difficile colonies on blood agar are identified
by chartruese fluorescence under UV light (< 360 nm wave-
length), colonial morphology (ground glass appearance),
and horse dandr color. Identification of uncertain isolates can
be achieved by Gram staining and detection of c-proline
anti-
body peptide (Knight et al., 2014), or more commonly, re-
cently, MALDI-TOF-MS (Kim et al., 2016).

Other assays for diagnosing CDI include commercially
available enzyme immunoassays (EIA) (Croebbe et al.,
2016). Despite the limitations associated with these tests
(Tahhan et al., 2010; Barnham and Carroll, 2013), they are
popular in laboratories because they are easy to use, relatively
cheap, and have a short turnaround time. Some ELISA are
designed to detect GDH in feces, the “common antigen” on
C. difficile strains, in addition to TcdA and TcdB, even though
there are reports of reduced sensitivity for these tests (Ten-
over et al., 2010). EIA that target GDH were initially said to
have a higher sensitivity than those that only target TcdA or
TcdB (Croebbe et al., 2016). Furthermore, some studies have
suggested that EIA vary in their ability to detect certain RIs
of C. difficile in human disease (Tennessee et al., 2011).

In addition, while there is no correlation between strain
type, toxin in feces, and disease severity both in human and
animals (Yang et al., 2002), a large study by Franke et al.
(2013) showed that the presence of toxins in feces predicted
poorer outcomes in humans. Although EIA that target GDH
and PCR methods that detect toxin genes have relatively high
sensitivity, they lack specificity for disease. Therefore, a two-
step diagnostic algorithm has been suggested that involves
retesting positive samples with a toxin EIA, which increases
specificity and positive predictive value. A complete diag-
nosis of CDI in pigs or indeed other animal species will
include a clinical history, toxigenic culture of C. difficile, and
detection of free toxins in feces or detection of toxin genes or
enzyme in isolates. Further, the European Society for Clinical
Microbiology and Infectious Diseases (ESCMID) recommends
testing feces that are not formed and are negative for
other enteropathogens (Croebbe et al., 2016). Currently, no
single standalone diagnostic test for CDI with suitable sen-
sitivity and specificity is available (Bloomfield and Ridley,
2016).

Most of the CDI diagnostic tests available on the market
have been validated for human medicine and they perform
suboptimally on animal samples. For example, some human
commercial molecular diagnostic assays showed low sensi-
tivity in the range of 25% to 50% on animal samples (Knight
et al., 2014). The reason for suboptimal performance of
molecular diagnostic tools in animal samples is unclear and
requires further research. Better diagnostic tools are crucial
for the early detection of many veterinary pathogens, in-
cluding C. difficile.

Epidemiology of C. difficile in Production Animals

Although diarrhea is common in neonatal livestock, there
are potentially many pathogens that may be involved apart
from C. difficile; such as enterotoxigenic Escherichia coli,
C. perfringens, Coccidia sp., Cryptosporidium sp., Giardia
sp., and rotovirus, among others. In Australia (Squire et al.,
2013), Europe, and North America (Yang et al., 2002; Hammitt
et al., 2008), it is rare for other pathogens to be present
with C. difficile in fecal samples, suggesting that C.
difficile alone was associated with diarrhea. However,
the importance of screening for other pathogens when un-
dertaking C. difficile surveys should not be ignored.

C. difficile in Pigs

The earliest published report of natural infection with C.
difficile in swine was that of two pigs diagnosed with
enterocolitis in the 1980s (Jones et al., 1983). A decade later,
there was a major outbreak of CDI at a farm in Canada with
a weekly mortality rate in the range 7% to 30% in pigs aged
1–14 days (Waters et al., 1998). CDI was isolated from
feces and toxins were detected, however, strain types were
not determined. Postmortem findings consistent with CDI,
such as mesenteric edema and typhlocolitis, were common.
The significance of CDI in pigs became prominent after a
12-year surveillance study of enteric pathogens in neonatal
pigs at the Iowa Veterinary Hospital. This study showed a
decline in the relative frequency of traditional enteric path-
ogens such as transmissible gastroenteritis virus, E. coli, and
C. perfringens type C from 70% to 21%, and an increase in C.
difficile (55%) (Yang et al., 2002).

The prevalence of C. difficile in pigs aged between 1 and
2 weeks has been reported in the range of 20% to nearly 100%
in asymptomatic pigs (Keel and Sengier, 2006; Weese
et al., 2010b; Moomo et al., 2016). This high prevalence is
followed by a gradual decline as pigs grow older (Norman
et al., 2009; Weese et al., 2010b; Moomo et al., 2016). Pigs
infected with C. difficile (diarrhetic) can be underweight by
10–15% and also have an extended weaning time (Sonier
and Ural, 2005). Squire et al. (2013) reported a mortality rate
of 14% in pigs. Even though sporadic outbreaks of CDI in
adult pigs are rare, they can have significant conse-
quences because adult pigs can also die from infection (Kiss
and Billet, 2005).
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**Risk factors for CDI in pigs**

Few studies have adequately investigated the risk factors contributing to CDI in pigs. Pig age is the most commonly reported factor associated with risk of CDI. The prevalence of *C. difficile* is typically highest in piglets up to 7 days post-partum (Norman et al., 2009; Wesser et al., 2010; Moono et al., 2016). Infection is likely to be acquired from the surrounding environment rather than by vertical transmission since piglets born by caesarean section were culture negative (Hopman et al., 2011). A longitudinal study in the United States found higher *C. difficile* prevalence in cooler months (16.2%) than in warmer months (10.3%) in a vertically integrated pig farm (Norman et al., 2009). Whether the seasonal variation in CDI seen in swine in North America is due to temperature, humidity, or other seasonal factors and whether this impacts exposure or host susceptibility is unclear. In addition, airborne dispersal of *C. difficile* spores in a pig barn has been reported (Keesiers et al., 2011).

Vermicide may play a role in the spread of *C. difficile* on pig farms. A survey on a pig farm in The Netherlands was undertaken to determine whether mice (*Mus musculus domesticus*) were competent vectors for *C. difficile* (Burt et al., 2012). Mice on the farm were trapped and their skin, muscles, and gut contents aseptically sampled for *C. difficile*. In addition, dead insects (dead flies, lesser house flies, and yellow meal worms) and birds were also sampled. The external body surface of mice had a culture prevalence rate of 51-66% compared to 8% for the gastrointestinal contents, with the predominant strain of *C. difficile* being RT 078. Although Burt et al. (2012) did not sample pigs, the finding of RT 078 in vermin is significant because it is a well-established animal pathogen. Given that the contamination rate of the body surfaces was higher than the gut, mice may be more likely to spread *C. difficile* mechanically in the environment than through the fecal route. The prevalence of *C. difficile* in wild bird droppings was 4%, in dead sparrows 66%, and in various insects 56-100% (Burt et al., 2012). *C. difficile* has since been isolated from urban rats, further highlighting the role vermin could play in dissemination of *C. difficile* in the environment (Hinneworth et al., 2014). Furthermore, a recent study showed that raccoons could play a role in *C. difficile* transmission at pig farms and the environment (Bondo et al., 2015).

**C. difficile in Cattle**

*C. difficile* was first isolated from cattle in the early 1980s. Preventing neonatal calf enterritis is a common problem in the cattle industry (Gunn, 2003). Although there are many pathogens associated with neonatal calf enteritis, *C. difficile* was only described as a potential causative agent in the early 2000s (Hammitt et al., 2008). In addition, a study found a poor recovery rate of pathogens from feces of diarrheic calves; 25% to 45% samples did not yield any pathogen (Gunn, 2003). This study was limited by the small number of pathogens covered in the surveillance program and, in particular, there was no *C. difficile* detection protocol in place. Using culture, Rodriguez-Palacios et al. (2006) reported a *C. difficile* prevalence rate of 7.6% (13/144) in diarrheic calves and 1.5% (203/1344) in control calves. Toxins were more likely to be detected in diarrheic calves, 39.6% (57/144) compared to 20.9% (20/100) in controls. In a subsequent study, Rodriguez-Palacios et al. (2007b) did not find an association between *C. difficile* colonization in calves and disease. Hammitt et al. (2008) reported a *C. difficile* prevalence of 25.3% (6/253) in feces of diarrheic calves compared to non diarrheic calves, 13% (7/53). Furthermore, 22.9% (58/253) of specimens from diarrheic calves were toxin positive compared to 30.2% (16/53) from non diarrheic calves (Hammitt et al., 2008). Although idiopathic enteritis in calves aged 1–14 days is well described, few studies have screened for *C. difficile* and those that did failed to find a correlation between *C. difficile* colonization and disease.

In the dairy industry, male calves are considered surplus and used for veal production. They are either slaughtered quite young (<4 weeks) or kept for ~6 months. Longitudinal studies of veal calves showed that young animals were colonized soon after birth, with the prevalence gradually declining as the calves grew older (Costa et al., 2011; Zidarić et al., 2012; Honser et al., 2012; Magistrati et al., 2015). Zidarić et al. (2012) reported a great diversity of *C. difficile* strains in veal calves (RTs 078, 126, 012, 045, 010, and 033) similar to Costa et al. (2011), with this diversity diminishing as they grew older. A cross-sectional study of 7-day-old veal calves conducted in Australia found a *C. difficile* prevalence of 96% with three predominant RTs (126, 033, and 127) (Knight et al., 2013). These RTs belong to clade 5 of *C. difficile*, as do RTs 078 and 237, and are frequently isolated from livestock and occasionally from human cases of CDI (Magistrati et al., 2015; Tsai et al., 2016). The high prevalence of *C. difficile* in calves could increase the risk of meat contamination at the abattoir.

**Risk factors for CDI in cattle**

Potentially risk factors for CDI in cattle include younger age and antibiotic use. Magistrati et al. (2015) found that veal calves aged 13–28 days were twice as likely to shed *C. difficile* than those aged 36–45 days (odds ratio 4.57 vs. 2.79). Elsewhere, calves reached a peak of *C. difficile* shedding by at least 14–18 days of age (Costa et al., 2011; Zidarić et al., 2012). Antimicrobial use appears to be a common practice in veal production in Europe (Zidarić et al., 2012; Magistrati et al., 2015) and United States (Costa et al., 2011). The use of multiple antimicrobials, or polymyxin E, or a beta-lactam antibiotic was highly associated with *C. difficile* shedding in veal calves (odds ratio 5.83) (Magistrati et al., 2015). Interestingly, Costa et al. (2011) in the United States demonstrated no association between *C. difficile* shedding by calves and housing type, however, this requires further investigation as production systems for veal calves vary immensely within and between countries.

**C. difficile in Goats and Sheep**

Although there are few studies on the prevalence of *C. difficile* in sheep and goats (0–8.5%) (Knight and Riley, 2013; Avberlek et al., 2015; Rodriguez et al., 2016), the available literature does not suggest that they pose a major risk of CDI in humans.

**C. difficile in Farmed Birds**

Prevalence studies show that poultry can be colonized with *C. difficile* (Simango, 2006; Simango and Mwakiramila, 2007).
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2008). In a cross-sectional study of poultry in Zimbabwe, the *C. difficile* culture prevalence was reported as 29% and, of the strains isolated, 90% were toxigenic (Simango and Mwakumbudza, 2008). However, these prevalence studies did not state the age of the chickens sampled and neither was there evidence of enteritis in the poultry. In addition, *C. difficile* has been reported in captive ostriches (Frazier et al., 1993; Shiva-prasad, 2003).

Risk factors for *CDI* in farmed birds

*CDI* in avian species appears similar to the manifestation in other animal species (Frazier et al., 1993; Shiva-prasad, 2003). In one study, 19-day-old captive ostrich chicks treated with amikacin, piperacillin, and enrofloxacain were diagnosed with *CDI* (Shiva-prasad, 2003). In another study, an outbreak of *CDI* in 9-day-old ostrich chicks treated with sulfaquinacine in North America was reported (Frazier et al., 1993). This is consistent with literature reporting the association of antibiotic therapy and *CDI* in humans (Simango and Riley, 2014).

Although *C. difficile* colonization has been reported in poultry (Simango, 2006; Simango and Mwakumbudza, 2008), there are no reports of enteritis associated with *C. difficile* colonization. Cephalosporins, which have been associated with *CDI* amplification in humans, are widely used in poultry production in North America (Webster, 2009). The relationship between exposure to antibiotics in poultry and *C. difficile* shedding needs further investigation.

C. difficile in Food Animals and Foodborne *CDI*

The majority of research about *C. difficile* in food has been conducted on meat and meat by-products, particularly, beef, poultry, and pork (Senger et al., 2009; Weese et al., 2010a; Rahimi et al., 2014; Varnhuyse et al., 2014). In these studies, the prevalence of strains of *C. difficile* associated with illness in hospitalized patients varied from high, predominantly in North America, to lower figures, usually in Europe. The common *RT*s from animal studies have been found in meat, suggesting that the contamination is occurring somewhere during processing, rather than from another external source (Rodriguez-Palacios et al., 2014). Contamination of meat likely results from gut content spillage during evisceration or perhaps accumulation of spores within abattoir environment (Housner et al., 2012). However, the data on *C. difficile* are limited within the abattoir environment. A recent study found high counts of *C. difficile* in 3 to 7-day-old veal calves with a median concentration of 2.5 x 10^9 cfu/ml (Knight et al., 2016). Furthermore, 16.7% (9/55) of the carcass samples were contaminated with the median count of *C. difficile* (5 x 10^9 cfu/ml) (Knight et al., 2016), suggesting that the abattoir environment could contribute to contamination of meat with *C. difficile* spores.

In North America (Varnhuyse et al., 2014) and elsewhere (Rahimi et al., 2014), the most prevalent *C. difficile* strain detected in meat is *RT* 078, although earlier studies highlighted significant levels of *RT* 027 (Rodriguez-Palacios et al., 2007a; Mardis et al., 2011). In Australia, harvesting meat from neonatal animals has been identified as a potential risk for community-associated *CDI* (Squire and Riley, 2013). The prevalence of *C. difficile* in production animals declines as they age (Knight et al., 2013) and meat from older animals may pose a minimal risk. This conclusion is supported by a recent study coordinated by the US Centers for Disease Control and Prevention showing no contamination of meat from adult animals (Lambgo et al., 2012). Nonetheless, future research should target the abattoir environment as it has great potential for risk reduction in the food chain with, for example, comprehensive disinfection protocols.

The use of effluent from animals on crops could contaminate vegetables (Squire and Riley, 2013). At Safe and Broyer (1996) detected *C. difficile* in raw vegetables 20 years ago. In 2009, *C. difficile* was detected in ready-to-eat organic and nonorganic salads in Scotland (Bakri et al., 2009). More recently, *C. difficile* has been detected in nonroot vegetables such as lettuce, green peppers and eggplant (Eckert et al., 2011; Rodriguez-Palacios et al., 2014). The overall prevalence of *C. difficile* in vegetables was reported as being up to 7.5% (Rodriguez-Palacios et al., 2014). The reason for the variations in *C. difficile* prevalence in vegetables is unclear, although differences in culture methods may be an important contributor.

The infectious dose and host factors are critical for disease manifestation in susceptible hosts. The infectious dose of *C. difficile* for humans is unknown and *CDI* is complicated further by the requirement for an insult to the gut microflora to occur before exposure. In addition, the frequency and quantity of contaminated food ingested might be a higher risk than the prevalence of *C. difficile* in food, and even low levels of contamination may be sufficient to cause CVD. Last, to address potential confounders such as laboratory contamination with *C. difficile* (March, 2013), highly discriminatory fingerprinting techniques like whole genome sequencing should be used in future studies.

Control of *C. difficile* in the Veterinary Environment

*C. difficile* spores can persist in the environment for more than 5 months (Kramer et al., 2006). Like other pathogenic organisms, lower temperatures (4°C), high humidity, and quantity of inoculum have been suggested as potential causes of persistence (Kramer et al., 2006). Although still controversial, some studies have shown that epidemic strains of *C. difficile* have a higher sporulation capacity than nonepidemic strains and may persist in the environment longer (Merrigan et al., 2010). However, a study conducted by Robinson et al. (2014) did not find a difference in the sporulation capacity between hypervirulent and nonhypervirulent strains. The thymidylate synthase gene in *RT* 027 strains could confer a growth advantage for its competitive fitness (Robinson et al., 2014), however, the majority of factors that enhance fitness among epidemic strains of *C. difficile* are unknown.

In the United States, Norman et al. (2003) reported *C. difficile* from human and swine composite sewage samples from closed integrated human and swine populations and a study in Australia found *C. difficile* in treated pig effluent (Squire et al., 2011). There is a high likelihood that animal and human effluent can contribute to environmental contamination. Studies investigating the efficacy of disinfectants and treatment regimens for effluent in livestock operations to achieve better control of *CDI* are needed.

Many humans treated with antibiotics for *CDI* experience a recurrence of infection and this has accelerated the need to identify alternative treatment regimens, including therapy that uses fecal microbiota transplantation (Britton and
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Young, 2014). In a recent study, Baffo et al. (2015) demonstrated that there is a specific microbiome that assists colonization resistance against infection by toxigenic strains of C. difficile than those parenterally fed. Kim et al. (2018) showed that tigecycline-altered microbiota balance of glutoclofobiotic pigs (increased Proteobacteria and reduced Firmicutes), but this did not predispose pigs to CDD. In contrast, others have found that mice treated with tigecycline or baritones with clindamycin were susceptible to CDD, despite showing a similar shift in microbiota to pigs (Peeters et al., 2012; Basisit et al., 2014). A recent study using metagenomics showed that the underlying ecological dynamics of gut microbiome (i.e., intra- and interspecies) communities are independent of host influence (Bahnh et al., 2016). Overall, it appears that no single community of microbiota determines the mechanism by which the gut provides colonization resistance (Theriot and Young, 2013), however, determining the beneficial components in the microbiota of production animals may be important.

There have also been studies of the potential benefits of probiotics (Schafer et al., 2015; Arrueta et al., 2016), although they showed varied performance against CDD in animals and humans (Collado et al., 2005). In addition, vaccines for humans are at various stages of development, although none is currently available for livestock.

Conclusions

C. difficile is an important pathogen of humans and animals. The fact that indistinguishable strains of C. difficile have been detected from humans, animals, and crops irrigated with manure suggests that C. difficile could be acquired from a common source or zoonotically transmitted. The development of tools that can accurately diagnose CDD in livestock will be crucial in improving our understanding of the evolving epidemiology of CDD and thus its control. However, the most important issue is likely to be the misuse of antimicrobials in production animals that is driving the amplification of C. difficile. Some research should be directed toward understanding the functionality of various host microbiomes as a treatment option for many infectious diseases, including C. difficile. In addition, misuse of antibiotics used on crops should be screened for C. difficile and appropriately treated to prevent community-acquired CDD. Surveillance of animal populations for C. difficile is needed to clarify the relationship between livestock-associated CDD, contamination of food or the environment, and human CDD. Sophisticated molecular techniques involving whole-genome sequencing will be required to prove these relationships. Ultimately, the promotion of a dialogue between physicians, veterinarians, and food scientists in the development of a One Health approach will be essential to control CDD.

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Persistence of Clostridium difficile RT 237 infection in a Western Australian piggery

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Clostridium difficile is commonly associated with healthcare-related infections in humans, and is an emerging pathogen in food animal species. There is potential for transmission of C. difficile from animals or animal products to humans. This study aimed to determine if C. difficile RT 237 had persisted in a Western Australian piggery or if there had been a temporal change in C. difficile diversity. C. difficile carriage in faeces without diarrhoea was investigated, as was the acquisition of C. difficile over time using colon surveys. Rectal swabs were obtained from pigs aged 1–10 days to determine prevalence of C. difficile carriage and samples were obtained from 20 pigs on days 1, 2, 7, 13, 20, and 42 of life to determine duration of shedding. Isolation of C. difficile from faeces was achieved by selective enrichment culture. All isolates were characterized by standard molecular typing. Antimicrobial susceptibility testing was performed on selected isolates (n = 29). Strain type was more likely to shed C. difficile than the non-diseased (p = 0.0015). In the cohort study, C. difficile was isolated from 46% samples on day 1, 50% on day 7, 20% on day 13, and 0% on days 20 and 42. All isolates were RT 237 and no antimicrobial resistance was detected. The decline of shedding of C. difficile to zero has public health implications because suckler age pigs have a low likelihood of spreading C. difficile to consumers via pig meat.

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1. Introduction

Clostridium difficile is a Gram positive, strictly anaerobic, spore forming bacterium commonly associated with healthcare-related infections [1]. C. difficile is responsible for 20% of all antibiotic-associated diarrhoea and colitis in humans [1]. The genome encodes two toxins, A (an enterotoxin) and B (a cytotoxin), which are the main virulence factors [2]. Some strains produce a third unrelated toxin, an ADP-ribose transferase (DNA toxin); the exact role for which is yet to be determined – although some studies suggest that it contributes to disease severity [3].

C. difficile is an emerging pathogen in food animals that has been recovered from the gastrointestinal tracts of multiple production animal species [2]. Piglets are colonized soon after birth, generally within 1–7 days [4]. Colonization is most common in younger piglets, with older pigs being culture-negative by 2 months of age [6]. Like other perineal enteric pathogens, C. difficile has been isolated from both non-diseased piglets and those with clinical diarrhoea [2, 3]. Strain type A and B, or just B alone, have been detected in both diarrhoeic and non-diarrhoeic piglets [7]. This suggests that several other factors are important in the manifestation of disease [3]. Infected piglets may succumb to diarrhoea and mortality rates of up to 50% have been reported in some outbreaks. Those that survive can be underweight by 10%–15%, which can delay weaning [8] and may affect profitability of pig farms.

Outbreaks of C. difficile in pig herds, and also humans, have been reported frequently since the early 2000s [9, 10]. Of particular interest was the rise in incidence of a so-called "hypervirulent" strain PCR ribotype (RT) 027 (also known as NAP1/BI, initially in North America and later in Europe) [10]. This coincided with C. difficile outbreaks in animals, although RT 078 was reported as the predominant
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strains colonizing cattle and pigs.[3,11,32] Increasingly, studies have shown genetic overlap between animal and human strains of C. difficile RT 078.[12,14], supporting the theory of zoonotic transmission.

In 2009, a farrow to finish commercial piggery in Western Australia was experiencing idiopathic diarrhea in up to 80% of neonatal pigs with mortality in the range 11–14%. The affected pigs had early-onset of diarrhea which was yellow, non-hemorrhagic, and pasty to watery. Untreated piglets had ill-thrift, became anorectic and dehydrated, and some died. Apparently healthy piglets (1–3 days old) were prophylactically treated with amoxicillin or penicillin. A cross-sectional study in the piggery found a C. difficile prevalence of 62% (114/185) in 5–7 day-old piglets[15]. In that study, molecular typing revealed all isolates of C. difficile recovered were an unusual RT 237, toxontype XXXI (toxin A, toxin B), binary toxin positive (cdtA/B) strain. Few studies have described the epidemiology of infections in livestock with RTs of C. difficile other than RT 078.[12,15–17]. This study aimed to determine if C. difficile RT 237 had persisted in the same piggery or whether there had been a temporal change in C. difficile diversity. C. difficile carriage in litters with and without diarrhea was investigated, as was the acquisition of C. difficile over time.

2. Materials and methods

2.1. Study design

The study designs used to address the aims were single cross-sectional and prospective cohort studies, with sampling conducted from October to December 2014. The piggery was located across two sites. The farrow-to-wean site had two holdings separated by a fence, with approximately 5000 sows; holding "A" consisted of older breeding sows (parity 1) and holding "B" consisted of gilts. Holding "C" was the finishing site some 20 km away. The sample size for the cross-sectional study was determined using Feiss methods with a continued correction factor[18]. We estimated that 47.4% of non-diarrheic piglets were shedding C. difficile and 92.8% of diarrheic piglets were exposed. The ratio of non-exposed piglets to exposed piglets was assumed to be 0.5, and with an odds ratio of 14, and a power of 80% to detect the difference if it existed, a sample size of 43 piglets was selected. Fresh fecal samples were collected via rectal swabs from 4 or 5 piglets randomly selected from each of 9 litters aged 1–10 days.

For the cohort study, we estimated a difference of 27% prevalence of C. difficile shedding between 1 day-old (77%) and 42 day-old piglets (50%) based on earlier studies[15]. Using a two tail Z-test for logistic regression, with n of 0.055 and power of 80%, we determined that a total sample of 88 piglets was required. To account for possible loss to follow up of 12%, 12 piglets were added to the sample to make a total of 100. Fecal samples (n = 20) were randomly obtained from 5 piglets from each of 4 litters as described above on days 1, 7, 13, and 20 at the farrow-to-wean holding and on day 42 at the finishing site. One day before weaning, 20 piglets were ear tagged to allow follow up at the finishing site. Among the four litters studied, two had 10 piglets each and the others had 14 piglets each. All swabs were transported in Ames transport medium with charcoal[Thermo Fisher Scientific, Waltham, MA, USA] in a cooler box at 4 °C to The University of Western Australia, School of Pathology and Laboratory Medicine, for processing within 24 h.

This piggery had a two stage in-series anaerobic pond system for treatment of effluent. The primary aerobic pond has an inlet design to facilitate easier desludging of the pond. After moving through the primary pond, effluent moved to a secondary pond which allowed reuse and storage. No chemical disinfection was applied to the water. Therefore, an additional four 30 ml specimen jars (Techno-Plus Pty Ltd, St Marys, Australia) of treated water held for under-pan flushing in storage tanks located adjacent to the farrowing shed, four 30 ml effluent samples from a drainage channel leading to the aerobic pond, and six shed floor swabs transported in Amies transport medium with charcoal were obtained from holding "X". The six floor swabs were obtained by directly swabbing the wet floor from six pens.

Additional data were collected such as the health status of the piglets, age, litter size, mortality, parity of sow and farrowing date. A piglet was considered diarrheic at the sampling time using the following criteria: 1) had yellow, non-hemorrhagic, and pasty to watery feces and 2) any piglet painted red at the dermis by personnel on the basis of diarrhea being observed, and that had a perineum soiled with watery feces. A litter was classified as diarrheic if one or more piglets had diarrhea at the time of sampling.

2.2. Isolates of C. difficile

C. difficile was isolated as previously described, with minor modifications[19]. Briefly, the swabs were cultured directly on ChromID agar (Becton, Dickinson and Co., Cockeysville, Maryland, USA) and in an enrichment broth containing cellobitin, cycloserine and gentamicin. Following alcohol shock when 1 ml of 48 h broth culture was mixed with 1 ml 10% tryptone broth (BBL) and left for 1 h, 0.01 ml of mixture was cultured on ChromID agar. Efficient and treated water samples (10 ml) were cultured directly on ChromID agar or following broth enrichment. An aliquot of 1 ml of either efficient or treated water was transferred to the enrichment broth and processed similarly to feces.

All cultures were incubated anaerobically (A35 anaerobic chamber, Den Whitley Scientific Ltd., Shipley, West Yorkshire, UK) at 37 °C, with an atmospheric gas composition of 80% N2, 10% CO2 and 10% H2. Two to three probable C. difficile colonies on ChromID agar were cultivated on blood agar and identified on the basis of their characteristic charnairsse fluorescence detected with UV light (~360 nm wavelength), coliform morphological characteristics (ground glass appearance) and horse dung odor. Identification of uncertain isolates was achieved by Gram staining and detection of β-proline aminopeptidase (Remel Inc., Lenexa, KS, USA).

2.3. Molecular characterization

All isolates were characterized by PCR to determine the presence of toxin A (toxA), B (toxB) and binary toxins (cdtA and cdtB) genes and changes in the repetitive region of the toxin A gene[20]. PCR ribotyping was performed on strains as described elsewhere[21]. RTs were identified by comparing their banding patterns with those in our reference library of animal and human C. difficile strains, consisting of a collection of 50 Anaerobe Reference Laboratory (ARL, Cardiff, UK) ribotypes that included 15 reference strains from the European Centre for Disease Prevention and Control (ECDC) and the most prevalent PCR ribotypes currently circulating in Australia[8, Elliott, I.V. Riley unpublished data].

2.4. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) for 14 antimicrobials were determined for a selection of isolates using the agar incorporation method as described by the Clinical and Laboratory Standards Institute (CLSI, M11-A7)[22]. A combination of CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints was used if available[23,24]. The quality control strains used were Bacillus subtilis ATCC 25240, Escherichia coli ATCC 25922, E. cloacae aerosolensis ATCC 25741, C. difficile ATCC 700657 and Eubacterium lentum ATCC 8055.350
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2.5. Statistical analysis

The Chi-squared test was used to evaluate the association between isolation of *C. difficile* and diarrhoea in the cross-sectional study. *C. difficile* shedding over time was evaluated by the generalized estimating equations (GEEs) for longitudinal data collected in clusters that are repeated measures. The outcome variable was considered as binary (presence or absence of *C. difficile* per sample) and fixed effects models were employed in GEEs to adjust for the response variable from within clusters (litters) as well as over time (6 weeks). In fitting the data to the model we used the independent working correlation structure as this implies that the within-litter correlation between all sampling was equal to zero. GEEs have been shown to be robust even when there is an error in specifying the working correlation structure [25]. All associations with a p value ≤ 0.05 were considered significant. All analyses were performed in Epi-info 7.1.40 statistical software from the Centers for Disease Control and Prevention (CDC) and R version 3.2.2.

Animal ethics committee approval. This study was approved by The University of Western Australia Animal Ethics Committee (reference number RA3/3000/75).

3. Results

3.1. *C. difficile* carriage in pigs

*C. difficile* was isolated from 39/43 (44.2%), 20/38 (52.6%), 23/38 (59.5%) and 20/34 (58.8%) faecal swabs by direct culture and 20/43 (46.5%), 55/95 (57.8%), 53/93 (56.9%) and 81/141 (57.4%) with enrichment media from holding “A”. Enrichment culture was significantly more sensitive than direct culture (p = 0.0002, χ²). Of the diarrhoeic pigs, 20 of 24 (83.3%) were *C. difficile* culture positive compared to 8 of 19 (42.1%) non-diarrhoeic pigs (p = 0.0124, χ²). *C. difficile* was isolated from piglets in 7 out of 8 litters (77.8%).

A total of 13/106 (12.2%) piglets died across the nine litters sampled in the cross-sectional study, however, the association between *C. difficile* positive status and mortality was not significant (p = 0.74). There were seven litters with one or more diarrhoeic pigs, and a total of 24 out of 43 diarrhoeic piglets. The comparison between parity and *C. difficile* positive status of piglets was made between parity 3 (referred) and combined piglets from sows with parity 4, 5 and 6 because of sparse data. *C. difficile* distribution in piglets by parity of sow was parity 3 (13/15; 86.7%); parity 4 (7/10; 70%); parity 5 (5/5; 100%); and parity 6 (4/5; 80%). All *C. difficile* isolates from piglets were RT 237.

3.2. The prospective cohort study

*C. difficile* was isolated from 8/20 faecal samples (40%) on day 1, 10/20 (50%) on day 7, 4/20 (20%) on day 13, 8/20 (40%) on day 20, and 0/20 (0%) on day 42 (Table 1). The multivariate model evaluated the following variables: age of piglets, litter size, mortality and diarrhoea (Table 1). There was no significant difference between *C. difficile* shedding on day 1 versus day 7 (p = 0.10); nor day 1 versus day 13 (p = 0.10). However, there was a significant difference in *C. difficile* shedding between 1-day-old piglets and piglets at 20 and 42 days of age (p < 0.0001). The regression coefficients were positively associated with *C. difficile* shedding on day 7 but were stronger and negatively (inversely) associated with shedding on day 13 to day 42 (Table 1). The risk of shedding *C. difficile* in the feces by piglets significantly declined from day 13 onwards, as the regression coefficients were negative (inverse) (Table 1). The overall prevalence of *C. difficile* was 22% (22/100). There was a total of 48 piglets from the four litters studied. This means that 42% of piglets were sampled at each time point indicating that each piglet had 42% chance of being sampled every week. *C. difficile* was isolated at least once from all study litters 100% (4/4).

*C. difficile* was not isolated from piglets aged 20 days and 42 days (n = 20) (Table 1). There was a total of 36/100 cases (36%) of diarrhoea among the sampled piglets. The cases of diarrhoea in piglets per sampling time were as follows: day 1 (8/20; 40%), day 7 (6/20; 30%), day 13 (11/20; 55%), day 20 (9/20; 45%) and day 42 (2/20; 10%). However, the association between *C. difficile* positive status and diarrhoea for all cases was not significant (p = 0.67).

Nine piglets from four litters died in this study, giving a 9% mortality rate. Seven of the piglets were from diarrhoeic sows where *C. difficile* was identified, while two were from non-diarrhoeic but *C. difficile* positive pens. The regression coefficient for mortality was positively associated with *C. difficile* shedding (p = 0.0001) (Table 1).

The toxin B gene (tdh) and but tdhA were detected by PCR in all *C. difficile* isolates from the 22 infected piglets, including both diarrhoeic and non-diarrhoeic animals. Both toxin genes (tdh and tdhA) were also detected in all isolates and all were RT 237.

3.3. Environmental samples

The effluent samples (n = 4) obtained from a drainage channel before the two-stage treatment ponds were all positive for *C. difficile* by enrichment culture. Additionally, two of the four samples of treated water collected from the farrowing sheds were positive. Furthermore, four of the six floor slab samples collected from some of the pens of diarrhoeic and non-diarrhoeic litters were positive (67%). All environmental isolates were RT 237.

3.4. Antimicrobial susceptibility profiles

MICs for 14 antimicrobials were determined for 29 isolates sourced from the cross-sectional study (Table 2). Despite the probability that these isolates were clonal, there were some small variations in susceptibility; however, all were susceptible to the antimicrobials for which breakpoints were available. There are no

<table>
<thead>
<tr>
<th>Variable</th>
<th>*C. difficile positive</th>
<th>*C. difficile negative</th>
<th>GEEs regression</th>
</tr>
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<tr>
<td></td>
<td>DP</td>
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<td>DP</td>
</tr>
<tr>
<td>Intercept</td>
<td>1.92</td>
<td>1.81</td>
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</tr>
<tr>
<td>Day 1</td>
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<tr>
<td>Day 7</td>
<td>3</td>
<td>7</td>
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</tr>
<tr>
<td>Day 13</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Day 20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Litter size</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mortality</td>
<td>7</td>
<td>2</td>
<td>9</td>
</tr>
</tbody>
</table>

Note: *C. difficile test*, *Dp* diarrhoea, *Dp* non-diarrhoea, *Total*.
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4. Discussion

This study aimed to determine if *C. difficile* RT 237 had persisted in the Western Australian piggy that we investigated in 2009 [15]. *C. difficile* RT 237 was found again and the prevalence in the cross-sectional study (67.4%) was similar to the earlier study (65%) [15], and the same as a national prevalence study conducted recently in 21 Australian piggy breeds (67%) [16]. In the Australian national survey RT 044, a strain commonly reported in human hospital settings [26,27], was the most prevalent RT found (36% / 154, 23%). Overall these prevalence results are consistent with findings in studies from Europe [17] and North America [11]. However, because no data are available concerning the presence of *C. difficile* in pigs in Europe, the prevalence of *C. difficile* in pigs in Australia is unclear. One possible explanation is that the piggy generates its own replacement breeding stock and this could have prevented introduction of new *C. difficile* strains from other piggy breeds. Our findings suggest that new strains of *C. difficile* are not commonly introduced from other sources such as rodents or birds on this piggy. An important factor could be the geographical location of the piggy both within the State of Western Australia, and within Australia generally where there is a large expanse of desert and great distances separating eastern and western Australia.

The prevalence of the cross-sectional study on holding "A" was 67.4% in piglets aged 1–10 days, and the overall prevalence of *C. difficile* from the cohort study was 22% (22/100). There was a gradual decline in *C. difficile* shedding in feces with increasing age of piglets in the cohort study on holdings "B" and "C". These findings are in agreement with similar studies from elsewhere [6,28] and with other cross-sectional studies [6,15–17,29] which reported a lower prevalence of *C. difficile* in older (>14 days) piglets than in younger piglets. Alvarez-Pérez et al. [8] reported a 26% prevalence of *C. difficile* in piglets aged 1–7 days in Spain but zero prevalence at 1–2 months, while a study conducted in an integrated swine production system in the USA found that fecal shedding of *C. difficile* was 50% in sudding piglets, 65% in weaner pigs (3–10 weeks old) and 3.5% in both farrowing (up to 32 weeks) and adult breeding herds and sows [29]. Another longitudinal study undertaken in Canada found a *C. difficile* prevalence of 74% (day 21), 55% (day 7), 40% (day 30), 23% (day 44) and 5.7% (day 62) [4]. These findings support the hypothesis that *C. difficile* colonization declines with increasing age, possibly due to interference from developing components of the normal intestinal microbiota in a phenomenon referred to as "colonization resistance" [30].

A high prevalence of *C. difficile* in slaughter age pigs could pose a risk of foodborne infection to humans through consumption of contaminated meat. The current study did not examine slaughter age pigs, but the overall prevalence found in younger pigs was 22% (22/100), lower than that reported in Canada (98%) [14] and in the Netherlands (90%) [15], but similar to that reported in Spain (75.6%) [28], although the RTs detected were different. Alvarez-Pérez et al. [20] found a peak prevalence of day 7 (50% / 10/20), but they sampled from the same piglets over time up to day 50 as opposed to sampling a subset of the same litterers over time. The decline in *C. difficile* shedding to zero by day 20 was earlier than reported in other studies [4,28], where *C. difficile* shedding continued up to day 30. Weese and colleagues [31] reported a farm level *C. difficile* prevalence of 6.5% (38/540) in slaughter age pigs in Canada. In that study, various strains of *C. difficile* were detected, but RT 078 was the predominant strain on farms, with a prevalence of 67% [31]. Many other studies have documented the presence of *C. difficile* in meat products such as retail beef, pork and turkey [32,33]. The fact that *C. difficile* was not isolated in elder pigs (6 weeks old) in the present study suggests that slaughter age pigs at this piggery are unlikely to pose a risk for human infection. However, there is a need to carry out further studies at local piggeries with different circulating RTs and in abattoir environments to be able to exclude local meat products as a source of *C. difficile*.

The contaminated farm environment may provide a source of *C. difficile* for human infection. *C. difficile* can be dispersed by wildlife [34], vermin (rats and flies on a piggy) [35], wind [36], and manure [33]. RT 078, a well-characterised animal pathogen, has increasingly been isolated from humans, particularly those living near pig farms in Europe [15,37]. Kiers et al. reported distinct genotypic strains of *C. difficile* RT 078 in pig farmers and pigs by whole genome sequencing technique [11]. In the present study, RT 237 was detected from the floor, treated water, and also from effluent from a drainage channel before the two-stage treatment pond at the piggery. Similarly, Squire and colleagues isolated *C. difficile* RT 237 from treated pig effluent planned for use in clearing the pig sheds [38]. However, RT 237 has been detected rarely in clinical specimens obtained from human patients in Western Australia [26,29], suggesting, perhaps, that it does not adapt well to a human host.

At the study piggery, a sporidical disinfectant (sodium hypochlorite) has been used in pig sheds for the last few years. An explanation for detection of *C. difficile* from pen floor and waterer was not obvious although substantial concentrations of the disinfectant used cannot be ruled out. *C. difficile* spores can persist in the environment for a long time, therefore additional control measures such as providing education to all working staff at the farm could further reduce the incidence of CDI. Overall, these findings suggest that sporidical disinfectants in pig sheds analogous to hospital environments may reduce piglet infections [40].

All the 10 *C. difficile* isolates sourced from the cross-sectional study had similar susceptibilities to a panel of antimicrobials, with no resistance detected (Table 2). This finding was expected because all isolates were most likely clonal. In an earlier smaller study of RT 237 isolates from the same piggery no resistance was detected [41]. In contrast, Peloza et al. [42] reported a 9% prevalence of metronidazole resistance (MIC = 256 μg/ml) and nearly 50% multidrug resistance in *C. difficile* swine herds in Spain. In general, there is a paucity of information on antimicrobial susceptibility of *C. difficile* in livestock.

---

Table 2

<table>
<thead>
<tr>
<th>Agent</th>
<th>MIC range (μg/ml)</th>
<th>Clinical breakpoints</th>
<th>Percentage distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>L</td>
<td>R</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1</td>
<td>≥2 NR</td>
<td>≥2 100 0 0</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>0.25–1</td>
<td>≤8 16</td>
<td>≥32 100 0 0</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>0.25–4</td>
<td>≤2 4</td>
<td>≥8 65 35 0 0</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>0.25–0.5</td>
<td>NR NR</td>
<td>≥8 NR NR NR NR NR</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>0.12–0.25</td>
<td>≤4 8</td>
<td>≥16 100 0 0</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>0.5</td>
<td>≤16 32</td>
<td>≤32 100 0 0</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>1</td>
<td>≤4</td>
<td>≥16 100 0 0</td>
</tr>
<tr>
<td>Meropenem</td>
<td>0.25–2</td>
<td>≤4 8</td>
<td>≥16 100 0 0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.12</td>
<td>≤4</td>
<td>≥16 100 0 0</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>2–4</td>
<td>≥32 64</td>
<td>≥128 100 0 0</td>
</tr>
</tbody>
</table>

Note. The susceptible (S), intermediate (I), and resistant (R) interpretive values were determined for resistance testing from the CLSI and EUCAST breakpoints available for the following antimicrobials: gentamicin had MIC range (32–64 μg/ml), spectinomycin (128 μg/ml), tobramycin (32–128 μg/ml), and tetracycline (32–64 μg/ml).
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5. Conclusions

RT-237 has persisted for at least 5 years and remains the predominant strain of C. difficile in pigs on a piggy in Western Australia. This unusual RT has been detected in human patients in Australia but not in high numbers. The decline of C. difficile shedding to zero by day 20 suggests that slaughter age pigs are unlikely to be greatly contaminated with C. difficile in this piggy. Further research is warranted to determine the sources of the persisting RT-237 on the piggy, and to reduce contamination levels in the piggy environments to limit piglet and potentially human exposure.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgement

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References

Aspects of *Clostridium difficile* infection in pigs

Peter Moona, Su Chen Lim, & Thomas V. Riley

*Clostridium difficile* is a well-established hospital pathogen. Recently, it has been detected increasingly in patients without hospital contact. Given this rise in community associated infections with *C. difficile*, we hypothesized that the environment could play an important role in transmission of spores outside the hospital. Lawn samples (313 collected in public spaces in the metropolitan area of Perth, Western Australia, from February to June 2016 were cultured for *C. difficile*. *C. difficile* was isolated from the samples by direct enrichment culture, and characterized by standard molecular methods using toxin gene PCR and ribotyping. The overall prevalence of *C. difficile* was 59%, new lawn (<4 months old) was twice as likely as old lawn (<4 months old) to test positive (OR: 2.3; 95% CI: 1.16-4.57, p = 0.015) and 35 *C. difficile* ribotypes were identified with toxigenic ribotype 014/020 (39%) predominating. The highest viable count from lawn soil samples was 1200 CFU/g. These results show that lawn in Perth Western Australia, harbor toxigenic *C. difficile*, an important finding. The source of lawn contamination is likely related to modern practice of producing “roll-out” lawns. Further work should focus on identifying specific management practices that lead to *C. difficile* contamination of lawn to inform prevention and control measures.

*Clostridium difficile* is a well-established hospital pathogen associated with outbreaks of severe gastroenteritis. *C. difficile* infection (CDI) in patients is acquired by oro-fecal ingestion of spores from the environment. The major virulence factors of *C. difficile* are two exotoxins, toxins A and B, with some strains producing a third binary toxin. There has been an increase in the incidence of community-associated CDI (CA-CDI) both in Australia and elsewhere. Because of this increase in CA-CDI, food sources have emerged as a potential reservoir of *C. difficile*. This paradigm is supported by studies that have reported detection of *C. difficile* in animals, meat, and root and leaf vegetables. However, the overall prevalence of *C. difficile* in foods is low.

Although foodborne transmission of CDI is plausible it has never been proven, and the environment may be another important source of CA-CDI. This is evident from many studies where ribotypes (RTs) of *C. difficile* have been detected from the environment including so-called hypervirulent strains (RT 001, 012, 045, 066, 078, and 121). It is possible that environmental contamination with *C. difficile* spores may be a more important source for CA-CDI than food.

Outbreaks of infectious organisms, other than *C. difficile*, associated with manure have been reported. In the USA, 50% of all bio solids produced each year is used in agriculture and landscaping. *C. difficile* has been isolated from raw and treated human bio solids, and the environment. The mesoprophilic properties of bio solids does not induce *C. difficile* spores to be killed and, therefore, application of bio solids to landscape including private and public space lawns could transmit *C. difficile* in the community. In Perth Western Australia, the construction of new housing and the development of new suburbs has seen an expansion of public and private lawns as has occurred in the USA.

The aim of this study was to determine the prevalence and concentration of *C. difficile* in newly established (NL) and older (OL) lawns in public spaces in Perth, and to characterize any *C. difficile* isolates by phenotypic and genotypic techniques. Second, we investigated factors such as the location and size of the lawn to see if they could predict *C. difficile* status.

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<table>
<thead>
<tr>
<th>Variable</th>
<th>Variable category</th>
<th>C. difficile number isolated (%)</th>
<th>Univariable model</th>
<th>Covariate Odds ratio (95% CI)*</th>
<th>Sampling site</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age*</td>
<td>Old (n = 115)</td>
<td>53 (47)</td>
<td>Referent</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>New (n = 149)</td>
<td>129 (90)</td>
<td>2.11 (1.52-3.34)</td>
<td>2.50 (1.24-5.07)</td>
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<tr>
<td></td>
<td>Extra-large (n = 85)</td>
<td>53 (62)</td>
<td>Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Large (n = 57)</td>
<td>26 (49)</td>
<td>0.55 (0.28-1.10)</td>
<td>0.49 (0.29-0.81)</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Medium (n = 101)</td>
<td>66 (69)</td>
<td>0.86 (0.49-1.50)</td>
<td>1.02 (0.42-2.51)</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Small (n = 72)</td>
<td>41 (58)</td>
<td>0.57 (0.47-0.73)</td>
<td>0.59 (0.32-1.05)</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td>Perth (n = 161)</td>
<td>98 (60.8)</td>
<td>Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Joondalup (n = 150)</td>
<td>64 (44)</td>
<td>Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fremantle (n = 224)</td>
<td>125 (56.0)</td>
<td>Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Animal (n = 195)</td>
<td>84 (55)</td>
<td>1.22 (0.78-1.88)</td>
<td>1.25 (0.62-2.53)</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human (n = 75)</td>
<td>47 (64)</td>
<td>0.77 (0.47-1.29)</td>
<td>0.87 (0.48-1.62)</td>
<td>0.32</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. The relationship between the prevalence of C. difficile in lawn and the age of the lawn, its size, sampling site, location, postcode, and season in Perth. *CI. The 95% confidence interval of odds ratio for the covariate estimates. "CI. The 95% confidence interval of odds ratio for the univariable estimates. Age of lawn; new lawn ≤ 4 months, old lawn > 4 months. *P-values are based on likelihood ratios and P<0.05 was considered significant. Univariable logistic regression model with random effect (site of sampling and postcode). The random effect term for postcode was not included in all the models because its addition or removal did not change the model estimates significantly.

Table 2. Viable counts of Clostridium difficile in soil from lawn samples using either phosphate buffer solution (pH 7.4) (PBS) or peptone saline (PST), both containing 0.1% Tween 20, as a diluent.

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Sample (table count log of soil)</th>
<th>No samples positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>1 2 3 4 5</td>
<td>6 7 8 9 10</td>
</tr>
<tr>
<td>PST</td>
<td>0 0 0 100</td>
<td>100 50 0 0</td>
</tr>
</tbody>
</table>

Results
Prevalence of C. difficile. The overall prevalence of C. difficile in lawn samples was 59% (162/271), varying from 0% to 70% by postcode. The prevalence of C. difficile was significantly higher in NLS (65% (120/189)) compared to 47% (53/114) in OLs (Table 1). In the unadjusted univariable model, C. difficile was more likely to be detected in NL than OL (OR = 2.11, 95% CI: 1.32-3.4, p = 0.001) compared to the adjusted model (OR = 2.30, 95% CI: 1.16-4.57, p = 0.015) (Table 1). In the multivariable analysis, age of lawn was also the only variable that was significantly associated with detection of C. difficile (OR = 1.92, 95% CI: 1.15-3.21, p = 0.013). The site and location of the lawns were not associated with detection of C. difficile and the study period was too short to observe any effect of season on recovery.

Quantitation of C. difficile in lawn. Viable counts were performed on 10 lawn samples taken from one positive area by direct culture on CedroChromID agar (Table 2). Samples were also concentrated by centrifugation. Using PBS or PST dilution, the highest number of C. difficile positive cultures was 8 out of 10 after centrifugation. C. difficile recovery was higher in PST than PBS by both direct culture (50% vs 40%) and after centrifugation (80% vs 60%). The highest viable count of C. difficile detected was 1200 CFU/g of soil and the lowest 50 CFU/g of soil (Table 2). There were three ribotypes detected, all non-toxigenic, QX 189, QX 501, and UK 01b.

Molecular characterization. There were 35 unique RIs detected in this study. Of the toxigenic (A - B - CDT+) RIs detected, 11 isolates harboured both A and B genes but no binary toxin genes (Table 3). 12 toxigenic RIs accounted for 47% (96/202) of the total and included internationally recognised RIs associated with hospital infections. The 24 non-toxigenic (A - B - CDT+) RIs accounted for 53% (96/182) of the total. The toxigenic strain RT 010/020 was the most predominant (39.9%), followed by the non-toxigenic RT 010 (20.3%) (Table 3). Toxigenic RIs isolated from NL accounted for 35.5% (64/182) of all isolates compared to 14.3% (29/202) in OL. In the NL among toxigenic strains only, RT 010/020 was over represented 78.8% (48/62), followed by RT 054, 056, 092, 018, QX 63, and QX 81 (all < 5%). In the OL among toxigenic strains only, RT 010/020 was also over represented at 88.5% (23/26), followed by RT 002, 010 and QX 409 (all < 5%).

Discussion
The aim of this study was to investigate the prevalence of C. difficile in public space lawns in Perth, Western Australia. The reasons for undertaking the project were three-fold. First, there has been an increase in the incidence of CA-CDI in Australia. Second, although many production animals carry C. difficile in their gut, particularly young animals, it is unlikely that most contamination plays a major role in CA-CDI. Last, anecdotaly we have heard that manure from pig farms was being used by turf farms for the production of lawn.
Aspects of *Clostridium difficile* infection in pigs

<table>
<thead>
<tr>
<th>Ribotype</th>
<th>tcdA</th>
<th>tcdB</th>
<th>cdtA/B</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>04/0284</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>71 (13.0)</td>
</tr>
<tr>
<td>010</td>
<td></td>
<td>-</td>
<td>-</td>
<td>377 (24.3)</td>
</tr>
<tr>
<td>QX 477</td>
<td></td>
<td>-</td>
<td>-</td>
<td>25 (7.2)</td>
</tr>
<tr>
<td>QX 259</td>
<td></td>
<td>-</td>
<td>-</td>
<td>7 (1.9)</td>
</tr>
<tr>
<td>039</td>
<td></td>
<td></td>
<td>-</td>
<td>22 (7.2)</td>
</tr>
<tr>
<td>QX 481</td>
<td></td>
<td></td>
<td>-</td>
<td>42 (1.5)</td>
</tr>
<tr>
<td>305</td>
<td></td>
<td></td>
<td>-</td>
<td>42 (1.5)</td>
</tr>
<tr>
<td>QX 142</td>
<td></td>
<td></td>
<td>-</td>
<td>42 (1.5)</td>
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<tr>
<td>042</td>
<td>+</td>
<td></td>
<td>-</td>
<td>42 (1.5)</td>
</tr>
<tr>
<td>Others*</td>
<td>Various</td>
<td>Various</td>
<td>-</td>
<td>35 (13.0)</td>
</tr>
</tbody>
</table>

Table 3. The frequency and toxin gene profile of *C. difficile* ribotypes in lanswes in Perth, Western Australia. *Others: QX 518, QX 611, QX 608, QX 610, RT 056, RT 054, QX 607, QX 606, QX 605, QX 603, QX 602, QX 550, QX 449, QX 409, QX 393, QX 210, RT 125, QX 121, RT 106, QX 072, QX 067, QX 054, RT 018, RT 012, RT 009 and RT 049.

The overall prevalence of *C. difficile* in lawn samples was 59%, varying from 0% to 79% by postcode. In the adjusted univariable model, *C. difficile* was more likely to be detected in NL than OL (OR = 2.30, 95% CI: 1.16–4.57, p = 0.015) (Table 1). Other studies have reported a high prevalence (30–68%) of *C. difficile* in the household environment and biosolid treatment plants8,19,20, similar to this study. The reason for the lower prevalence of *C. difficile* in OL (>4 months) is not clear, although an effect of natural exposure of germinated spores to oxygen or ultraviolet light cannot be excluded. It is unlikely that *C. difficile* can multiply to any great extent in the lawn and the counts detected were not very high. A recent study has shown that ultraviolet light device can be used as sanitizers in hospitals to reduce pathogenic organisms including *C. difficile*. Another study found that *C. difficile* spores can survive up to 5 months in the environment21. Therefore, the difference in prevalence between NL and OL could be explained by environmental factors such rain, wind, and ultraviolet light over time, and contact with people and animals which could help disperse the initial spore load, particularly on the surface of the lawn. The majority of the NL in this study was located within recently built suburbs in Perth. The relationship between new suburb buildings and lawn expansion is well studied22. Our findings suggest that new suburbs and consequent expansion of NL could be contributing to dispersal of *C. difficile* in the environment and variables other than NL were not significantly associated with isolation of *C. difficile*.

Nearly 25% of global disease burden has been attributed to environmental risk factors23. Animal manure is widely used in both agriculture and landscaping24-26, and anecdotal evidence suggests this practice is widespread in Australia. In the USA, manure that has been composted is exempted from federal or state rule regarding pathogen levels if it is going for land application27. The major problem which is often overlooked in applying animal manure or biosolids to land is consideration of the duration of persistence of pathogens on plants28. Animal effluent treatment involves removal of solid wastes and then holding liquid effluent in anaerobic ponds under mesophilic anaerobiosis. The treatment method for human raw effluent in Switzerland involved grid separation, primary sedimentation, and secondary biological treatment (an activated sludge process)29. The major problem in animal manure treatment is the separation of feces from young animals versus older animals. Young animals are associated with shedding high levels of pathogenic organisms including, in particular, *C. difficile*. 30,31. Currently, there is pressure on the turf industry to meet the demand for lawns in newly built suburbs both private and public laws. This has led the industry to develop the lawn farming system of roll-out lawns which can easily be transported to clients on demand. The lawn, like other plants, is grown on manure or biosolids for rapid growth and early maturation. The fact that even composted manure or treated animal effluent does not inactivate *C. difficile* spores implies that lawns could be heavily contaminated with *C. difficile* and our data suggest this is the case. Even compost, manure or biosolids that have been produced with specific standards32 have caused disease outbreaks in the USA33,34. In this regard, manure, compost and biosolids should be screened for pathogenic microorganisms such as *C. difficile* before being applied to land for recreational or agricultural use.

Although the infections dose for *C. difficile* is unknown, there are suggestions that it could be low (100–1000 spores)35. In addition, in some high-risk individuals infection could be driven mainly by host factors. In this study, we found that the highest *C. difficile* spore concentration in lawn samples was 1.000 CFU/g of soil (Table 2). The concentration of *C. difficile* in feces of cattle has been reported to be 2.5 × 10^6 CFU/mL36, and in human feces as 6.66 ± 0.27 log10 CFU/mL37. It is unclear how much exposure to lawns is required for a human or animal to be contaminated with sufficient spores that could lead to infection with *C. difficile*. The fact that ingestion of *C. difficile* from the environment does not quickly result in infection is due to the complex nature of the pathogenesis of disease that requires the gut microflora to be perturbed, usually in the form of antibiotic exposure38. Although we did not find any seasonal fluctuations of *C. difficile* in lawn samples in this study, it is likely that recreational exposure to *C. difficile* could be higher in some seasons when people regularly use public spaces and lower in low activity periods. Therefore, studies that quantitate spore load on surfaces such as shoes are needed because they may help explain the rise of CA-CDI39.

There were 35 unique RTs detected in this study, including the clinically important RTs 014/020, 056, 054, 002, 018, 012 and 043 (Table 3). The high prevalence of RT O4/020 in this study was an interesting and important
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finding given that RT 014/020 is the most predominant RT in laboratory samples from diarhetic patients in Australia\textsuperscript{35,36}. RT 04/020 is also the main ribotype currently found in pigs in Australia\textsuperscript{37} suggesting that pig manure may be responsible for the contamination of lawn in Perth. Other researchers have reported detection of RT014/020 in river water\textsuperscript{38} and treated sludge\textsuperscript{39}.

The detection of RTs 012, 002, and 018 was unexpected because they are not routinely detected from the environment\textsuperscript{40,41}, although they are increasingly associated with CA-CDI\textsuperscript{40,41}. Recently, RTs 056, 010, 078, 213, 009 and 020 were detected in dogs\textsuperscript{42}. RT 056 was also recently detected in gastrointestinal tracts of veal calves at slaughter\textsuperscript{43}. Several of the other RTs detected in our study (056, 054, 018 and 002) have been associated with human CDI in Europe\textsuperscript{44,45}, Japan\textsuperscript{46} and Australia\textsuperscript{47}. These findings suggest that all lawns may be getting contaminated with animal strains of C. difficile and that this could be contributing to an increase in CA-CDI.

Interestingly, no binary toxin producing C. difficile isolates were cultured in this study although such strains have been recovered previously in high numbers from production animals in Australia\textsuperscript{18-20}. The reasons for this are unclear. Our earlier animal studies have suggested that different strains of C. difficile tend to infect different classes of animals in particular geographic areas\textsuperscript{18,21} and these are not always binary toxin producers, such as RT08/020 in pigs\textsuperscript{47}. The lack of binary toxin producers in the current study may just reflect a relatively small sample size and the fact this study was undertaken in Western Australia only.

The lawns sampled were located within the metropolitan area of Perth suggesting that people and dogs walking on lawns could transfer C. difficile into the households\textsuperscript{48}. A high prevalence (32.3%) of C. difficile in household environs has been reported, particularly on the soles of shoes\textsuperscript{49}. It is also possible that shoes could be vehicles by which C. difficile could be introduced in health care facilities. The detection of C. difficile in lawn could help explain the substantial proportion of cases (45%) of CDI originating from unknown reservoirs as recently described by others\textsuperscript{50,51}.

This study has some limitations. First, we only sampled a relatively small number of public spaces (n = 20) and the study was undertaken only in Western Australia which may impact its generalizability to other regions of Australia and the world. However, all lawnturf producing companies have access to a range of manures in Western Australia, suggesting that the problem could be similar throughout the metropolitan area of Perth. The age of some of the lawns was estimated with a series of photos of lawns with a known age and this could have resulted in misclassification bias. However, we would not have affected the overall outcomes of the study as both OL and NL had relatively high prevalence of C. difficile. We investigated management practices of commercial lawn farms, however, the frequency of maintenance of the lawns was not established.

In conclusion, the high prevalence of C. difficile in lawn is potentially an important finding, however, it is difficult to estimate the risk of CDI through exposure to C. difficile in lawns. There is a need for further detailed and structured studies to examine how modern lawns are grown, and to investigate the role of any manure and biosolids applied as fertiliser as a source of CA-CDI. The relevance of these findings should be further confirmed by studies comparing the relatedness of isolates in this study to those CA-CDI cases using whole genome sequencing.

Methods

Background. Lawns in Western Australia can either be raised as seeding lawn or "instant turf" in the form of rolls of pre-grown lawn\textsuperscript{52}. Seeded lawn has lower cost to plant compared to instant turf, however, it takes longer to mature and is more labour intensive than turf rolls. The advantage of turf rolls is that they are fast to grow and easy to lay, and can be used within a week, hence the current high demand. Soft leafed buffalo grass is a widely used variety in Western Australia because it withstands drought; most lawns are established in early autumn or late summer to avoid extreme temperatures in summer\textsuperscript{53}.

Lawns in Australia are considered young up to 4 months by the turf industry\textsuperscript{54}. Therefore, we used this time-point to distinguish between newly established (NL) and older (OL) lawns (NL <4 months) and OL (>4 months). From autumn through winter, older lawns tend to lose colour and become dormant. We initiated this study during this period when it should have been relatively easy to identify lawns that were new.

Study location and samples. NL and OL samples were collected from 20 public spaces within 11 postcode areas in Perth and surrounding areas in February–June 2016. When information on the age of lawns in some areas was unanswerable, photographic identification was employed. A NL was identified by the presence of a visible line between two or more strips of laid down lawn which remained visible for up to 4 months. Lawns that did not have these assumed to be old\textsuperscript{55}. This involved comparing photographs with a series of photographs taken of lawns of known age (NL appeared bright green, luxuriant and with sharp edges). This technique has been widely used in the USA while some researchers have even employed remote sensing imaging\textsuperscript{56}. We sampled within 5–30 km north and 5–80 km south of Perth city. We categorized public spaces by size (small [0.5–1 km²], medium [1.1–2 km²], large [1.6–2.9 km²], and extra large [3–9 km²]) based on area data from local council authority websites in each study area or, where no information was available, we estimated the area of the space in relation to other known areas. All the public spaces that met the size cut off and had a grass variety that was commercially available were included in the sampling frame, however, sampling was performed on the basis of convenience. We estimated that 150 samples were appropriate to detect 18% difference in C. difficile prevalence between NL and OL (α = 0.05, power 80%, 95 CI). To account for sampling clustering, we multiplied 150 samples obtained by simple random sampling by 2 design effect. A total of 311 NL or OL samples were collected in sterile 200 mL specimen jars. Each public space was divided into four quadrants with the centre generally being a children’s play facility. Four samples were obtained per quadrant starting from the centre and moving outwards. The lawn sample consisted of grass and its root system with attached soil, and was obtained using a new set of sterile examination
gloves and a sterile tongue depressor which was used to dig out grass and its root system. Each lawn sample weighed approx. 50g and those were transported at ambient temperature to the laboratory.

**C. difficile isolation.** Ninety ml brain heart infusion broth supplemented with cycloserine and colistin, were pre-reduced for 4h in an anaerobic chamber (A.S. Don Whitley Scientific Ltd, Shipley, West Yorkshire, UK) at 37°C. The lawn samples were aseptically cut into ~2 cm² pieces weighing approx. 5g, inoculated into the broth and gently shaken, and then incubated at 35°C in an A35 AnaeroBroth Workstation (Don Whitley Scientific Ltd., UK) for 5 days with the lid loose. A 5 ml aliquot of culture broth was mixed with 5 ml of equal volume 90% ethanol and incubated for 1h before being centrifuged at 3000g for 10min. The pellet was plated onto C. diff. ChromID agar (bioMérieux, Marcy Etoile, France) and the plates incubated anaerobically for 48h. C. difficile was identified on the basis of its characteristic charantiana fluorescence detected with UV light (~360 nm wavelength), morphological characteristics (ground glass appearance) and also lambdoid (horse-dung). Identification of uncertain isolates was achieved by Gram staining and detection of L-proline aminopeptidase (Remel Inc., Lenexa, KS, USA).

**Quantification of C. difficile in lawn samples.** A visible count of C. difficile was performed on a subset of lawn samples using previously described methods with some modifications. Briefly, approx. 5g of soil was aseptically obtained from an approx. 10g lawn sample by shaking the root system and placed in a stomacher bag (Cobertec, Lendrum, UK) containing 25 ml of phosphate buffered saline at pH 7.4 supplemented with 0.1% Tween 20 (PBST) or peptone saline also containing 0.1% Tween 20 (PSST) and shaken for 60s. A 100μl aliquot was removed and directly inoculated onto C. diff. ChromID agar, spread using a sterile hockey stick (InterPath Services Pty Ltd, West Victoria, Australia) and incubated anaerobically. The remaining volume was centrifuged at 3000g for 10min, the supernatant discarded, the pellet resuspended in 1000 μl of either PBST or PSST and directly inoculated on C. diff. ChromID. Viable colony counts were performed at 48h. A negative control (either PBST or PSST) was used in each assay to monitor for potential contamination. We performed molecular typing on all positive samples using tcdC PCR and PCR-RiboTyping to confirm the isolates. The detection limit for the viable count was >1 CFU per gram of soil.

**Molecular characterization.** All isolates were characterized by PCR to determine the presence of toxin A (tcdA), B (tcdB), and binary toxin (cdtA and cdtB) genes. Novel primers were multiplexed with tcdA primers NK2 and NK3 to detect the tcdA repeat region A3 fragment. Briefly, 4 μl of DNA extract was added to a PCR mixture containing 2μM MgCl₂, 50mM KCl, 1.5mM Tris-HCl (pH 8.3), 200μM of dNTP, 0.2μM of each primer NK2, NK3 and BE tcdA 1 (FP: 5’CGATCACGGATGGGGAATTT 3’) and BE tcdA 2 (RP: 5’AAAGCATTAGGCTTACGAC 3’) (B. Elliott, unpublished data). Detection of both the tcdA 1 and tcdA 3 fragment was required for an isolate to be considered tcdA positive. Amplifications were carried out in a 2720 Thermal Cycler (Applied Biosystems, Foster City, USA).

PCR-RiboTyping was performed on isolates as described elsewhere. K1s were identified by comparing their banding patterns with those in our reference library from the Ancestral Reference Laboratory (ASL, Castell, UK) ribotypes that included 15 reference strains from the European Centre for Disease Prevention and Control (ECDC) and the most prevalent PCR ribotypes currently circulating in Australia (B. Elliott, T. V. Riley, unpublished data). Isolates that could not be identified with the international reference library were designated with local (QX) nomenclature.

**Statistical analysis.** Univariable random effect logistic regression analysis was used to describe the relationship between C. difficile culture status (response variable), and independent variables: age of lawn (new vs. old), sampling site, location (north vs. south), postcose, site of playground (small, medium, large, and extra-large) and season (autumn: March, April, May and winter: June, July August). Two random effects, postcode and sampling site were included in the model to account for clustering on a 3×4×2×2×2-level sampling site and postcode. Univariable models were constructed including one main effect and two random effects per model, then a backward stepwise multivariable model including the interactions terms, main effects and random effects. In the multivariable model, variables that were not statistically significant at α = 0.05 were excluded from the model with the assumption that they were not confounders. Variables were retained in the model if they were significant or part of their interaction terms was significant or they were considered as confounders. Random effects were excluded from the model if they explained very little from the models based on the change in Akaike’s Information Criterion (AIC), Bayesian Information Criterion and deviance χ². Model selection was also evaluated by the likelihood ratios using the generalized linear model (GLM) framework using R version 3.3.1.

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Author Contributions
TVR conceived the study. EM collected samples and performed culture and DNA extraction. SGL performed molecular characterization and contributed to drafting the manuscript. TVR and EM performed data analysis and interpretation of results and drafted the manuscript. All authors approved the final manuscript.

Additional Information
Competing financial interests: The authors declare no competing financial interests.

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