Clostridium difficile and idiopathic neonatal diarrhoea in Australian piglets

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This thesis is presented for the degree of Doctor of Philosophy of
The University of Western Australia

Microbiology and Immunology
School of Pathology and Laboratory Medicine

26 March 2015

Abstract

Clostridium difficile has emerged in pork producing countries worldwide as a leading cause of enteric disease in piglets less than 7 days of age. Outside Australia this is primarily due to a single ribotype, RT 078. While this association has been well studied elsewhere, nothing is known about porcine CDI in Australia despite reports of idiopathic scour. It was hypothesised that *C. difficile* would be present in Australian pig herds but the epidemiology would be different due to our geographic isolation, rigorous import restrictions on live animals and low pig stocking density, limiting the applicability of available data to the local setting.

To understand this organism in the Australian context, epidemiologic approaches were used to evaluate *C. difficile* in Australian farrowing units, including prevalence and risk factors such as environmental contamination. Genetic analyses were employed to characterize the unique Australian strains isolated in these studies and determine the most reliable diagnostic tools for a genetically diverse and heterogeneous population. The relationship between Australian porcine *C. difficile* strains and enteric disease was assessed in a mouse and piglet model of infection.

Prevalence studies revealed *C. difficile* was commonly found in Australian piggeries, with 60% prevalence in a retrospective analysis of diagnostic samples and 67% in a period prevalence study of scouring and non-scouring neonatal herds. These rates are higher than that reported in diagnostic and period prevalence studies from major pork producing countries. Key aspects of CDI were confirmed, including age-dependent colonisation of piglets ≤ 7 d of age and asymptomatic carriage in affected herds, similar to other porcine enteropathogens. RT 078 was not isolated from Australian piglets. Instead there was a heterogeneous mix of RTs, the majority of which (71 and 61%, respectively) had not been previously described in animals or humans either locally or outside Australia. Strains were overwhelmingly toxigenic (87%) and A-B+ variant strains were common. There was overlap between PCR ribotypes isolated from humans, piglets and other animals but an epidemiological link was not obvious.

Environmental contamination with *C. difficile* spores was examined prospectively in a 9-month study in a newly commissioned farrowing shed. Spore density was 1.2×10^4 spores/ pen in 61% of pens 1 month after baseline experiments revealed spore numbers

were below the detectable limit. Contamination increased to 4.08 x 10^5 spores/ pen in 82% of pens by the end-point. There was evidence that an extraneous source of spores was driving contamination; scouring illness was minimal and spore load in pens containing scouring piglets and their near environment was not significantly greater than other pens in the shed. The finding that *C. difficile* resisted pond-based effluent treatment and was likely disseminated into the environment via effluent by-product recycling practices such as hosing and flushing of farrowing pens confirmed this.

Comparative genomic analysis of a representative clade 5 ST 11 strain, AI 35 (RT UK 237, A-B+CDT+), revealed a novel PaLoc structure, with *tcdA* and *tcdC* deleted and a novel *tcdE*. *tcdB* was intact but AI 35 produced a variant CPE in cell culture, consistent with other *tcdB*-variant *C. difficile* strains that have the same cytotoxic potency as the highest toxin producing *C. difficile* strain, VPI 10463. The AI 35 CdTLoc was complete and contained an intact copy of the CDT expression regulator cdtR, unlike RT 078. This suggested that AI 35 was a more proficient binary toxin producer than RT 078 but this was not proven experimentally. AI 35 retained a fragment of the *cdd1* gene whose acquisition has been phylogenetically dated to about 1,300 years ago, making it older than RT 078. AI 35 was further characterized by toxin B quantitation in Vero cells and virulence potential in a mouse model of infection. AI 35 expressed toxin B at low levels; approximately 25-fold less than RT 027 and RT 078 strains, but similar levels to strain 630, a low toxin producing strain. This did not correlate perfectly with clinical virulence in the mouse model; AI 35 produced more weight loss than a RT 078 strain, suggesting that toxin quantity is not associated with clinical outcome, or that CDT was intrinsic to virulence.

Five assays were evaluated for their suitability in detecting *C. difficile* in piglet feces. The diverse strain population, broad geographic distribution of sampling sites, and sample transport logistics in Australia provided a unique scenario for assessing the local performance of assays for detecting CDI in piglets. The assays comprised a loop-mediated isothermal amplification (LMIA)-PCR for *tcdA* (illumigene *C. difficile*; Meridian), a real-time PCR for *tcdB* (GeneOhm Cdiff; Becton Dickinson), two-component enzyme immunoassays (EIA) for *C. difficile* glutamate dehydrogenase (GDH) (EIA-GDH) and TcdA/TcdB (EIA-TcdA/TcdB) (C. diff Quik Chek; Alere), and direct culture (DC) (*C. difficile* chromID agar; bioMerieux). The assays for detection of

the organism were compared against enrichment culture (EC), and assays for detection of toxins/toxin genes were compared against EC followed by PCR for toxin genes (toxigenic EC [TEC]). The recovery of *C. difficile* by EC was 39.5% (n = 62/157), and TEC revealed that 58.1% (n = 36/62) of isolates were positive for at least one toxin gene (*tcdA*/*tcdB*). Compared with those for EC/TEC, the sensitivities, specificities, positive predictive values, and negative predictive values were, respectively, as follows: DC, 91.9, 100.0, 100.0, and 95.0%; EIA-GDH, 41.9, 92.6, 78.8, and 71.0%; EIA-TcdA/TcdB, 5.6, 99.2, 66.7, and 77.9%; real-time PCR, 42.9, 96.7, 78.9, and 85.4% and LMIA-PCR, 25.0, 95.9, 64.3, and 81.1%.

Direct faecal culture on CA outperformed toxin- and molecular-based assays in detecting *C. difficile* in piglet faeces. This was true across all RTs. This method had a number of additional benefits including simplicity of use, low-cost, rapid turnaround and ability to isolate strains for toxin gene profiling and genotyping.

Spores of unique Australian strains of toxigenic *C. difficile* isolated from scouring piglets were inoculated into newborn piglets in a snatch farrowed model of infection. Clinical manifestations of disease including classic microscopic lesions of porcine CDI (caecal and colonic lesions and mesentritis), mesocolonic oedema and faecal toxin were identified significantly more often in culture positive animals than culture negative. CDI lesions were also significantly more severe in culture-positive animals. Microscopic luminal “volcano” lesions, the hallmark of severe CDI in piglets were identified in 5 animals, positive for toxigenic strains.

An RT 078 endemic strain infected some piglets. Although RT 078 produced a numerically greater mean CDI lesion score, the mean microscopic lesion score in *C. difficile* positive piglets was not significantly different between toxigenic strains with more than 1 score/ strain. (RT 078: 8, AI 35: 3, VP27: 5, p = 0.344). This suggested that strain-dependent virulence was similar. Scouring was not a good indicator of disease; it did not correlate with culture-positive animals. Although this has been previously reported in natural infection and previous infection experiments, it may be a consequence of the feeding regime chosen for this experiment.

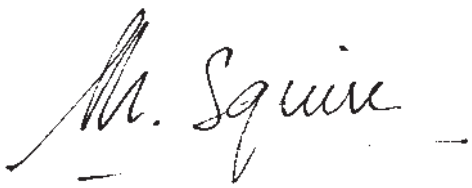
This is the first comprehensive study of *C. difficile* in Australian piglets. Collectively this data demonstrate that genotypically unique strains are prevalent in the neonatal

piglet population in Australia and the farrowing environment, presenting a transmission risk. Australian strains of *C. difficile* are capable of producing clinical manifestations of CDI in neonatal piglets. Local veterinary practitioners now have a case definition for CDI and verified, easy to use laboratory techniques to diagnose infection with this organism in piglets.

Declaration

Unless otherwise indicated, experiment design, data collection and analysis related to this thesis was conducted by the author.

This thesis was professionally edited by Dr Margaret Johnson of The Book Doctor, in accordance with the guidelines established by the Institute of Professional Editors and the Deans and Directors of Graduate Studies.



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Abbreviations

ABARES	Australian Bureau of Agricultural and Resource Economics and Sciences
ACT	Australian Capital Territory
ADP	Adenosine triphosphate
AGRF	Australian Genome Research Facility
ANOVA	Analysis of variance
AMR	Antimicrobial resistance
APIQ	Australian pork industry quality assurance program
APL	Australian Pork Limited
APSA	Australasian Pig Science Association Inc.
ARU	Anaerobe reference laboratory
ASTM	American society for Testing and Materials (International)
ATCC	American type culture collection
BA	Blood agar
BHIB	Brain heart infusion broth
BI	Restriction endonuclease group BI
bp	Base pair(s)
BSA	Bovine serum albumin
BSL	Biosecurity level
BU	Breeder unit
CA	ChromID® *C. difficile* agar
CA-CDI	Community-acquired *C. difficile* infection
CARD	The comprehensive antibiotic resistance database
CCFA	Cycloserine cefoxitin fructose agar
CDAD	*C. difficile*-associated diarrhoea
CDC	US Centers for Disease Control
CDCD	Caesarean derived colostrum deprived
CDI	*Clostridium difficile* infection
CDT	Binary toxin of *C. difficile*
CdtLoc	Binary toxin locus of *C. difficile*
cdtA	Gene encoding binary toxin subunit A of *C. difficile*
CdtA	Binary toxin subunit A (enzymatic component) of *C. difficile*
cdtB	Gene encoding binary toxin subunit B of *C. difficile*

CdtB	Binary toxin subunit B (catalytic component) of *C. difficile*
cdtR	Gene encoding CdtR regulatory response protein of *C. difficile*
cfu	Colony forming unit(s)
CPE	Cytopathic effect
CO_2	Carbon dioxide
CRC	Cooperative research centre
CTn	Conjugative transposon(s)
^0C	Degrees Celsius
d	Days
DepC	Diethyl pyrocarbonate
DC	Direct culture
dH_2O	Deionised water
dNTP	deoxynucleoside triphosphate
EC	Enrichment culture
ECDC	European Centre for Disease Prevention and Control
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
EPA	US Environmental Protection Agency
ETEC	Enterotoxigenic *E. coli*
FCS	Foetal calf serum
FMD	Foot and mouth disease
FTF	Farrow to finish
g	Gram(s)
g	Gravity
GDH	Glutamate dehydrogenase
GIT	Gastrointestinal tract
GTP	Guanosine triphosphate
GVP	Gross value of production
h	Hours
H_2O	Water
HA-CDI	Healthcare-acquired *C. difficile* infection
HP H_2O	High purity water
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ISU	Iowa State University

JETACAR	Joint Expert Technical Advisory Committee on Antibiotic Resistance (Australia)
KCL	Potassium chloride
L	Litres
LCT	Large clostridial toxin(s)
m	Months
MIC	Minimum inhibitory concentration
min	Minute(s)
$MgCl_2$	Magnesium chloride
MGE	Mobile genetic element(s)
MHA	Mueller-Hinton agar
mL	Millilitre(s)
MLS_B	Macrolide-lincosamide-streptogramin B
MLST	Multi-locus sequence typing
MLVA	Multi-locus variant analysis
mM	Millimole(s/ar)
MRSA	Methicillin-resistant Staphylococcus aureus
MSDS	Material safety data sheet(s)
NaCl	Sodium chloride
NAP1	North American pulsotype 1
nm	Nanometres
NPV	Negative predictive value
NSW	New South Wales
OD	Optical density (at 360 nm unless otherwise specified)
PaLOC	Pathogenicity locus of *C. difficile*
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PHAST	Phage search tool
PMC	Pseudomembranous colitis
PMN	Polymorphonuclear leucocytes
Pork CRC	Cooperative Research Centre for High Integrity Australian Pork
PPV	Positive predictive value
PRRS	Porcine reproductive and respiratory syndrome
QLD	Queensland

QUAST	Quality assessment tool for genome assemblies
RCM + GCC	Robertson's cooked meat medium + gentamycin, cycloserine, cefoxitin
REA	Restriction endonuclease analysis
rpm	Revolutions per minute
rRNA	Ribosomal ribosenucleic acid
RT	Ribotype
RT-PCR	Real time PCR
SA	South Australia
SLPs	Surface layer proteins
SNAR	Snatch farrowed artificially reared
SNP	Single nucleotide polymorphism(s)
SSCC	Sterile site culture collection
TA	Taurocholic acid
TC	Toxigenic culture
TCCFA	Cycloserine cefoxitin fructose agar with 0.1% taurocholic acid
tcdA	Gene encoding toxin A of *C. difficile*
TcdA	Toxin A of *C. difficile*
tcdB	Gene encoding toxin B of *C. difficile*
TcdB	Toxin B of *C. difficile*
tcdE	Gene encoding tcdE protein (putative holin) of *C. difficile*
TcdE	TcdE protein (putative holin) of *C. difficile*
tcdC	Gene encoding tcdC of *C. difficile*
TcdC	Negative regulator of *C. difficile* toxin production (controversial)
tcdR	Gene encoding tcdR of *C. difficile*
TcdR	Positive regulator of *C. difficile* toxin production
TcsL	Lethal toxin of *C. sordellii*
TEC	Toxigenic enrichment culture
TLR	Toll-like receptor
Tris	Trishydroxymethylaminomethane
Tris-HCl	Trishydroxymethylaminomethane-buffered hydrochloric acid
µg	Microgram(s)
µl	Microlitre(s)
µM	Micromole(s)
UPGMA	Unweighted pair group method with arithmetic means

UWA	The University of Western Australia
v/v	Volume to volume
VIC	Victoria
WA	Western Australia
WGS	Whole genome sequencing

Contents

Tables

Figures

Acknowledgements

This journey began with a visit to a piggery. Forty years of life experience could not have prepared me for the sensory assault of noise, smell and sights that ensued. Thousands of plump pink piglets squealing and squabbling in their pens created quite a spectacle. The onslaught settled over several hours until we reached a farrowing unit that operated as a de facto hospital wing. This place was differentiated by a complete lack of noise. Instead, row after row of moribund piglets lay motionless, silent and covered in faeces in their pens, besieged by an enteric disease that no one could identify.

I dedicate this work to those stock hands who worked tirelessly, also covered in faeces, to offer supportive treatment to the piglets, and the veterinarians and farmers who still face the challenge of identifying this disease today. And to the Australian Pork Industry who funded my investigation via The Australian Biosecurity Cooperative Research Centre for Emerging Infectious Disease. In particular I would like to thank Dr Pat Mitchell and Dr Hugo Dunlop for their support. I hope I have provided some answers. The mathematician Mark Kac stated, ‘A proof is that which convinces a reasonable man.’ I believe I have presented such proof herein.

Writing a thesis is a satisfying and mostly solitary indulgence that would never have been completed if it were not for my husband Philip and children Olivia and Angus whose formidable work ethic is inspirational. I am indebted to my supervisor Thomas Riley, mostly for his patience, but also for his support, humour and broad worldview. I have had the pleasure of working with some amazing people in the Riley Lab, but Daniel Knight, Stacey Hong and Su Chen Lim deserve special mention. I wish you every success as you embark on your PhD journey and will enjoy watching your careers unfold.

Publications

As with all multi-author papers, it is sometimes difficult to accurately appraise each author's role. In all the papers listed below, authorship was based on the National Health & Medical Research Council/Australian Vice-Chancellors' Committee guidelines, i.e. I made a significant contribution to: a) conception and design, or analysis and interpretation of data; and b) drafting the article or revising it critically for important intellectual content; and c) final approval of the version to be published. In addition, for all publications on which I am the first author, I was responsible for writing the manuscripts in question with editorial assistance from my co-author(s). The contribution to authorship as a percentage appears below:

Elliott B, **Squire MM**, Thean S, Chang BJ, Brazier JS, Rupnik M, Riley TV. New types of toxin A-negative, toxin B-positive strains among clinical isolates of *Clostridium difficile* in Australia. J Med Microbiol. 2011; 1108-11.

Wrote introduction and assisted with final editing of paper, performed C. difficile UK 237 characterisation work (30% contribution).

Squire MM, Lim SC, Foster NF, Riley TV. Detection of *Clostridium difficile* after treatment in a two-stage pond system. van Barneveld R J, editor. Adelaide, Australia: APSA Biennial Conference, Australasian Pig Science Association; 2011. p. 215.

Designed experiments, assisted with sample collection and analysis, wrote paper (70%).

Hensgens MP, Keessen EC, **Squire MM**, Riley TV, Koene MG, de Boer E, Lipman LJA, Kuijper EJ, and the European Society of Clinical Microbiology and Infectious Diseases Study Group for *Clostridium difficile* (ESGCD). *Clostridium difficile* infection in the community: a zoonotic disease? Clin Microbiol Infect. 2012; 635-45.

Contributed to writing of the paper by clarifying/ adding aspects relating to animals and C. difficile (10%).

Squire MM, Riley TV. *Clostridium difficile* infection: the next big thing! Microbiology Australia. 2012; 135.

Wrote the initial draft of the paper, revised it critically (90%).

Squire MM, Riley TV. *Clostridium difficile* Infection in humans and piglets: a 'One Health' opportunity. Curr Top Microbiol Immunol. 2013; 299-314.

Wrote the initial draft of the paper, revised it critically (90%).

Boseiwaqa LV, Foster NF, Thean SK, **Squire MM**, Riley TV, Carson KC. Comparison of ChromID *C. difficile* agar and cycloserine-cefoxitin-fructose agar for the recovery of *Clostridium difficil*e. Pathology. 2013; 495-500.

Performed laboratory work relating to piglet strains on ChromID C. difficile agar (20%).

Squire MM, Carter GP, Mackin KE, Chakravorty A, Noren T, Elliott B, Lyras D, Riley TV. Novel molecular type of *Clostridium difficile* in neonatal pigs, Western Australia. Emerg Infect Dis. 2013 May; 790-2.

Performed all experiments related to isolation and molecular characterisation of C. difficile strain AI 35, confirmed genetic analyses, wrote the initial draft of the paper (70%).

Knight DR, **Squire MM**, Riley TV. Nationwide surveillance study of *Clostridium difficile* in Australian neonatal pigs shows high prevalence and heterogeneity of PCR ribotypes. Appl Environ Microbiol. 2015; 119-23.

Knight D, **Squire MM**, Riley TV. Prevalence and molecular characterisation of *Clostridium difficile* in neonatal piglets in Australia. Manipulating Pig Production XIV, Australasian Pig Science Association 2013:198.

Designed experiments, part supervision of sample collection, laboratory work and data analysis, extensive contribution to writing of papers and report for funding body (40%).

Knight DR, **Squire MM**, Riley TV. Laboratory Detection of *Clostridium difficile* in Piglets in Australia. J Clin Microbiol. 2014; 3856-62.

Designed experiments, part supervision of sample collection, laboratory work and data analysis, extensive contribution to writing of paper and report for funding body (40%).

Presentations

Oral presentations

May 2010: Telethon Institute for Child Health Research breakfast meeting, Perth, WA. Title: Porcine *C. difficile* in Australia.

May 2010: Bacteriology Research Group, Microbiology & Immunology, UWA, Perth, WA. Title: Porcine *C. difficile* associated disease in Australia.

August 2010: Australian Council for Quality and Safety in Healthcare National *C. difficile* Workshop, Sydney, NSW. Title: *C. difficile* in animals.

September 2010: 3rd ICDS, Bled, Slovenia. Title: A novel molecular type of *C. difficile* in neonatal pigs in Australia.

January 2011: Pacific Biotekindo/Departemen Patologi Klinik *C. difficile* seminar & workshop, Jakarta, Indonesia. Title: *C. difficile* in animals.

March 2011: PathWest Continuing Education Program, Perth, WA. Title: *C. difficile* in pigs: is this the aporkalypse?

July 2011: Australian Society for Microbiology National Scientific Meeting, Hobart, Tasmania. Title: A novel molecular type of *C. difficile* in neonatal pigs in Australia.

August 2011: Centre for Nursing Education, Graduate Certificate in Infection Prevention and Control course, Perth, WA. Title: *C. difficile* infection: implications for infection control.

October 2011: Animal Disease Diagnostic Division, Ministry for Food, Agriculture, Forestry and Fisheries, Seoul, Republic of Korea. Title: *C. difficile* in piglets.

November 2011 (presented by Tom Riley due to family illness): Australasian Pig Science Association (APSA) Conference, Adelaide, SA. Title: *C. difficile* in piggery effluent.

May 2014: Meat and Livestock Australia (MLA) Antimicrobial Resistance Symposium, Canberra, ACT. Title: *C. difficile* and antibiotic use.

Poster presentations

May 2010: Australian Biosecurity Cooperative Research Centre for Emerging Infectious Diseases National Workshop, Fraser Island, Qld. Title: Prevalence and molecular characterisation of *C. difficile* in piglets with and without diarrhoea in Australia.

May 2010: Pan Pacific Pork Expo, Gold Coast, Qld. Title: A pilot study of *C. difficile* prevalence in neonatal piglets with and without scours in Australia (poster session cancelled prior to meeting).

August 2010: Combined Biological Sciences Meeting, Perth, WA. Title: A pilot study of *C. difficile* prevalence in neonatal piglets with and without scours in Australia.

September 2012: 4th International *Clostridium difficile* Symposium (ICDS), Bled, Slovenia. Title: A novel molecular type of *Clostridium difficile* in neonatal pigs in Australia lacks *tcdA* and *tcdC* but causes greater morbidity than 078 strains.

September 2011: 7th International Meeting on the Molecular Biology and Pathogenesis of Clostridia (ClostPath), Ames, Iowa. Title: Detection of *Clostridium difficile* in piggery effluent after treatment in a two-stage pond system.

January 2013 (with Daniel Knight): 8th International Meeting on the Molecular Biology and Pathogenesis of Clostridia (ClostPath), Port Douglas, Australia. Title: evaluation of diagnostic assays for routine laboratory identification of *Clostridium difficile* in the faeces of Australian porcine neonates.

Other achievements

Awards

Travel Grant awarded by Becton Dickinson for travel to the Australian Society for Microbiology National Scientific Meeting in Hobart, Tasmania.

$3000 Travel Grant awarded by Australian Pork Limited for travel to the 3rd International *Clostridium difficile* Symposium (ICDS) in Bled, Slovenia.

Grants

Riley TV, Squire MM, 2011/12. Title: Evaluation of diagnostic tests to detect *Clostridium difficile* in piglets. Funded by: CRC for High Integrity Australian Pork (Pork CRC) Innovation Project. $AUD 50,000

Riley TV, Squire MM, Dunlop H, de Boer B, 2012. Title: The prevalence *Clostridium difficile* in Australian piggeries and the role of *C. difficile* in neonatal scours. Funded by: Australian Pork Limited (APL). $AUD 76,000.

Riley TV, Squire MM, 2013. Title: Quantitative detection of *Clostridium difficile* in piggery effluent treated in covered and uncovered anaerobic ponds and prevalence in biosolid byproducts (including land application and compost). Funded by: APL. $AUD 90,000.

Teaching

GENE2204 Principles of Genetics, UWA: Laboratory demonstrating

MICR8814 Microbiology for Nurses, UWA: Laboratory demonstrating

Student supervision

Miss SuChen Lim: Master of Infectious Diseases, UWA. Title: Detection of *Clostridium difficile* in treated piggery effluent after two-stage pond treatment system.

Miss Hsueh-En (Stacey) Hong: 2A Honours in Microbiology, UWA. Title: The role of passive immunity in porcine *Clostridium difficile* infection.

Research Associate supervision

Mr Daniel Knight: *C. difficile* Research Associate, UWA.

Chapter 1 Introduction

1.1 Emergence of *Clostridium difficile*

Clostridium difficile is an anaerobic Gram positive spore-forming bacterium first described in 1935 as part of the normal gastrointestinal flora of human neonates (Hall and O'Toole, 1935). For over 40 years after its discovery, *C. difficile* led a life of relative obscurity with occasional reports of infections. Following a period of intense investigation in the 1970s *C. difficile* was finally shown to be the organism responsible for pseudomembranous colitis (PMC) an often fatal gut disease that occurred usually after the administration of antimicrobials, particularly clindamycin (Larson, Price et al., 1978; Tedesco, Barton et al., 1974). Shortly after, many cases of antibiotic-associated diarrhoea were also shown to be caused by *C. difficile* and, during the 1980s and 1990s, *C. difficile*-associated diarrhoea (CDAD, as it was known) became a significant hospital-acquired infection, driven by the widespread use of broad spectrum (third generation) cephalosporin antimicrobials (Gerding, Johnson et al., 1995).

Another major change in the epidemiology of *C. difficile* infection (CDI) occurred around the beginning of this millennium. Not seen previously, major epidemics of severe CDI were reported in North America and Europe following the emergence of a fluoroquinolone-resistant 'hypervirulent' strain of *C. difficile* (Kuijper, Coignard et al., 2006; McDonald, Killgore et al., 2005; Pepin, Valiquette et al., 2004). Concurrently, although less widely publicised, large outbreaks of enteritis were occurring in neonatal piglets in USA and later Europe (Debast, van Leengoed et al., 2009b; Songer, 2004). Perhaps most significant were reports that the strain of *C. difficile* that predominantly infected piglets in North America and Europe was now infecting humans (Goorhuis, Bakker et al., 2008) and was the third most commonly isolated strain of *C. difficile* in human CDI in Europe (Bauer, Notermans et al., 2011). CDI is clearly a disease that crosses the boundaries between human health, animal health, the environment and the science of microbiology.

1.2 CDI in humans

C. difficile is the leading cause of infectious diarrhoea in hospitalised humans. It is spread oro-faecally through ingestion of metabolically inactive bacterial spores, which are significant environmental contaminants due to their resistant nature. *C. difficile* can

be isolated from soil, water and the gastrointestinal tract of many animals, although it is not considered commensal. Our understanding of animal CDI is based on studies of the disease in humans.

1.2.1 Clinical features

CDI is essentially a disease of the colon; involvement of the small intestine is rare. Human disease is characterised by a spectrum of clinical manifestations encompassing asymptomatic carriage without toxin production, at its mildest form, to severe PMC and, rarely, fulminant colitis with toxic megacolon and intestinal perforation. CDI typically presents as non-haemorrhagic watery diarrhoea, accompanied by fever, abdominal pain and leucocytosis commencing 48–72 hours post infection (Gebhard, Gerding et al., 1985). Non-diarrhoeal presentation with acute abdomen is also possible. This occurs with gastrointestinal ileus where faecal fluid collects in loops of dilated, atonic colon (Kelly and LaMont, 1998).

Histologic lesions include bowel wall oedema, erythematous/granular mucosa, friability and inflammation. Colonic pseudomembranes, if present, are pathognomonic for *C. difficile* disease. These are characteristic yellow mucosal plaques produced following enterocytic actin cytoskeleton disruption resulting in shallow ulcerations in the mucosa with leucocytic infiltrates and mucus and fibrin exudates (Gebhard, Gerding et al., 1985).

Extraintestinal *C. difficile* infections including bacteraemia, soft tissue infections, abscesses of abdominal organs and pleural effusion/empyema have also been reported (Elliott, Reed et al., 2009; Jacobs, Barnard et al., 2001).

1.2.2 Pathogenesis

The fundamental requirements for development of CDI include (i) disruption or absence of protective colonic microbiota, (ii) presence of the organism in the environment, and (iii) production of the major virulence factors, toxins A and B (Figure 1.1). *C. difficile* possesses other virulence factors that may contribute to pathogenesis by facilitating colonisation or immune evasion. The spectrum of CDI severity may be explained by strain-dependent variations in expression of virulence factors along with differences in host immunity.

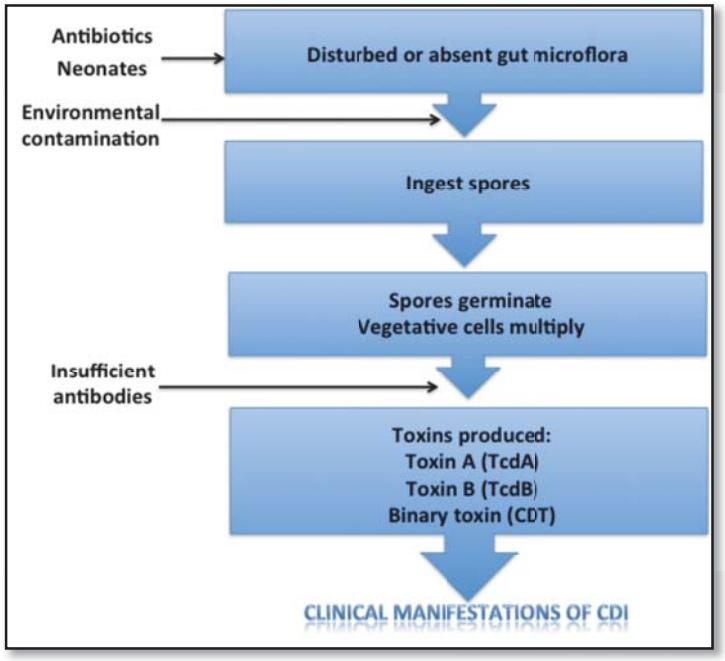

Figure 1.1 Pathogenesis of *C. difficile* infection

1.2.3 Colonisation resistance

C. difficile infection occurs opportunistically when the niche usually occupied by endogenous intestinal flora is disrupted, allowing spores to germinate in the gut and produce toxins. Antibiotic-mediated alteration of colonic flora was established in the 1940s but its association with CDI was not confirmed until the 1980s (Britton and Young, 2012; Wilson and Freter, 1986; Wilson, Silva et al., 1981). These studies in mice and hamsters showed that *C. difficile* colonisation is suppressed by endogenous colonic flora, a protective mechanism known as colonisation resistance. Conversely, CDI can be experimentally induced following administration of antimicrobials in animal models (Chen, Katchar et al., 2008; Razaq, Sambol et al., 2007). Loss of colonisation resistance may increase also the risk of CDI associated with use of chemotherapeutic agents (Cudmore, Silva et al., 1982) and inflammatory bowel disease (Ananthakrishnan, Issa et al., 2009). Similarly, neonates are susceptible to *C. difficile* colonisation because of an immature colonic flora (McFarland, Brandmarker et al., 2000).

Several mechanisms have been postulated to explain the protective effect of colonisation resistance. These include (i) negative regulation of bile acid derivatives metabolised by gut flora and required for *C. difficile* spore germination, (ii) physical

exclusion of *C. difficile* and successful competition for nutrients, (iii) inhibition of *C. difficile* growth through production of bacteriocins by gut flora, and (iv) stimulation of innate host immune response by microbiota-induced TLR signalling (Britton and Young, 2012).

Recent distal-gut microbiome studies have demonstrated the profound impact of ciprofloxacin on human colonic flora (Dethlefsen, Huse et al., 2008; Dethlefsen and Relman, 2011). Temporal analysis showed that microbial communities do not commence recovery until four weeks post-treatment and may not re-establish completely, or in their original composition, in particular taxonomic diversity. Failure to re-establish colonic flora may also be associated with recurrent CDI. Using deep 16S rRNA sequencing, Chang and colleagues showed reduced diversity in microbial gut taxa where patients presented with recurrent versus an initial episode of CDI (Chang, Antonopoulos et al., 2008). The importance of normal colonic microbiota in CDI is underscored by successful treatment regimens for recurrent CDI involving restoration of colonisation resistance. These include tapered or pulsed administration of vancomycin with or without a probiotic adjunct, or faecal microbiota replacement (Bakken, Borody et al., 2011; McFarland, Elmer et al., 2002; O'Horo, Jindai et al., 2014). Fidaxomicin, the first in a new class of narrow spectrum macrocyclic antibiotics, has recently been developed to treat CDI. In clinical trials there was a significantly lower rate of CDI recurrence with fidaxomicin than with vancomycin treatment, possibly due to preserved faecal microbiota (Louie, Miller et al., 2011).

1.2.4 Virulence factors

1.2.4.1 Toxins

C. difficile produces two major virulence factors, toxins A (tcdA) and B (tcdB), that are responsible for the characteristic symptoms of CDI. These exotoxins glucosylate and inactivate Rho-subtype GTPases of host cells to disrupt tight junctions between intestinal epithelia and actin cytoskeleton assembly. This mediates enterocytic necrosis and initiates host immune cell activation and the release of proinflammatory cytokines that lead to acute inflammation and further enterocyte destruction (Kelly and Kyne, 2011; Pothoulakis, 2000; Voth and Ballard, 2005). The key feature in animal models of *C. difficile* toxin A-induced enterocolitis is an acute inflammatory infiltrate characterised by migration of neutrophils into the intestinal mucosa (Pothoulakis, 2000).

Systemic effects of CDI may be attributable to toxins A and B as they disseminate systemically and produce extraintestinal symptoms in mouse and hamster experiments (Steele, Chen et al., 2011).

In recognition of their role as the primary virulence factors, tcdA and tcdB, and the genes that encode them, are targets for CDI diagnosis. The majority of *C. difficile* strains produce both toxins A and B (A+B+). Early animal experiments concluded that toxin A was essential for disease as toxin B alone failed to produce symptoms (Lima, Lyerly et al., 1988; Lyerly, Saum et al., 1985). This led to diagnostics based solely on toxin A and the erroneous belief that strains producing only toxin B due to a deletion in the repeating domain of *tcdA* (A-B+) did not cause disease. This model was challenged when an A-B+ strain was isolated from a nosocomial outbreak of CDI (Alfa, Kabani et al., 2000). Subsequent analyses showed increased disease severity in A-B+ outbreaks (Johnson, Kent et al., 2001) and an apparent increase in prevalence (Drudy, Fanning et al., 2007; Kim, Riley et al., 2008); recent advances in genetic manipulation of *C. difficile* toxin genes will allow the relative contribution of each toxin to disease to be determined (Heap, Pennington et al., 2007; Kuehne, Collery et al., 2014; Lyras, O'Connor et al., 2009).

Some strains produce an additional binary actin-ADP-ribosylating toxin (CDT), the role of which is not as well elucidated although it is postulated to assist with colonisation. CDT is a binary toxin consisting of two components: cdtB, which binds to cells and translocates cdtA, which catalyses the actin-ADP ribosylation reaction. Strains that produce only CDT and not tcdA or B (A-B-CDT+) colonise the gut but do not cause symptomatic disease in hamsters (Geric, Carman et al., 2006). This is supported by recent evidence suggesting that CDT depolymerises the cell cytoskeleton to produce microtubule cell protrusions to facilitate bacterial adhesion to intestinal epithelia (Aktories, Schwan et al., 2012; Schwan, Stecher et al., 2009). Binary toxin-producing strains are increasing in prevalence, independent of the emergence of the CDT-positive BI/NAP1/027 epidemic strain (Barbut, Mastrantonio et al., 2007; Bauer, Notermans et al., 2011; Spigaglia and Mastrantonio, 2004). They are also associated with community-acquired infection and more severe disease (Barbut, 2005). Between 20 and 100% of animal strains produce binary toxin, compared with <10% of human isolates (prior to the BI/NAP1/027 outbreak) (Rupnik, 2007).

1.2.4.2 Molecular organisation of toxin genes: PaLoc and CdtLoc

Toxin A and toxin B are encoded by the genes *tcdA* and *tcdB* that reside on a 19.6 kb region of the chromosome known as the Pathogenicity Locus (PaLoc) (Figure 1.2) (Braun, Hundsberger et al., 1996; Hammond and Johnson, 1995). The PaLoc also contains the regulatory genes *tcdR* and *tcdC* that positively and negatively regulate toxin production, respectively, by altering transcription rates in response to environmental stimuli, although the role of tcdC is now controversial (Bakker, Smits et al., 2012; Cartman, Kelly et al., 2012). The gene *tcdE*, a putative holin-expression gene, is also located on the PaLoc and may be involved in toxin transport (Figure 1.2a). A 115 bp non-coding fragment replaces the PaLoc in non-toxigenic strains (Braun, Hundsberger et al., 1996; Dupuy, Govind et al., 2008; Dupuy and Sonenshein, 1998). The two components of CDT are encoded by the genes *cdtA* and *cdtB*, with both required for toxicity. CDT genes are co-located on a separate chromosomal locus (CdtLoc) with *cdtR*, a positive regulator of CDT production (Carter, Lyras et al., 2007) (Figure 1.2b).

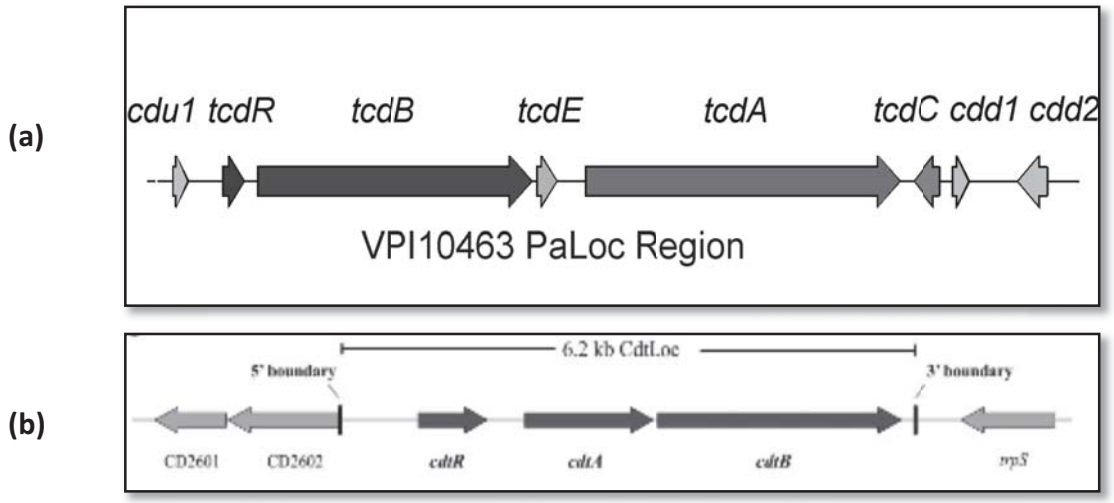

Figure 1.2 Genetic organisation of the pathogenicity loci of *C. difficile*

(a) the 19.6 Kb Pathogenicity Locus (PaLOC), and (b) the 6.2 Kb Binary Toxin Locus (CdtLoc)

Source: (Carter, Lyras et al., 2007)

1.2.4.3 Sporulation

C. difficile is a strict anaerobe and produces metabolically dormant spores as a survival mechanism when exposed to oxygen or otherwise stressed. Toxins are secreted when spores are ingested from the environment and germinate in the jejunum in response to

bile salts (Sorg and Sonenshein, 2008). During disease CDI spores are excreted into the environment by infected individuals and spread by direct contact and environmental contamination (Samore, Venkataraman et al., 1996). Spores can persist for long periods of time as they are resistant to UV, heat, desiccation and commonly used disinfectants (Gerding, Muto et al., 2008). Endogenous spore persistence occurs through resistance to both host immune attack and CDI treatments (McFarland, 2005; Paredes-Sabja, Cofre-Araneda et al., 2012), although fidaxomicin inhibits spore production (Babakhani, Bouillaut et al., 2012); hence the *C. difficile* endospore is considered the infective agent of CDI. This is supported by evidence that *C. difficile* strains defective in spore production are unable to be transmitted between infected mice (Deakin, Clare et al., 2012).

1.2.4.4 Other virulence factors

C. difficile possesses a range of other virulence factors. These include flagella, proteolytic enzymes that facilitate penetration of the intestinal mucus layer, and surface layer proteins (SLPs) associated with enterocytic adhesion. Capsule production has also been identified (Borriello, Davies et al., 1990). Recent evidence suggests that *C. difficile* is capable of biofilm production and sporulation within the biofilm. These mechanisms may contribute to the traditional biofilm functions of colonisation and avoidance of host defences, but there may also be a novel function involving endogenous spore exposure in recurrent disease (Semenyuk, Laning et al., 2014). Table 1.1 provides a complete list of putative and experimentally confirmed non-toxin virulence factors identified in *C. difficile*.

Table 1-1 Putative and experimentally confirmed non-toxin virulence factors in *C. difficile*.

Adapted from: (Vedantam, Clark et al., 2012)

Function	Description	Reference
Motility and Secretion		
Putative type IV pilus	Putative type IV pilus biosynthesis & function	(Varga, Nguyen et al. 2006)
Capsule	Poly-gamma-glutamate biosynthesis (required for capsule formation)	(Stecher and Hardt 2008)
Flagella	Flagellar biosynthesis operon and flagellin glycosylation	(Twine, Reid et al. 2009)
Adhesion & Immune Evasion		
Collagen binding proteins	Putative recognition of extracellular matrix collagen	(Sebaihia, Wren et al. 2006)
Fibronectin binding proteins	Putative recognition of extracellular matrix fibronectin	(Hennequin, Janoir et al. 2003, Barketi-Klai, Hoys et al. 2011)
Thrombospondin domain containing protein	Putative recognition of extracellular matrix fibrinogen	(Sebaihia, Wren et al. 2006)
Von-Willebrand Factor binding proteins	Putative von-Willebrand Factor binding – possibly to facilitate bacterial aggregates in serum	(Sebaihia, Wren et al. 2006)
Sortase	Class B sortase	(Sebaihia, Wren et al. 2006)
Major surface layer protein (SlpA)	Cleaved into high and low molecular weight S-layer proteins, phase variable. Contributes to host-cell adherence	(Calabi, Ward et al. 2001, Merrigan, Venugopal et al. 2013)
Cysteine protease (Cwp84)	Cleaves SlpA, possible degradation of host extracellular matrix proteins	(Janoir, Pechine et al. 2007, de la Riva, Willing et al. 2011)
Adhesin (Cwp66)	Putative adherence to host cells	(Waligora, Hennequin et al. 2001)
Haemagglutinin/ Adhesin	Putative haemagglutinin	(Sebaihia, Wren et al. 2006)
Phase-variable cell wall protein (CwpV)	Bacterial aggregation, putative immune evasion	(Reynolds, Emerson et al. 2011)
Heat shock protein (GroEL)	Host-cell adhesion	(Hennequin, Porcheray et al. 2001)
Other proteins		
Cell lysis	Putative haemolysin-like protein	(Sebaihia, Wren et al. 2006)
Collagen-specific protease	Putative degradation of collagen	(Sebaihia, Wren et al. 2006)
Global transcription regulator (Spo0A)	Positive regulation of sporulation genes, flagella pathways, metabolic pathways including glucose fermentation	(Pettit, Browne et al. 2014)

1.2.5 Host immunity

CDI presents clinically with a spectrum of symptoms and outcomes, which may be explained by variability in host immunity and innate immune response to toxin-mediated inflammation. Increased incidence of CDI in immunocompromised individuals such as the elderly and those with comorbid medical conditions is good evidence of the correlation between the inability to mount a robust systemic immune response and the severity of clinical infection (Loo, Bourgault et al., 2011). Colonised hosts with high levels of serum immunoglobulin G to toxin A (anti-tcdA IgG) are more likely to become asymptomatic carriers than to develop fulminant disease (Kyne,

Warny et al., 2000). Serum antitoxin B antibodies are also higher in these cases. Individuals with higher anti-tcdA IgG at day 12 of an initial episode of *C. difficile* diarrhoea are less likely to suffer disease recurrence (Kyne, Warny et al., 2001).

Host immune responses to non-toxin virulence factors, specifically SLPs, may be protective against recurrent CDI. Patients with recurrent CDI have lower serum anti-SLP IgM antibodies than those presenting with a single episode (Drudy, Calabi et al., 2004; Kyne, Warny et al., 2001).

Components of the innate immune system may protect against CDI, although this has not been studied in humans. Lawley and colleagues (Lawley, Clare et al., 2009) demonstrated that Myd88-depleted mice succumb to fatal systemic CDI. This suggests that the TLR-$NF_{\kappa}B$ pathway that Myd88 participates in may be a protective mechanism against CDI. Wild-type mice experienced milder, self-limiting disease. Several animal studies have shown that anti-inflammatory agents can reduce intestinal injury (Anton, O'Brien et al., 2004; Chen, Kokkotou et al., 2006; Kim, Kokkotou et al., 2005; Kim, Rhee et al., 2005; Kokkotou, Espinoza et al., 2009).

1.2.6 Hypervirulence

CDI rates throughout Canada (Pepin, Valiquette et al., 2004), USA (McDonald, Owings et al., 2006), and Europe (Kuijper, Barbut et al., 2008) began to rise alarmingly in the early 2000s. This was largely due to the emergence of epidemic strains belonging to restriction endonuclease type BI, North American pulsed field type 1 and PCR ribotype 027 (BI/NAP1/027). RT 027 is significantly associated with more severe disease and denoted as 'hypervirulent' (Pepin, Valiquette et al., 2004). The genetic basis of increased virulence was reported (now controversially) as an 18 base pair deletion in *tcdC* resulting in dysregulation of toxins A and B (Carter, Douce et al., 2011; Warny, Pepin et al., 2005) as well as CDT production, and a *gyrA* mutation conferring fluoroquinolone resistance (Drudy, Kyne et al., 2007). Increased sporulation efficiency in vitro has also been reported but remains controversial (Burns and Minton, 2011). Other 'hypervirulent' strains such as RT 017 (Drudy, Harnedy et al., 2007; Kim, Riley et al., 2008) and RT 078 (Goorhuis, Bakker et al., 2008) have been associated with severe disease outbreaks. RT 078 is an A+B+CDT+ strain possessing a 39 base pair *tcdC* deletion. It causes disease that presents with similar severity to RT 027-mediated infection but is associated with community-acquired disease in younger patients. It is

also the predominant strain isolated from pigs and cattle outside Australia (Goorhuis, Bakker et al., 2008). Human and pig strains of RT 078 *C. difficile* are genetically identical by whole genome sequence analysis, suggesting interspecies transmission (Knetsch, Keessen et al., 2013).

1.2.6.1 Genetic basis of increased virulence

Strain variation in pathogenicity ranges from the so-called 'hypervirulent' epidemic strains RT027, RT078 and RT017 to non-toxigenic strains (Drudy, Harnedy et al., 2007; Goorhuis, Bakker et al., 2008; Kim, Riley et al., 2008; Pepin, Valiquette et al., 2004). The genetic basis of increased virulence is reported as *tcdC* deletions resulting in dysregulation of toxins A and B, a novel *tcdB* that confers broad receptor binding and enhanced cell entry, CDT production, and a *gyrA* mutation conferring fluoroquinolone resistance (Carter, Douce et al., 2011; Drudy, Kyne et al., 2007; Lanis, Barua et al., 2010).

C. difficile MLST genotypes (STs) fall into one of four clades, with RT078 (ST11) forming a genetically distinct fifth clade; these lineages are conserved across microarray analysis (Stabler, Gerding et al., 2006) and WGS (He, Sebaihia et al., 2010). Clade 1 contains intermixed human and animal (porcine, equine, murine) strains, hypervirulent STs (RT027) form clade 2, animal strains (pig and bovine) with a small number of human strains fall into clade 3, and A-B+ strains are typically clade 4 (RT017, ST37, A-B+CDT-), with a sub-clade (C-I) of A-B-CDT- strains (Dingle, Elliott et al., 2014; Griffiths, Fawley et al., 2010; Stabler, Gerding et al., 2006). Clade-specific genetic differences correspond to niche-adapted virulence factors such as antibiotic resistance, motility, adhesion and metabolism (Stabler, Gerding et al., 2006).

Mobile genetic elements (MGE) constitute 11% of the *C. difficile* genome (Sebaihia, Wren et al., 2006) comprising conjugative transposons and bacteriophages integrated into the genome (prophages). These carry antibiotic resistance and virulence factor genes. He et al. suggest horizontal gene transfer and recombination events account for increased virulence in outbreak ('hypervirulent') strains (He, Sebaihia et al., 2010).

Bacteriophages ('phages') are viruses that specifically infect bacteria. Phage contribution to virulence in other pathogens is well documented, such as *E. coli* outbreak strains O157:H7 and O104:H4, where shiga-toxin is carried on *Stx2*-encoding phages (Fortier and Sekulovic, 2013; Muniesa, Hammerl et al., 2012). Phages are not as

well studied in *C. difficile*, but a diverse assortment of prophages has been identified (Shan, Patel et al., 2012) that may contribute to host specificity, fitness and virulence. For example, phage-mediated toxin regulation in *C. difficile* has been reported (Govind, Vediyappan et al., 2009), as has phage transduction of antibiotic resistance (Goh, Hussain et al., 2013).

Conjugative transposons (CTn) integrate into and excise from the host genome and transfer between bacteria via self-encoded genetic machinery. They contain accessory genes not involved in transfer that typically encode functions enhancing fitness in the host, especially antibiotic resistance (Brouwer, Warburton et al., 2011). Several CTn mediating antimicrobial resistance are encoded in the *C. difficile* genome. *Tn*5397 mediates tetracycline resistance (Mullany, Wilks et al., 1990) and transfers readily between *C. difficile* and several bacterial species including *Enterococcus faecalis, Bacillus subtilis* and *Streptococcus* spp. due to its preference for a ubiquitous insertion domain. *Tn*4453a and *Tn*4453b confer chloramphenicol resistance and are closely related to Tn4451 of *C. perfringens*, suggesting the possibility of mobilisation between the two clostridia. *Tn*5398 and *Tn*6194 are responsible for MLS_B resistance via *ermB*. *Tn*5398 is transferable between *B. subtilis, Staphylococcus aureus* and *C. difficile*, whereas Tn6194 is transferable between *C. difficile* and *E. faecalis* (Mullany, Allan et al., 2015). Other putative *C. difficile* CTn are closely related to either the *Tn*916 family of MGE that confers tetracycline resistance via *tetM* or *Tn*1549 responsible for vancomycin resistance via the *vanB* operon (Sebaihia, Wren et al., 2006).

1.2.7 Diagnostics

Culture of *C. difficile* from faecal specimens is straightforward with the correct media and atmospheric conditions, but does not differentiate asymptomatic carriers from those with CDI, or toxigenic from non-toxigenic strains; hence toxigenic culture (culture of *C. difficile* and demonstrating the organism is toxigenic) with detection of toxins A and B in faeces or intestinal contents is generally accepted to be diagnostic for *C. difficile* disease, and remains the gold standard for CDI diagnosis in the laboratory (Planche and Wilcox, 2011). The generally accepted anaerobic culture method uses selective media with sodium taurocholate, cycloserine, cefoxitin and fructose (TCCFA) (Foster and Riley, 2012; George, Sutter et al., 1979). This is usually accompanied by selective broth enrichment with ethanol shock to enhance spore recovery (Arroyo, Rousseau et al.,

2005; Riley, Brazier et al., 1987). A chromogenic medium has recently become available that outperforms TCCFA in terms of turnaround time and selectivity (Carson, Boseiwaqa et al., 2013).

Cell-culture cytotoxicity neutralisation has traditionally been the reference method for toxin detection because of its sensitivity and specificity; however, its long turnaround time is not ideal in outbreak situations. Commercial toxin detection enzyme immunoassay (EIA) kits provide rapid results but are considered inadequate for diagnosing CDI when used alone, and few kits have been validated for use in animals (Keessen, Hopman et al., 2011; Post, Jost et al., 2002). A PCR-based method to evaluate the presence of the toxin-encoding genes *tcdA*, *tcdB* and *cdtA/B* is currently recommended in USA (Brecher, Novak-Weekley et al., 2013), either alone or in conjunction with EIA testing (Goldenberg, Cliff et al., 2010; Keessen, Hopman et al., 2011; Swindells, Brenwald et al., 2010).

Typing of isolates is important for epidemiological purposes and a number of techniques are used, most commonly PCR ribotyping, multilocus variable number tandem repeat analysis (MLVA) and pulsed field gel electrophoresis (PFGE) (Knetsch, Lawley et al., 2013) (Figure 1.3).

PCR ribotyping
PCR ribotyping exploits differences in the spacer regions of 16S and 23S ribosomal RNA. Specific primers are used for PCR-mediated amplification of the DNA that encodes these RNA regions. This method generates a few DNA bands as visualized by gel electrophoresis; the DNA band patterns are referred to as ribotypes.

Pulsed field gel electrophoresis (PFGE)
PFGE involves using an enzyme that cuts the bacterial genome infrequently, resulting in large DNA fragments. The fragments are then slowly separated in a polyacrylamide gel that is submitted to an electrical field in which the voltage repeatedly switches. This enables the large DNA fragments to migrate varying distances through the gel according to their size. The fragments are then visualized by DNA staining to reveal differences in banding patterns that are sometimes referred to as pulsovars.

Multilocus variable number tandem repeat analysis (MLVA)
MLVA is a method of counting the numbers of repeat alleles in the genome for a series of predefined, conserved loci that are amplified by PCR. This method requires expensive equipment but is highly discriminatory, and produces a consistent numerical result (code) for each strain that should be comparable between different laboratories. This method is well known in forensic science, as it is the basis of DNA fingerprinting in humans.

Restriction endonuclease analysis (REA)
REA relies on more frequent cutting of the bacterial genome than PFGE, resulting in large numbers of DNA fragments. These fragments are separated by electrophoresis in an agarose gel. This method is usually highly discriminatory, but produces complex DNA banding patterns that can be difficult to interpret and reproduce.

Other methods
Other methods that are used for typing *C. difficile* include toxinotyping (BOX 2); multilocus sequence typing (MLST), which is similar in principle to MLVA; and amplified fragment length polymorphism (AFLP), which uses restriction enzymes to cut genomic DNA, followed by ligation of adaptors to the ends of the restriction fragments. A subset of the restriction fragments are then amplified using primers that are complementary to the adaptor and part of the restriction site fragments, with the DNA visualized following gel electrophoresis.

Figure 1.3 Summary of *C. difficile* typing methods

Source: (Rupnik, Wilcox et al., 2009)

Whole genome sequencing (WGS) has recently become more accessible, both in availability of technology/expertise and cost (Metzker, 2010). It is the most discriminatory genotyping method and is being used more frequently for *C. difficile* surveillance. Whole genomes can be compared at the single nucleotide polymorphism (SNP) level, which is particularly useful to determine transmission pathways in outbreaks where conventional typing methods have identified a single strain type (Eyre, Golubchik et al., 2012). Evolutionary dynamics can also be determined by analysing the rate of SNP accumulation or other sequence acquisition in the genome (Dingle, Elliott et al., 2014; He, Sebaihia et al., 2010). This technology has provided new insights into *C. difficile* dogma regarding transmission (Eyre, Cule et al., 2013).

1.2.8 Treatment and prophylaxis

Therapy of CDI has typically relied on removing the inciting antibiotic (or changing it to one with a narrower spectrum) and treating with the antibiotics metronidazole or vancomycin. Metronidazole treatment failure is an emerging problem (Al-Nassir, Sethi et al., 2008; Baines, O'Connor et al., 2008), as is selection of vancomycin-resistant *Enteroccocus faecium* clones (McFarland, 2005). Another limitation is ineffectiveness of these treatments against spores. Fidaxomicin has recently been added to the antibiotic treatment arsenal. This new antibiotic has the advantage of treating fulminant disease, sparing normal microflora and decreasing spore shedding (Babakhani, Bouillaut et al., 2012; Louie, Cannon et al., 2012). Several other new antibiotics are currently undergoing clinical trials (Baldoni, Gutierrez et al., 2014; Ivarsson, Leroux et al., 2014).

Non-antibiotic treatment strategies may be useful, particularly to treat recurrent or severe CDI. Treatment with *Saccharomyces boulardii* reduces *C. difficile* recurrence, but only when used as an adjunct to standard antibiotic treatment (McFarland, Surawicz et al., 1994; Surawicz, McFarland et al., 2000). Prophylaxis with *Lactobacillus* spp. probiotics is more promising (Gao, Mubasher et al., 2010; Hickson, D'Souza et al., 2007) but there is still insufficient evidence to move these products into the mainstream. Targeted probiotics in the form of orally administered spores of non-toxigenic *C. difficile* strain VP 20621 have also been successful (Villano, Seiberling et al., 2012) but clinical trials have been hampered by in vitro evidence that non-toxigenic strains can acquire the PaLoc from toxigenic strains (Brouwer, Roberts et al., 2013). Faecal microbiota transplantation has been adapted from veterinary medicine for use in humans, and is the most successful form of bacteriotherapy for CDI. Faeces from

healthy donors are transplanted into recipients where it restores colonisation resistance. The success rate of this treatment in 100 patients with recurrent CDI is close to 90% (Bakken, 2009). Faecal transplant is significantly more effective for the treatment of recurrent *C. difficile* infection than vancomycin (van Nood, Vrieze et al., 2013).

Passive antibody approaches have also been explored. Monoclonal antibodies directed towards toxins A and B have reduced CDI recurrence and severity in 200 patients in phase 2 clinical trials when administered with standard antibiotic therapy (Lowy, Molrine et al., 2010). Intravenous immunoglobulin (specifically, anti-TcdA IgG) has been used to bolster passive immunity in a small number of patients with intractable disease where surgical options are limited, or in paediatric patients (Shahani and Koirala, 2012; Wilcox, 2004) Overall, the treatment is successful, but studies so far have been uncontrolled and the numbers too small to be significant. Passive immunotherapy may provide an option for those with inadequate immunity and severe disease.

Prevention of primary CDI with active immunisation is the preferable strategy as it negates the need for antibiotics completely. Several active vaccines are currently in human trials; a toxoid vaccine consisting of inactivated toxins A and B (ACAM-CDIFF, Sanofi-Pasteur) and a recombinant toxin A and B protein (IC84, Intercell).

1.2.9 Epidemiology

1.2.9.1 Risk factors - Antibiotics

More than 90% of CDIs occur in conjunction with antimicrobial therapy, making this the most important risk factor for development of CDI in humans. This is primarily through perturbation of gut flora but also because *C. difficile* is resistant to multiple antibiotics, allowing it to colonise during treatment (Rupnik, Wilcox et al., 2009). Almost all antimicrobials have been implicated in the development of CDI, especially when given in combination (Owens, Donskey et al., 2008). As expected, broad-spectrum antimicrobials, particularly clindamycin, cephalosporins, penicillins, quinolones and the newer fluoroquinolones, are most commonly reported in association with CDI, usually in a temporal relationship with the popularity of use of a particular antimicrobial class (Boone, Goodykoontz et al., 2011). Studies examining the association between various antimicrobials and CDI may be flawed, however, as a result of poor study design (Thomas, Stevenson et al., 2003). Reliable data on the

association between nosocomial CDI and clindamycin, penicillins and cephalosporins is available (Slimings and Riley, 2014).

1.2.9.2 Asymptomatic carriage and neonates

Toxigenic *C. difficile* was first isolated from an asymptomatic neonate (Hall and O'Toole, 1935) and colonises as many as 70% of healthy neonates. Despite high colonisation rates with toxigenic strains, studies have failed to show a consistent association between *C. difficile* colonisation and neonatal CDI. Fulminant disease does occur, although less often than adult CDI (Jangi and Lamont, 2010), but the incidence of HA-CDI and CA-CDI in paediatric populations has increased in recent years (Pant, Deshpande et al., 2013). One explanation for lack of symptomatic disease development is protection by maternal placental or lactogenic antibodies.

Neonates are particularly susceptible to *C. difficile* colonisation as their gut flora does not fully establish until at least 12 months of age. *C. difficile* may be acquired within the first days of life as peak colonisation rates occur in neonates under seven days of age (Bolton, Tait et al., 1984; Pant, Deshpande et al., 2013). Children older than three years show asymptomatic colonisation frequencies similar to adults (1% - 4%) (McFarland, Brandmarker et al., 2000). The association between colonisation decrease and advancing age suggests that the establishment of normal gut flora displaces *C. difficile* and impedes the development of protective antibodies.

Genotyping studies of isolates from colonised infants and their hospital room or nursery suggest that environmental contamination is the usual source. Temporal studies confirm that colonisation rate increases with exposure to an environmental source. *C. difficile* has not been isolated from maternal vaginal cultures, and there is no correlation between colonisation rates and method of delivery or feeding (Bolton, Tait et al., 1984; Delmee, Verellen et al., 1988). Despite the absence of clinical disease, colonised neonates may play a role in transmission of *C. difficile* either directly or through environmental contamination (Hecker, Riggs et al., 2008). Between 4% and 15% of healthy adults may be asymptomatically colonised with toxigenic *C. difficile* (Eyre, Griffiths et al., 2013). Asymptomatic adults shed spores into the environment in smaller quantities than symptomatic patients (Riggs, Sethi et al., 2007). These individuals are a potential reservoir for *C. difficile* transmission (Eyre, Cule et al., 2013).

1.2.10 Changing epidemiology of human CDI

1.2.10.1 Community acquired CDI

It is now accepted that CDI is not limited to the hospital setting and disease can be acquired in the community (CA-CDI). This represents an additional *C. difficile* healthcare burden, with hospitalisation rates for those with CA-CDI approximating 40% (Khanna, Pardi et al., 2012a; Naggie, Miller et al., 2011). Disease can be severe, with one study reporting similar attributable complication rates for community and hospital onset cases and a ~3% case fatality rate (compared with ~5% for hospital acquired CDI) (Khanna, Pardi et al., 2012b).

Our understanding of the true incidence of CA-CDI is limited by few studies with inconsistent study parameters, most crucially the definition of 'community-acquired'. Reported incidence ranges from ~8 cases per 100,000 person days in the 1990s (Hirschhorn, Trnka et al., 1994) to a more recent study reporting 46 per 100,000 in 2006 (Kutty, Woods et al., 2010). Despite the apparent temporal increase in CA-CDI incidence, this conclusion may be unreliable, given differences in study design, population characteristics and diagnostic methods. Recent evidence suggests that CA-CDI incidence is increasing. A population-based US study demonstrated a four-fold increase in CA-CDI from 1991–2005 (Khanna, Pardi et al., 2012b). In Australia, CA-CDI rates doubled during 2011 and increased by 24% between 2011 and 2012 (Slimings, Armstrong et al., 2014).

Whilst reported incidence varies widely, independent studies concur that risk factors for CDI differ between hospital and community cohorts, although CA-CDI risk factors are not as clearly delineated. CA-CDI is significantly associated with younger, otherwise healthy people (particularly females), often without prior exposure to antimicrobials (Bauer, Goorhuis et al., 2008; Kutty, Woods et al., 2010; Naggie, Frederick et al., 2010; Wilcox, Mooney et al., 2008). Only one study has reported a relationship between CA-CDI acquisition and contact with a hospitalised patient (Naggie, Miller et al., 2011). Spillover of hospital strains does not fully explain CA-CDI as predominant hospital strains such as RT 027 have rarely been reported in the community setting. Community strains are also more heterogeneous, consisting of many previously unidentified PCR ribotypes (Bauer, Veenendaal et al., 2009); this suggests that other reservoirs of infection contribute to CA-CDI. A four-year WGS study of isolates from 1250 patients

with CDI at hospitals and in the community in the Oxfordshire region of England found that 45% of the 1223 isolates successfully sequenced were genetically diverse and distinct from all previous cases. This suggests a reservoir of *C. difficile* outside healthcare centres (Eyre, Cule et al., 2013). One possible explanation is exposure to animal sources of *C. difficile*.

1.3 Animal and food sources of *C. difficile*

1.3.1 *C. difficile* in animals

C. difficile is a recognised enteric pathogen in a variety of animals, including companion animals (cats, dogs, horses) and food animals (cattle, sheep, goats, pigs). Natural infection has also been described in non-human primates, Kodiak bears, prairie dogs, ostriches, camels, donkeys, seals, snakes, penguins, and elephants (Keel and Songer, 2006; Rupnik and Songer, 2010). CDI has been experimentally reproduced in piglets (Steele, Feng et al., 2010), foals (Arroyo, Weese et al., 2004) and laboratory rodents (rats, rabbits, hamsters, guinea pigs, mice) (Chen, Katchar et al., 2008; Lyerly, Saum et al., 1985). *C. difficile* has been isolated from chicken faeces in the absence of symptomatic infection, although this may be a function of study design rather than failure to produce enteric symptoms in this species (Indra, Lassnig et al., 2009; Simango and Mwakurudza, 2008; Zidaric, Zemljic et al., 2008).

Clinical presentation in animals, as in humans, encompasses a spectrum of disease ranging from asymptomatic carriage to fulminant haemorrhagic enterocolitis. Diarrhoea ('scouring') is a hallmark of most animal disease (Keel and Songer, 2006). Animal lesions vary in severity and location within the gastrointestinal tract according to species and age, although they are histologically similar to human lesions.

The risk of developing CDI in animals may be age-related. Asymptomatic carriage of toxigenic strains is commonly reported in young animals. Unlike human neonates, however, the young of some animal species commonly develop symptomatic disease. Indeed, CDI in pigs and cattle is almost exclusively a disease of neonates (Rodriguez-Palacios, Borgmann et al., 2013; Songer and Anderson, 2006). It is possible that all young animals are colonised with *C. difficile* due to the organism's ubiquity and the lack of host colonisation resistance. Longitudinal assessment shows that *C. difficile* colonisation rates decrease with age in pigs (Weese, Wakeford et al., 2010), chickens

(Zidaric, Zemljic et al., 2008), horses (Baverud, Gustafsson et al., 2003) and cattle (Rodriguez-Palacios, Koohmaraie et al., 2011). Adult horses, dogs and cats, like humans, are more likely to acquire *C. difficile* after hospitalisation and administration of antibiotics (Clooten, Kruth et al., 2008; Ruby, Magdesian et al., 2009; Songer, Trinh et al., 2009a).

There is little evidence to explain age-dependent disease development, and work thus far has focused primarily on toxin A. Neonatal rabbits do not develop symptomatic CDI and lack toxin A receptors (Eglow, Pothoulakis et al., 1992) whereas neonatal pigs possess abundant toxin A receptors and demonstrate internalisation of toxin A in vitro (Keel and Songer, 2010; Keel and Songer, 2007). Despite the extreme sensitivity of adult hamsters to *C. difficile* toxins, neonatal hamsters do not develop disease, but the binding kinetics of toxins A and B when compared with adult hamsters are not statistically different, suggestive of a mechanism other than receptor expression contributing to age-related susceptibility (Rolfe, 1991).

Although there is heterogeneity amongst animal isolates, particularly chickens and horses, the predominant genotype isolated from food production animals outside Australia is RT 078, Toxinotype V, NAP 7/8, REA group BK (Songer, Trinh et al., 2009b). This ribotype has not been isolated from animals in Australia, presumably due to import restrictions on live animals and geographic isolation. Binary toxin positive strains are also more prevalent in animals. Approximately 40% of horse isolates, 80% of pig isolates and 100% of calf isolates are binary toxin positive (Rupnik, 2007).

1.3.2 *C. difficile* in food

Concomitant with the emergence of *C. difficile* in food production animals was the finding that it contaminates retail food, including meat products, seafood, ready-to-eat salads, salad leaves and vegetables (Bakri, Brown et al., 2009; Metcalf, Avery et al., 2011; Metcalf, Costa et al., 2010; Rupnik and Songer, 2010). The first published report of *C. difficile* in retail meat was in a 2005 Canadian study (although the possibility of foodborne transmission was mooted more than 20 years earlier). Twenty per cent of beef and veal samples contained toxigenic *C. difficile* after enrichment for spores. The dominant ribotype was not identified (Rodriguez-Palacios, Staempfli et al., 2007). The prevalence was higher (42%) in retail meat samples from a single geographical location in the USA (Songer, Trinh et al., 2009b). Much lower levels of *C. difficile*

contamination have been found in European enrichment-based studies in meat products (<5%) which may be a result of different study methodologies or slaughter and food handling practices (Bouttier, Barc et al., 2010; Bouttier, Barc et al., 2007; Indra, Lassnig et al., 2009; Jobstl, Heuberger et al., 2010; Von Abercron, Karlsson et al., 2009). A 2009 Canadian study without enrichment confirmed low-level spore contamination of meat samples (Weese, Avery et al., 2009). Recent studies showing low rates of *C. difficile* colonisation in food production animals just prior to slaughter support this finding, although the degree of colonisation (4.8 log cfu/g of faeces) in individual animals may be high (Rodriguez-Palacios, Koohmaraie et al., 2011). RT 078 predominates in meat products, seafood and vegetables, indicating a possible association with animals or animal faeces, although the genotyping methods used in these studies lack the discriminatory power of whole genome sequencing. Contamination could feasibly occur through spillage of gut contents at slaughter or direct contamination by food handlers during processing or retailing.

There is abundant evidence that food products intended for human consumption contain toxigenic strains of *C. difficile*, but food-borne transmission remains unproven. Preliminary studies show that *C. difficile* spores of animal origin survive the recommended cooking temperature for ground meat (71^0C), which requires heating to 96^0C for 15 minutes to destroy spores (Rodriguez-Palacios and Lejeune, 2011; Rodriguez-Palacios, Reid-Smith et al., 2010).

1.3.3 Is *C. difficile* a zoonosis? Overlapping genotypes in humans, animals and food

Although the status of CDI as a zoonosis is indeterminate, several recent findings have emerged that suggest interspecies transfer is a possibility. There may be increasing genotypic overlap between epidemiologically linked isolates of *C. difficile* from humans, animals and food.

Investigations into the potential for zoonotic transmission of *C. difficile* have been fuelled by the increasing prevalence of RT 078 in humans, food production animals and food products. This ribotype dominates in *C. difficile* isolates from food production animals worldwide, and is now the third most common European human ribotype (Bauer, Notermans et al., 2011). In the Netherlands, where infections with RT 078 increased more than four-fold from 2005 to 2008, patients infected with this ribotype

were younger and acquired *C. difficile* in the community more frequently, particularly if they lived in rural, pig-producing areas (Goorhuis, Bakker et al., 2008). In the USA, the prevalence of RT 078 infections in humans has increased from 0.02% to 1.3% (pre-2001 to 2006) and RT 078 is increasingly associated with CA-CDI. These strains are indistinguishable or very closely related to animal RT 078 strains by PFGE analysis (Jhung, Thompson et al., 2008); similarly, RT 078 strains from Dutch humans and pigs are indistinguishable by the greater discriminatory power of MLVA subtyping (Debast, van Leengoed et al., 2009b). However, a 2011 study reported that these subtyping methods lack the necessary power to discriminate between transmission events in RT 078 strains from humans, animal and food sources (Marsh, Tulenko et al., 2011). A recent study using the greater discrimination of WGS showed that human and pig strains of RT 078 *C. difficile* were genetically identical (Knetsch, Keessen et al., 2013).

Derivation from a common source of organisms is a possibility. Airborne *C. difficile* spores up to 20 metres from a pig facility have been reported (Keessen, Donswijk et al., 2011).

No confirmed animal-to-human transmission has been reported to date. Evidence of human-to-animal transmission has been demonstrated by isolation of toxigenic *C. difficile* (including RT 027) from the faeces of hospital pet therapy dogs that had prior negative bacteriologic cultures for *C. difficile*. In addition, dogs that visited hospitals were >2 times more likely to be colonised with *C. difficile* than dogs not visiting hospitals (Lefebvre, Reid-Smith et al., 2009).

1.4 *C. difficile* in neonatal pigs

1.4.1 Emergence of *C. difficile* in neonatal pigs

Although natural infection was first reported in 1983, *C. difficile* is emerging worldwide in swine-producing areas as a major cause of enteritis in neonatal pigs (birth to seven days of age). It has now become the most diagnosed cause of enteritis in pigs in this age group in the USA (Songer and Anderson, 2006). Disease-associated mortality in neonatal piglets can reach 50%, although it is generally much lower due to good stockmanship. Surviving piglets remain, on average, 10%–15% underweight, and take additional time to wean (Songer and Uzal, 2005).

C. difficile and its toxins can be found in approximately 79% of apparently healthy piglets (Yaeger, Kinyon et al., 2007). Like human neonates, piglets do not exhibit colonisation resistance, being gnotobiotic at birth until normal microflora starts to establish around five days of age (Salminen, Isolauri et al., 1995). Consequently *C. difficile* colonisation frequency in piglets decreases from 74% at two days of age to 3.7% at 62 days of age (Weese, Wakeford et al., 2010): this suggests that all piglets in an affected farrowing facility may be colonised soon after birth. Unlike human neonates, piglets develop enteric disease following *C. difficile* colonisation. CDI symptoms and lesions have been reproduced in gnotobiotic six-hour-old colostrum-deprived piglets after oral inoculation with *C. difficile* spores and vegetative cells (Steele, Feng et al., 2010) as well as in conventional piglets obtained from farms (Arruda, Madson et al., 2013; Lizer, Madson et al., 2013). The parallels with human disease are such that a piglet model of human CDI has been developed (Steele, Feng et al., 2010).

The mechanism by which toxin-positive piglets remain asymptomatic, despite having toxin A receptors, is not yet understood. The newborn piglet is profoundly immunodeficient, and completely reliant on ingested maternal colostral antibodies and immune factors for protection; an intrinsic immune response cannot be mounted until at least three weeks of age (Stokes, Bailey et al., 2004). Differential intake of maternal colostral anti-tcdA IgG, one of the key determinants in development of symptomatic versus asymptomatic infection in humans, may provide a plausible explanation for asymptomatic carriage in neonatal piglets. Overwhelming challenge with *C. difficile* spores in the environment is another hypothesis that warrants further investigation.

1.4.2 Clinical features and diagnosis

Porcine disease is characterised by profuse non-haemorrhagic yellow pasty-to-watery scouring, although diarrhoea alone in individual animals is not a good predictor of CDI (Yaeger, Kinyon et al., 2007). Individual piglets with colitis and *C. difficile* toxin in intestinal contents are more likely to present with constipation than diarrhoea (Yaeger, Kinyon et al., 2007), but a herd history of scouring is usually the primary impetus for microbiological investigation. Extra-intestinal symptoms such as anorexia, dehydration, ascites/hydrothorax, scrotal oedema and dyspnoea have also been described, which may be attributable to systemic sepsis (or possibly toxin dissemination: (Steele, Chen et al.,

2011). Symptoms are limited to neonatal piglets and generally commence soon after birth (Songer, Post et al., 2000; Waters, Orr et al., 1998).

Necropsy findings include colitis and typhlitis. Mesocolonic oedema is a uniquely porcine lesion, although not pathognomonic for *C. difficile*. Severe oedema (≥ 3mm between loops) correlates strongly with production of *C. difficile* toxins (Yaeger, Kinyon et al., 2007). Small intestinal lesions have not been described, suggesting that porcine CDI, like human CDI, is a disease of the caecum and colon (Songer, Post et al., 2000). Indeed, colonic lesions and colitis may be CDI-specific in pigs as they are not associated with the usual enteric pathogens in this age group except for *C. perfringens* type C infections where colitis is accompanied by small intestine necrosis. Colitis is also significantly associated with the presence of *C. difficile* toxins in intestinal contents (Yaeger, Funk et al., 2002).

Microscopically, multifocal suppurative lesions ('volcano lesions') that are typical of human CDI can be seen in caecal and colonic superficial lamina propria. These are described histologically as having mucus, PMN and fibrin exudates into the lumen. Segmental erosion of the mucosa is also a histological finding as well as neutrophil aggregates in the mesocolon (Songer and Anderson, 2006).

Microbiological diagnosis is problematic; culture of the organism alone from affected animals is not diagnostic due to high asymptomatic carriage rates. A confirmatory diagnosis of CDI requires positive bacteriologic culture and toxin detection from intestinal contents accompanied by characteristic gross and histopathologic lesions at necropsy examination. Co-infection with other enteric pathogens needs to be excluded. *C. difficile* is also notoriously difficult to culture and commercially available EIA and molecular diagnostics designed to detect toxins or toxin genes in human faeces perform poorly with pig faeces (Keessen, Hopman et al., 2011).

Predisposing antimicrobials may not be required for development of CDI in piglets, although penicillin and cephalosporins have been implicated (Yaeger, Funk et al., 2002). There is evidence that antibiotic administration does not contribute to colonisation of neonates with immature gut flora but may contribute to higher rates of colonisation in three- to ten-week-old nursery animals (Arruda, Madson et al., 2013; Susick, Putnam et al., 2012). The use of ceftiofur, a third-generation veterinary

cephalosporin to which *C. difficile* is intrinsically resistant, is likely a high-risk practice, especially if administered as protective gut flora is developing.

No controlled studies of antimicrobial treatment for piglet CDI have been conducted and no commercial immunoprophylaxis is available. Treatment is largely supportive, with attention to rehydration.

1.4.3 Epidemiology

1.4.3.1 Environmental contamination

Contamination of the environment with *C. difficile* spores plays a critical role in transmissibility. In the human hospital setting, the role of environmental reservoirs in the transmission of *C. difficile* is well established (Gerding, 2009). Several groups have applied this knowledge to explore the epidemiology of porcine CDI, finding that gross contamination of swine facilities with *C. difficile* spores is commonplace. A 2011 study reported that *C. difficile* could be isolated from the faeces of piglets one hour after birth, presumably ingested from their environment, as vertical transmission was ruled out. Within two days of birth 100% of piglets had acquired *C. difficile* of the same molecular type that was found in sow faeces, sow teats, farrowing pens and air on the farm. (Hopman, Keessen et al., 2011). Asymptomatic colonisation of sows has also been reported (Norman, Harvey et al., 2009).

1.4.3.2 Piggery effluent

In the USA, residential proximity to high-density pig operations or effluent-applied crops is associated with increased community-acquired MRSA (Casey, Curriero et al., 2013) but the relationship with CA-CDI has not been investigated. Pig farm effluent is nutrient-rich and a valuable and cost-effective fertiliser and water source. The goal is sustainable animal waste treatment with minimal exposure of animals and humans to pathogens.

In Australia, effluent re-use is governed by the APL National Environmental Guidelines for Piggeries, Second Edition (Australian Pork Limited, 2011).The majority (90%) of Australian piggeries treat effluent in on-site anaerobic ponds to remove pathogens, and water is re-used to wash sheds or applied to agricultural or recreational land. Screened solids and pond sediment is removed and composted, or stockpiled on site. Ponding systems generally involve two stages; biological inactivation of pathogens via an

interconnected primary anaerobic pond followed by a facultative pond. An additional evaporative (aerobic) pond can constitute a final storage stage at some sites. On-site composting is the accepted treatment of neonatal pig mortalities (carcasses). Carcass compost is applied to land after a full bund undergoes a three-week composting period (Australian Pork Limited, 2011).

Survival of non-spore-forming pathogens in treated effluent and effluent-irrigated soils has been reported (Chinivasagam, Thomas et al., 2004) but there are no data on the survival of *C. difficile*. The role of *C. difficile*-contaminated effluent by-products in piggery contamination dynamics or the risk to public health, if any, has not been investigated.

1.4.3.3 Asymptomatic carriers

Asymptomatic animals in the farrowing shed environment may also be important. *C. difficile* spores and vegetative cells are shed into the immediate environment in the faeces of both scouring and non-scouring pigs (Hopman, Keessen et al., 2011). Asymptomatic sows may also shed spores, much like human carriers. This carrier state is emphasised in mouse studies where spore shedding increases when antibiotics are administered to asymptomatic carrier mice. Subsequent spore-mediated transmission to immunosuppressed mice has led to severe intestinal disease (Lawley, Clare et al., 2009). *C. difficile* spores persist in the human hospital environment for months and are resistant to many commonly used disinfectants.

1.5 Problem definition

1.5.1 The Australian pig meat industry

Australia is a small pork producer on the world stage, accounting for only 0.4% of world pig meat production. However Australian pork is viewed as ‘clean’, based on its freedom from the common pig diseases porcine reproductive and respiratory syndrome (PRRS) and foot and mouth disease (FMD), particularly in the lucrative South East Asia export market.

Local pig meat production consists of an estimated 2,200 pork producers and approximately 2.3 million pigs, including a national breeding herd of approximately 260,000 sows (Australian Pork Limited, 2013-2014; Australian Pork Limited, 2012-

2013) (Table 1.2). All fresh pork meat in Australia is from Australian animals; the import of live animals has been banned since the 1980s.

The estimated gross value of production (GVP) for Australian pork production was $932 million for the period 2012–13, representing approximately 2% of total Australian farm production *C. difficile* and Australian piglets

Table 1-2 Australia's pigmeat industry – distribution by total pig herd size and state

Source: (Australian Pork Limited, 2012-2013)

		Distributed by Total Pig Number					
		1– 49	50 – 99	100 – 499	500 – 999	1,000 +	Total
NSW	Sows	1,262	889	3,264	3,150	47,460	56,024
	Establishments w/Sows	332	48	75	28	46	528
	Total Pigs	5,207	3,289	16,451	21,784	439,447	486,178
	All Establishments	498	49	80	29	56	712
VIC	Sows	742	379	1,010	2,097	51,354	55,583
	Establishments w/Sows	168	20	26	15	62	291
	Total Pigs	2,823	1,496	8,454	14,784	477,499	505,055
	All Establishments	268	22	35	20	85	431
QLD	Sows	701	305	1,202	1,437	56,716	60,362
	Establishments w/Sows	192	25	33	13	71	333
	Total Pigs	3,312	2,043	9,428	19,435	604,721	638,939
	All Establishments	288	28	38	26	106	485
SA	Sows	265	269	1,958	1,618	45,726	49,836
	Establishments w/Sows	76	23	49	19	63	229
	Total Pigs	1,530	1,797	11,014	14,479	344,415	373,235
	All Establishments	115	26	52	20	69	281
WA	Sows	249	189	1,559	1,543	34,188	37,728
	Establishments w/Sows	76	12	18	16	33	156
	Total Pigs	1,129	923	5,743	11,548	249,592	268,935
	Sows	110	12	22	16	41	201
TAS	Sows	163	92	158	248	1,022	1,684
	Establishments w/Sows	36	7	5	2	4	54
	Total Pigs	606	550	1,417	1,706	8,553	12,833
	All Establishments	71	8	6	2	4	92
NT	Sows	6					6
	Establishments w/Sows	2					2
	Total Pigs	40					40
	All Establishments	5					5
Australia	Sows	3,387	2,122	9,152	10,094	236,467	261,222
	Establishments w/Sows	881	134	205	94	279	1,594
	Total Pigs	14,647	10,097	52,508	83,735	2,124,227	2,285,214
	All Establishments	1,355	146	232	114	361	2,208

In 2007, toxigenic *C. difficile* was isolated from 10/37 (27%) faecal samples from scouring Western Australian piglets. Four PCR ribotypes were identified, with RT 014 predominating (Riley TV, unpublished). No RT 078 isolates were detected, possibly due to Australia's geographical isolation and strict quarantine laws on the importation of livestock.

There are reports from specialist pig veterinarians and pork producers Australia-wide of herds with long-standing, high-morbidity, idiopathic scour in neonates that presents with clinical features of CDI. Although reports are not yet widespread, the impact of thousands of scouring animals is profound in the affected farms. A diagnosis of CDI cannot be confirmed in these cases due to the lack of diagnostic capacity in Australia, including a lack of awareness of *C. difficile* as a pathogen in this age group. The presumptive diagnosis is generally intractable ETEC colibacillosis, for which ceftiofur is the drug of choice.

No systematic studies of *C. difficile* in the Australian pork industry have been undertaken. The importance of CDI in Australian piglets, and the public health risks this poses, if any, are impossible to assess without accurate epidemiological and clinical data. Further studies are needed to understand the epidemiology, pathogenesis, most likely reservoirs of infection, and potential methods of treatment and control. There is also a need to develop better tools for diagnosing *C. difficile* in these animals.

1.6 Research objectives

This project will evaluate the following in Australian neonatal piglets:

- the epidemiology of *C. difficile* in farrowing units including risk factors and prevalence;
- the characteristics of *C. difficile* strains isolated;
- association between *C. difficile* and enteric disease, and;
- appropriate methods for diagnosis of CDI.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Culture media

Culture media and manufacturers are listed in below. Unless otherwise specified, media were stored at 4^0C and brought to 37^0C before use.

Media manufactured by PathWest Laboratory Medicine Media (Mt Claremont, Australia)

Blood Agar (BA)

Cycloserine cefoxitin fructose agar with 0.1% sodium taurocholate (TCCFA)

Chocolate agar

Robertson's cooked meat broth + gentamicin (5mg/L), cycloserine (200mg/L) and cefoxitin (10mg/L) (RCM + GCC)

Brain heart infusion broth (BHIB)

BHIB + 15% glycerol

Media manufactured by bioMérieux (Marcy l'Etoile, France):

ChromID® *C. difficile* agar (CA)

BHIB germination media:

Powdered brain heart infusion extract (Difco™, BD Pty Ltd, Franklin Lakes, NJ, USA) 37 g/L and 5 g/L yeast extract were dissolved in 800 mL of water then brought to a final volume of 1 L. This was autoclaved at 121^0C for 15 m then allowed to cool to room temperature before adding 10 mL of filter-sterilised 10% (w/v) l-cysteine, 1 mL of 10% taurocholic acid and 5 mL foetal calf serum (FCS) to each bottle. Bottles were reduced in an anaerobic chamber (Don Whitley Scientific Ltd, North Gosford, Australia) overnight then stored at room temperature until use.

2.1.2 Buffers and solutions

Buffers and solutions prepared during this study are listed below. For sterilisation, solutions were either autoclaved (121°C/15 min) or filtered through a sterile 0.2 µm-pore size syringe filter (Merck Millipore, Darmstadt, Germany). Solutions were stored at room temperature unless otherwise indicated. Pre-prepared solutions and their manufacturers are listed in Table 2.1.

10% Taurocholic acid

Taurocholic acid sodium salt hydrate	3 g
Deionised H_2O (dH_2O)	27 mL

The solution was mixed until completely dissolved then sterilised by autoclaving.

DepC-treated water

Diethyl pyrocarbonate (DepC) (0.1% v/v)
High-purity water (HP H_2O)

The solution was stirred for a minimum of 12 h and sterilised by autoclaving.

5% Chelex-100™ solution

Chelex-100™ resin	50 g
DepC-treated H_2O	1000 mL
HP H_2O	60 mL

The resin was washed three times with DepC-treated H_2O over a period of 7 d to remove inhibitors then resuspended in 1000 mL of fresh DepC-treated H_2O and stored in 20 mL aliquots at 4^0C. Immediately before use a 10% Chelex aliquot was washed with 20 mL HP H_2O, vortexing three times over 15 min. Washed 10% Chelex was diluted to a 5% solution with 40 mL HP H_2O. 5% Chelex aliquots were stored at 4^0C until use.

0.85% saline solution

Sodium chloride	0.85 g
dH_2O	100 mL

The solution was mixed until completely dissolved then sterilised by autoclaving at 121^0C/15 min.

Table 2-1 Pre-prepared solutions used in this study and their manufacturers

Chemicals and reagents	Manufacturer
100 bp DNA ladder	Invitrogen, Life Technologies, Vic, Australia
0.1% peptone solution	PathWest Media, Mt Claremont, WA
AmpliTaq Gold® polymerase	Applied Biosystems, Foster City, USA
Bovine serum albumin (BSA)	Sigma-Aldrich, St Louis, MO, USA
Chelex-100	Sigma-Aldrich, St Louis, MO, USA
Diethyl pyrocarbonate (DepC) (0.1% v/v)	Sigma Aldrich, St Louis, MO, USA
dNTP mix	FisherBiotech, Wembley, WA, Australia
L-proline aminopeptidase	Remel Inc, KS, USA
Magnesium chloride ($MgCl_2$)	Applied Biosystems, Foster City, USA
PBS	PathWest Media, Mt Claremont, WA
Potassium chloride (KCL)	Applied Biosystems, Foster City, USA
QIAxcel Alignment marker	QIAGEN, Germantown, MD, USA
QIAxcel DNA size marker	QIAGEN, Germantown, MD, USA
QIAxcel DNA dilution buffer	QIAGEN, Germantown, MD, USA
QIAxcel separation buffer	QIAGEN, Germantown, MD, USA
QIAxcel wash buffer	QIAGEN, Germantown, MD, USA
QIAxcel mineral oil	QIAGEN, Germantown, MD, USA
QIAxcel intensity calibration marker	QIAGEN, Germantown, MD, USA
Reaction buffer II	Applied Biosystems, Foster City, USA
Ringer's solution	PathWest Media, Mt Claremont, WA
Taurocholic acid sodium salt hydrate	Sigma-Aldrich, St Louis, MO, USA
Tris	Invitrogen, Life Technologies, Vic, Australia
Tris HCl	Invitrogen, Life Technologies, Vic, Australia

2.1.3 PCR primers

All primers were supplied by GeneWorks Australia. Desiccated primers were stored at -80^{0}C. Primers were reconstituted in a volume of HP H_2O appropriate to their concentration. To minimise freeze-thawing cycles 10 µl working aliquots were removed from the original suspension and stored at -20^{0}C. Any remaining resuspended primer was stored at -80^{0}C.

2.2 Bacterial strains

2.2.1 Strains used in this study

All strains of *C. difficile* used in this study, including reference and control strains and their source, are listed in Table 2.2.

Table 2-2 *C. difficile* strains used in this study

Sources: (Delmee, Homel et al., 1985; Kato, Kato et al., 1999; Lefebvre, Arroyo et al., 2006; Rupnik, Avesani et al., 1998)

***C. difficile* Strain**	**RT**	**Toxin profile**	**Origin**	**Purpose**	**Reference/ Source**
ATCC 43593	060	A-B-	Faeces, human, Belgium	Control, chamber anaerobiosis	(Delmee, Homel et al., 1985)
SSCC 28297	027	A+B+CDT+	Faeces, dog, Canada	Control, PCR ribotyping	(Lefebvre, Arroyo et al., 2006)
SE 844	-	A+B+CDT+	Faeces, human, France	Control, *tcdAB*, *cdtAB* PCR	(Rupnik, Avesani et al., 1998)
ES 173	017	A-B+	Faeces, human, Australia	Control, *tcdA* rep PCR	(Kato, Kato et al., 1999)
M 7404	027	A+B+CDT+	Faeces, human, Canada	Virulence studies	D. Lyras, Monash University
JGS 6133	078	A+B+CDT+	Faeces, piglet, USA	Virulence studies	D. Lyras, Monash University
JGS 753	-	A-B-CDT-	Faeces, piglet, USA	Control, piglet challenge	J.G. Songer, ISU
AI 35	237	A-B+CDT+	Faeces, piglet, Australia	Test strain, piglet challenge	This study
VP 27	QX8	A+B+CDT+	Faeces, piglet, Australia	Test strain, piglet challenge	This study
QP 6	QX3	A+B+CDT-	Faeces, piglet, Australia	Test strain, piglet challenge	This study

2.3 Methods

2.3.1 Recovery of *C. difficile* from piglet faeces ('clinical samples')

2.3.1.1 Rectal swab collection

Faecal samples were collected by rectal swab of piglets using Transwab® sterile rayon swabs in Amies transport medium (Medical Wire and Equipment, Wiltshire, England). Samples were collected by specialist pig veterinarians or piggery stock hands, and transported under ambient conditions to the laboratory where they were stored at 4^0C until analysis.

2.3.1.2 Gut content sample collection

Faecal samples were collected during necropsy from the gastrointestinal tract directly into sterile containers (the type of container varied by veterinarian). Samples were collected by specialist pig veterinarians only. Samples were transported under ambient conditions to the laboratory where they were stored at 4^0C until analysis.

2.3.1.3 Isolation of C. difficile *from clinical samples*

Unless otherwise stated, all incubations took place in an anaerobic chamber (Don Whitley Scientific Ltd, North Gosford, Australia) at 37°C in an atmosphere containing 80% N_2, 10% CO_2 and 10% H_2, with 75% relative humidity. The control strain *C. difficile* ATCC 43593 was used to monitor anaerobiosis. All solid media, with the exception of CA, were pre-reduced for a minimum of 2 h and liquid media for a minimum of four h prior to inoculation (Sorg and Dineen, 2009).

Solid media

C. difficile strains were cultured directly from faecal samples onto TCCFA or CA plates. The plates were incubated for 24–48 h. Putative *C. difficile* isolates on solid media were subcultured onto BA and grown under the same conditions (Bliss, Johnson et al., 1997; George, Sutter et al., 1979; Perry, Asir et al., 2010).

Spore enrichment media

Spores were enriched in faecal samples by inoculating a 20 µl loopful of faeces or faecal swab into RCM + GCC enrichment broth. The broths were incubated for 24 h in the anaerobic chamber, then sealed and transferred to a 37^0C room for 48 h. Spores were selected by adding 1 mL of enrichment broth to an equal volume of 96% alcohol, and incubating at room temperature for 60 min before inoculating a 20 µl loopful onto solid media (Borriello and Honour, 1981).

Liquid media

Spores were enriched in *C. difficile* strains by harvesting pure *C. difficile* growth from the entire surface of a lawn inoculated plate with a swab and inoculating into BHIB. The broths were incubated anaerobically for five days (Smith, Markowitz et al., 1981).

2.3.1.4 Identification of C. difficile

C. difficile cultured on TCCFA was identified by colony morphology (yellow, ground glass appearance), odour (horse dung smell) and chartreuse fluorescence under long-wave UV light (~360 nm). The identity of uncertain isolates was confirmed by Gram stain and the presence of L-proline aminopeptidase activity (Remel Inc., KS, USA) (Fedorko and Williams, 1997; George, Sutter et al., 1979).

2.3.2 Recovery of *C. difficile* from environmental samples

2.3.2.1 Farrowing shed sample collection

Environmental samples were collected using either Polywipe™ 10 cm pre-moistened sponges (Medical Wire and Equipment, Wiltshire, England) or Transwab® in 10 mL of neutralising buffer (Medical Wire and Equipment, Wiltshire, England). The Polywipe™ sponge was wiped three times (reversing direction each time) over a 100 cm^2 sampling area within a sterile template (ThermoFisher Scientific MA, USA) using a fresh pair of gloves for each sample. Sponges were placed in a sterile resealable bag and sealed. Transwab® samples were taken by holding the swab at a 30^0 angle to the sampling surface and rubbing slowly and thoroughly over the 100 cm^2 sampling area within the sterile template. The sample area was swabbed three times, reversing direction between strokes and rotating the swab tip. All samples were transported and stored at ambient temperature until use.

The number of samples required was calculated to detect circular hot spots (positive for *C. difficile*) with 95% confidence using a square grid sampling pattern (Department of Environment and Conservation, 2001):

Grid size calculation (G):

G = R/0.59
R = radius of smallest hot spot that the sampling intends to detect (in metres)
0.59 = factor derived from 95% detection probability

Number of sampling points calculation (*n*):

$n = A/G^2$
A = area to be sampled (in square metres)
G = calculation from Part (a) (in metres)

2.3.2.2 Isolation and quantitation of C. difficile *from Polywipe™ sponges*

The sponge was aseptically placed into a Stomacher® bag (Seward Ltd, West Sussex, UK) with 50 mL of ¼ strength Ringer's solution added, and processed for 30 s in a stomacher. Excess liquid was squeezed from the sponge before removing it from the bag with sterile forceps. The remaining liquid was decanted into a sterile container and stored at ambient temperature until use.

Solid media

A 1 mL aliquot of stomacher liquid was passed through a 0.45 μm pore size cellulose membrane filter (Merck Millipore, Darmstadt, Germany) and the filter cultured anaerobically on CA plates for 24-48 h (al Saif and Brazier, 1996; Dubberke, Reske et al., 2007). Putative *C. difficile* colonies were counted to obtain a viable count of cfu/mL. Colonies were confirmed as *C. difficile* by subculture onto BA and identification as per Section 2.3.1.4.

Spore enrichment media

A 1 mL aliquot of stomacher liquid was passed through a 0.45 μm pore size cellulose membrane filter (Merck Millipore, Darmstadt, Germany) and the filter inoculated into RCM + GCC and incubated for 5–7 d (al Saif and Brazier, 1996). Spores were selected as above. Putative *C. difficile* colonies were counted to obtain a viable count of cfu/mL. Colonies were confirmed as *C. difficile* by subculture onto BA and identification as per Section 2.3.1.4.

2.3.2.3 Isolation and quantitation of C. difficile *from Transwabs®*

The Transwab® tube was vortexed for 15 s and the contents decanted into a sterile 10 mL centrifuge tube (Sarstedt, Nümbrecht, Germany) which was stored at room temperature until use. The tube was vortexed and 200 μl of its contents inoculated onto pre-reduced TCCFA using the spread plate method. An additional 200 μl was inoculated into RCM + GCC and treated as above. Putative *C. difficile* colonies were counted to obtain a viable count of cfu/mL. Colonies were confirmed as *C. difficile* by subculture onto BA and identification as per Section 2.3.1.4.

2.3.2.4 Piggery effluent sample collection

Effluent samples were collected using a sterile 28 mL specimen jar (Techno-Plas Pty Ltd, St Marys, Australia) from moving effluent at a depth of at least 10 cm. Samples were taken from pre-treatment influent (outlet from farrowing sheds) and treated effluent (evaporative pond, storage tanks, inlet to storage tanks). All samples were transported and stored at ambient temperature until use.

2.3.2.5 Isolation and quantitation of **C. difficile** *from piggery effluent*

A 1 mL aliquot of each sample was diluted in 10 mL of 0.1% peptone solution and filtered through a 0.45 μm pore size cellulose membrane filter using a vacuum manifold supplied by the PathWest Laboratory Medicine (WA) Waters Laboratory. Filters were removed with sterile forceps and placed directly onto CA plates and cultured anaerobically for 24–48 h. Putative *C. difficile* colonies were counted to obtain a viable count of cfu/mL. Colonies were confirmed as *C. difficile* by subculture onto BA and identification as per Section 2.3.1.4.

2.3.3 *C. difficile* spore preparations

2.3.3.1 Spore preparation for ambient transport/storage

C. difficile isolates were cultured on chocolate agar plates for five days then all the growth was carefully scraped off with a swab and suspended in 1 mL sterile saline. This was incubated with 1 mL of 90% ethanol for 1 h then 100 μl dropped onto a 6 mm Whatman sterile filter disc (GE Lifesciences, Rydalmere, Australia) and allowed to dry at room temperature (Sorg and Dineen, 2009). Inoculated discs were then sealed in sterile aluminium foil and stored at room temperature until use.

2.3.3.2 Spore preparation for cryopreservation

C. difficile isolates were cultured on BA for four days then the entire growth was inoculated into BHIB + 15% glycerol and stored at -80^{0}C (Sorg and Dineen, 2009).

2.3.4 Genotyping of *C. difficile*

2.3.4.1 DNA extraction for ribotyping/toxin gene PCR

Chromosomal DNA was extracted and purified from overnight *C. difficile* cultures on BA. A 1 μl loopful of culture was suspended in 100 μl of 5% Chelex®100 resin freshly

prepared in DepC-treated water. The suspension was vortexed and heated at 100°C in a dry heating block for 12 min before centrifugation at 20, 817*g* (Eppendorf 5417C microfuge, Hamburg, Germany) for 12 min to pellet cell debris. A 50 µl volume of the supernatant (containing chromosomal DNA) was removed and stored at -20 °C until use as template DNA in genotyping reactions (O'Neill, Ogunsola et al., 1996). If required, DNA concentration was determined with a ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA), using the elution solution as the blank.

2.3.4.2 PCR ribotyping (amplification of 16S-23S intergenic spacer region)

Source: (O'Neill, Ogunsola et al., 1996)

Primers (Stubbs, Brazier et al., 1999)

CD16S: 5'-CTGGGGTGAAGTCGTAACAAGG-3'
CD23S: 5'-GCGCCCTTTGTAGCTTGACC-3'

Reactions

Reactions (total volume 50 µl/tube) were prepared containing 5 µl of 1x reaction buffer II, 4 mM $MgCl_2$, 0.4 µM of each primer, 0.4 mM of each dNTP, 3.75 units AmpliTaq Gold *Taq* polymerase, 0.02% BSA and 10 µl template DNA.

PCR conditions

PCR was performed on a Gene Amp® PCR system 1700 thermocycler (Applied Biosystems, Foster City, CA, USA) with the following conditions: initial denaturation of 95°C for 10 min, followed by 25 amplification cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, with a final extension cycle of 72°C for seven min. A negative control containing the reaction mix without template DNA and a positive control with DNA extracted from *C. difficile* RT 027 (SSCC 28297) was included in each experiment.

Purification of PCR products

PCR amplification products were purified using a MinElute PCR Products Purification Kit (Qiagen, Germantown, MD, USA) as per the manufacturer's instructions.

2.3.4.3 PCR assay for toxin genes tcdA, tcdB, cdtA, cdtB

Source: (Kato, Ou et al., 1991; Stubbs, Rupnik et al., 2000)

Primers

Table 2-3 Toxin gene PCR primers

Source: (Kato, Ou et al., 1991)

Gene	Primers	Sequence (5' to 3')	Position	Size of product (bp)
tcdA	NK2	CCCAATAGAAGATTCAATATTAAGCTT	2479-2505	251
	NK3	GGAAGAAAAGAACTTCTGGCTCACTCAGGT	2254-2283	
tcdA rep	NK9	CCACCAGCTGCAGCCATA	8043-8060	1,265
	NK11	TGATGCTAATAATGAATCTAAAATGGTAAC	6795-6824	
tcdB	NK104	GTGTAGCAATGAAAGTCCAAGTTTACGC	2945-2982	203
	NK105	CACTTAGCTCTTTGATTGCTGCACCT	3123-3148	
cdtA	cdt Apos	TGAACCTGGAAAAGGTGATG	507-526	375
	cdt Arev	AGGATTATTTACTGGACCATTTG	882-860	
cdtB	cdt Bpos	CTTAATGCAAGTAAATACTGAG	368-389	510
	cdt Brev	AACGGATCTCTTGCTTCAGTC	878-858	

Reactions

Reactions (total volume 20 µl/tube) were prepared containing 2 µl of 1x reaction buffer II, 2 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM of each dNTP, five units AmpliTaq Gold™ *Taq* polymerase, 0.01% BSA and 2 µl template DNA.

PCR conditions

PCR was performed on a Gene Amp® PCR system 1700 thermocycler with the following conditions: 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* strains SE844 and ES173 was included in each experiment.

2.3.4.4 Visualisation of PCR products

PCR products were analysed by automated high-resolution capillary electrophoresis using the QIAxcel Advanced System (Qiagen, Germantown, MD, USA) and viewed using ScreenGel software (Qiagen, Germantown, MD, USA). Multiple assays of the same strain were performed to ensure reproducibility.

2.3.4.5 Analysis of ribotyping banding patterns

Analysis of banding patterns and electropherograms was performed using BioNumerics™ software package v.6.5 (Applied Maths, Saint-Martens-Latem, Belgium). Dendrograms were generated using an unweighted-pair group method UPGMA and Dice coefficient. PCR ribotypes were identified by comparison with banding patterns in our reference library, which consisted 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 (B. Elliott, T. V. Riley, unpublished data). Isolates that could not be identified with the available reference library were designated with internal (QX) nomenclature. Strains matching the reference ribotypes were assigned ARL (UK) nomenclature.

2.3.5 *C. difficile* toxin detection

2.3.5.1 Enzyme immunoassay for toxins A/B

EIA was performed using a C. diff Quik Chek Complete kit (Alere North America Inc., Orlando, FL, USA). All EIAs were carried out according to the manufacturer's instructions.

2.3.6 Piglet challenge experiment

2.3.6.1 Spore inoculum preparation

The growth from pure cultures of three toxigenic strains and one non-toxigenic strain of *C. difficile* on BA was used to lawn inoculate BA plates. These were incubated anaerobically for 7 days to produce spores. The plates were washed with 5mL cold PBS and the resultant suspension centrifuged (Eppendorf 5424 microfuge, Hamburg, Germany) at 10,000*g* for 20 min at room temperature. The pellet was washed with 180 mL 1 M KCL:0.5 M NaCl, resuspended in 100 mL 50 mM Tris-HCl (pH 7.2) with 10

mg lysozyme per mL and incubated for 1 h. Spores were then washed three times with 100 mL HP H_2O and stored in 2 mL aliquots (~2 x 10^9 cfu/mL) at -80^0C until use.

Immediately prior to inoculation of piglets, 5 mL of the spore preparation was centrifuged (Eppendorf 5424 microfuge) at 13*g* for 10 min at room temperature to pellet. After removing the supernatant, the pellet was washed twice in 1 mL of sterile 1X PBS and the supernatant discarded. An aliquot of 10 µl of well-vortexed spore preparation was diluted 1:100 with 1X PBS and spores were counted using a haemocytometer. This suspension was made up to 10 mL with sterile 1X PBS then heated at 56^0C for 15 min. Then 3.3 mL of pre-reduced BHIB germination media was added to each tube, which was incubated anaerobically at 37^0C for 1 h to commence germination. A volume of 1.5 mL was drawn up into a separate syringe for each piglet.

Sham inocula consisted of 10 mL of sterile 1X PBS heated at 56^0C for 15 min. Then 3.3 mL of pre-reduced BHIB germination media was added to each tube and incubated anaerobically at 37^0C for 1 h with spore preparations. A volume of 1.5 mL was drawn up into a separate syringe for each piglet.

Purity plates consisting of pre-reduced BA were streaked with 20 µl of each spore preparation and incubated anaerobically for 24 and 48 h. A volume of 100 µl was inoculated onto TCCFA using the spread plate method and incubated anaerobically for 24 and 48 h to perform viable counts.

2.3.6.2 Spore counts—haemocytometer

Spore inocula were prepared as described in 2.3.6.1. Spores were enumerated using a Neubauer haemocytometer (Bright-Line, Sigma-Aldrich, St Louis, MO, USA). A volume of 10 µl of spore inoculum was loaded onto each side of the haemocytometer and visualised by phase-contrast microscopy. Spores were counted in the five marked regions of the haemocytometer and expressed as spores/mL.

2.3.6.3 Quantitative C. difficile *culture—viable spore counts*

Enumeration of viable spores in challenge inoculum was assessed by viable counts. A volume of 100 µl of each spore inoculum in BHIB was plated onto pre-reduced TCCFA as per Section 2.3.1.3. In addition, serial 10-fold dilutions were prepared in 1X PBS and

10 µl plated onto pre-reduced TCCFA. Counts were performed at 24 and 48 h after anaerobic incubation at 37^0C.

2.3.6.4 Intragastric administration of challenge inocula

Challenge inocula were administered intragastrically 4 h after birth using a fresh sterile 8-gauge Foley catheter (Sovereign, Tyco Healthcare, Mansfield, MA, USA) for each piglet. Each catheter was flushed post-inoculation with 25 mL of puppy milk replacer (Esbilac Milk Replacer Liquid, PetAg, Hampshire, IL, USA).

2.3.6.5 Necropsy and sample collection

After euthanasia, each piglet was placed onto a clean disposable tray and assessed blind by two ISU veterinary staff for gross intestinal and systemic lesions as per the scoring rubric (Table 2-4). Fresh tissue for histology was collected using instruments flamed in 70% alcohol between each necropsy. The following tissue sections were collected: ileum, jejunum, descending colon, cecum, and a cross section of spiral colon containing 4–5 loops. Pooled colon and caecal contents were collected in a sterile container for toxin ELISA. Swabs from the ileum and colon were taken for routine aerobic and anaerobic culture for *Salmonella* spp, *E. coli* and *C. perfringens*.

2.3.6.6 Specimen processing

Histopathology

Fresh tissue sections obtained at necropsy were fixed in 10% formalin for 48 h then stored in 70% ethanol until paraffin embedding, sectioning and staining with hematoxylin and eosin. Slides were then assessed blind by two ISU veterinary staff as per the scoring rubric (Table 2-5).

Table 2-4 Clinical features scoring rubric

Gross morphology and clinical features of piglets were scored blind by two ISU veterinarians at necropsy, 72 h post-inoculation with *C. difficile* spores

Score	Clinical feature
Body condition	
0	normal
1	thin
2	emaciated
Hydration status	
0	normal
1	mild
2	moderate
3	severe
Perineal staining	
0	none
1	mild
2	moderate
3	severe
Distal SI and LI contents	
0	firm
1	normal
2	pudding-like
3	watery
Necrotizing lesions in SI and LI	
0	none
1	mild
2	moderate
3	severe
Mesocolonic oedema	
0	none
1	mild
2	moderate
3	severe
Stomach contents	
0	empty
1	half-full
2	full

Table 2-5 Histopathology scoring rubric

Tissue samples taken from each piglet at necropsy, 72 h post-inoculation with *C. difficile* spores, were scored blind by two ISU veterinarians

Score	Lesion
Goblet cell loss	
0	normal
1	less than 25% goblet cells in 2 or more adjacent glands
2	30-50% glands with <25% goblet cells
3	>50% of glands with < 25% goblet cells

Neutrophils (PMNs in colonic superficial lamina propria)	
0	0-3
1	<20
2	21-50
3	>50

Mucosal alterations	
0	normal
1	rare mucosal erosions (≤4)
2	≥5 erosions
3	≥1 ulceration/s

Mesentritis	
0	none/normal
1	mild infiltrate
2	moderate
3	severe

C. difficile toxin detection

Toxin detection (tcdA/B) was performed on pooled colon and caecal contents using a commercial EIA kit (*C. difficile* Tox A/B II, Techlab, Blacksburg, VA, USA) according to manufacturer's instructions. Intestinal contents were stored at 4^0C until toxin testing.

Bacteriology

Large intestine and small intestine swabs were tested at the ISU Veterinary Diagnostic Laboratory (IA, USA) using routine enrichment and culture methods for *Salmonella* spp., *E. coli*, and *C. perfringens*. Faecal swabs were stored at -20^0C before export to Australia for routine *C. difficile* culture and typing as described in Sections 2.3.1 and 2.3.4.

2.3.7 Diagnostic evaluation study

2.3.7.1 Sample preparation for diagnostic tests

Faecal swabs from piglets were suspended in 800 µL PBS. The samples were vortexed briefly to create a homogenous suspension and split into 200 µL aliquots. These were stored at -20°C until use, at which point a single freeze thaw cycle was implemented as per the assay recommendations.

2.3.7.2 Loop-mediated isothermal amplification (illumigene® LAMP) test for tcdA

The illumigene® *C. difficile* amplification assay (Meridian Bioscience Inc., Cincinnati, OH, USA) detects toxigenic *C. difficile* by targeting a conserved 5` 204 bp sequence of *tcdA* (Noren, Alriksson et al., 2011). Assays were performed on the illumi*pro-10*™ according to the manufacturer's instructions and recorded as positive or negative using the illumi*pro-10*™ software.

2.3.7.3 Real time PCR assay (GeneOhm Cdiff Assay) for tcdB

The GeneOhm™ Cdiff Assay (Becton Dickinson, La Jolla, CA, USA) is RT-PCR technology that amplifies a conserved region of *tcdB.* Detection of the amplified products is achieved by using fluorogenic target-specific hybridisation probes (Terhes, Urban et al., 2009). Assays were performed on a SmartCycler® (Cepheid, Buckinghamshire, UK) according to the manufacturer's instructions. The SmartCycler software recorded the results of the PCR assay as positive, negative, or unresolved.

2.3.8 Virulence investigation

2.3.8.1 Mouse challenge experiment

In vivo virulence of *C. difficile* strains was assessed using a mouse model of CDI (Chen, Katchar et al., 2008). Six- to eight-week-old male C57/B6 mice (n = 15, five mice per strain) were challenged by oral gavage of 10^7 spores of one of three toxigenic strains of *C. difficile*: RT 027 human strain (M 7404), RT 078 animal strain (JGS 6133), or RT 237 neonatal piglet strain (AI 35).

Spore inocula were prepared by culturing *C. difficile* strains on BA for 5 to 6 days. Growth was harvested into 10 mL of PBS, washed in PBS, and heat-shocked at 56°C for 10 min to kill surviving vegetative cells. The spores were pelleted by centrifugation,

resuspended in Dulbeccos's Modified Eagle Medium (DMEM), and stored at -80°C. Spores were quantified by plating 10-fold serial dilutions of the spores onto TCCFA plates without cycloserine and cefoxitin and counted after overnight incubation at 37^0C. Spores were diluted in DMEM before inoculation to a final count of 10^7.

Mice were pre-treated with antibiotics at day zero. The antibiotic mixture was delivered via drinking water and comprised kanamycin (40 mg/kg), gentamicin (3.5 mg/kg), colistin (4.2 mg/kg), metronidazole (21.5 mg/kg), and vancomycin (4.5 mg/kg).

Animals were individually housed at a Monash University facility and handled to prevent cross-contamination.

Mice were monitored daily throughout the four day experiment for weight loss and signs of disease such as diarrhoea and hunched posture. In the 027 group, animals that had not died after 48 h were culled due to severe weight loss in accordance with the Monash University animal ethics guidelines.

2.3.8.2 Toxin B quantitation assay (Vero cell cytotoxicity)

A quantitative measure of toxin B production from *C. difficile* strains M 7404, JGS 6133 and AI 35 was performed using a Vero cell cytotoxicity assay (Lyras, O'Connor et al., 2009).

C. difficile strains were grown in 90 mL of Tryptone-Yeast broth for 3 days, and the cells pelleted by centrifugation at 10,000*g* for 15 min at room temperature. The supernatants were filter sterilised and stored on ice before use.

Vero cells were cultured in minimum essential medium (MEM alpha medium: GIBCO, Invitrogen), containing 10% heat-inactivated foetal calf serum (FCS), 100 units/mL penicillin and 100 μg/mL streptomycin in culture flasks at 37°C in 5% CO_2. The cells were grown to a confluent monolayer and subcultured by incubation in 1 to 2 mL of 0.1% trypsin in 1 mM EDTA. The cells were counted and resuspended in fresh medium at a concentration of 0.25×10^5 cells/mL.

One mL of the cell suspension was seeded into each well of 24-well plates. The plates were incubated for 20–24 h and the culture medium removed, after which cells were

washed with PBS. Serial two-fold dilutions of the *C. difficile* culture supernatants were made in PBS and 100 µl added to each well, followed by 400 µl of MEM or McCoy's medium containing 1% heat-inactivated FCS. Negative controls were treated with 500 µl of fresh medium. The plates were incubated at 37°C in 5% CO_2.

The morphological changes were observed by microscopy after 24 h. The cytopathic effect (CPE) was determined on a scale from 0 to +4 in comparison to the negative control wells. The end point was scored as the last dilution at which 100% or 4+ CPE was observed. The assays were performed in triplicate on independent culture supernatants. An Olympus 1X71 inverted microscope was used to visualise the cells at 10× and 20× magnifications.

2.3.9 Bioinformatics – strain AI 35

2.3.9.1 Whole genome sequencing

Sample DNA was prepared and genome shotgun sequencing was performed using the Illumina HiSeq2000 platform (Australian Genome Research Facility, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia).

2.3.9.2 Genome assembly

Paired-end reads of 31,085,914 bp were concatenated into 117 contigs using the Velvet software suite (Zerbino and Birney, 2008). Sequencing reads were aligned and annotated with Wasabi software against the genome sequence of the *C. difficile* reference strain 630 (RT 012, ST54, A+B+CDT-) (Victorian Bioinformatics Consortium, Monash University, Australia).

2.3.9.3 Sequence metrics

Sequence metrics were analysed by QUAST (Gurevich, Saveliev et al., 2013).

2.3.9.4 Prophage analysis

Prophage sequence identification was by PHAST (Zhou, Liang et al., 2011)

2.3.9.5 Sequence comparison

Sequence comparison was by BLAST searches against GenBank (Altschul, Gish et al., 1990), specifically previously sequenced PaLocs for *C. difficile* strains 8864

(Accession: AJ011301.1) and 1470 (Accession: X93158.1), and the genome of *C. sordellii* strain VPI 9048 (Reference sequence: NZ_AQGJ01000000) (Sirigi Reddy, Girinathan et al., 2013).

2.3.9.6 Antimicrobial resistance gene analysis and antibiogram phenotyping

Searches for acquired antimicrobial resistance genes were by CARD (McArthur, Waglechner et al., 2013) and ResFinder 2.1 (Zankari, Hasman et al., 2012).

Minimum inhibitory concentrations (MIC) for fourteen antimicrobials were determined for AI-35 using the agar incorporation method as described in standard M11-A7 (Clinical and Laboratory Standards Institute, 2011). A combination of breakpoints from CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used (Clinical and Laboratory Standards Institute, 2013; European Committee on Antimicrobial Susceptibility Testing, 2014).

Chapter 3 *C. difficile* prevalence in Australian piglets

3.1 Introduction

Despite its importance to swine operations elsewhere, we knew nothing about the epidemiology of *C. difficile* in Australian piglets, particularly its relationship to idiopathic neonatal scour and association with human disease, if any.

Studies performed in North America and Europe have reported the prevalence of *C. difficile* in neonatal piglets in the range of 29 to 73%, with a single PCR ribotype (RT), RT 078, predominating in the majority of cases (Avbersek, Janezic et al., 2009; Goldova, Malinova et al., 2012; Keel, Brazier et al., 2007; Keessen, Leengoed et al., 2010; Noren, Johansson et al., 2014; Schneeberg, Neubauer et al., 2013).

Retrospective analyses of diagnostic samples have been performed using various *C. difficile* isolation and identification methods. These studies report prevalence rates from older pigs in the Netherlands (25%, enrichment culture) (Koene, Mevius et al., 2012), neonatal piglets in Canada (48%, EIA + culture + histopathology) (Chan, Farzan et al., 2013), and farrowing-unit samples from the USA (34%, enrichment culture) (Susick, Putnam et al., 2012).

3.2 Diagnostic sample prevalence study

The aim of this study was twofold: (1) to determine *C. difficile* prevalence from convenience (diagnostic) samples from herds of scouring neonatal piglets, and (2) to evaluate *C. difficile* isolated from these piglets using toxin gene PCR and PCR ribotyping.

Samples were collected by veterinarians or piggery stock hands. The secondary use of diagnostic samples from piglets was approved by The University of Western Australia Animal Ethics Committee (Use of Animal Tissue, FA/3/500/). Data related to individual pork producers was de-identified to maintain confidentiality.

Results of this investigation were presented at the 3rd International *C. difficile* Symposium, Bled, Slovenia, 22–24 September 2010. Financial support to attend the conference was provided by APL (Distinguished Visitor and Travel Award No. 00206).

3.2.1 Experiment design

3.2.1.1 Study population

This was the first piglet *C. difficile* prevalence study conducted in Australia. To obtain baseline data, we utilised readily-available samples submitted to our laboratory for investigation of idiopathic neonatal scour. Little was known about porcine *C. difficile* in Australia at this time so samples from all piglets with veterinary-diagnosed idiopathic scour were included in the study, regardless of age or type of scour.

A total of 423 faecal samples were obtained by rectal swab from live piglets (n = 404) or GIT contents from necropsied piglets (n = 19) between June 2009 and June 2011. Samples represented major Australian pig-producing regions: Western Australia (WA, six farms, n = 305), South Australia (SA, two farms, n = 12), Queensland (QLD, three farms, n = 34), Victoria (VIC, seven farms, n = 66) and New South Wales (NSW, one farm, n = 6) (Table 3.1).

All farms had veterinary-diagnosed idiopathic neonatal scour in the farrowing herd prior to sampling, with the exception of two herds in WA (Farms A4, B1). Common pork production methods in Australia were represented, including farrow-to-finish (n = 15) and sow breeder units (n = 3); however, all samples were from piglets in farrowing, not grower, units. There was one outdoor facility (farm A4); the remainder were conventional facilities. The number of samples per farm varied widely (range: 1-113).

3.2.1.2 Methods

After sampling, swabs were placed immediately in Amies transport medium (Thermo Fisher Scientific, Waltham, MA, USA) and transported under ambient conditions to The University of Western Australia, where they were stored at 4°C and processed within 24 h. Swabs were received in the laboratory between 1–7 days from time of sampling, depending on farm location.

C. difficile was isolated and identified using the methods outlined in Section 2.3.1. All isolates were screened by PCR for the presence of toxin A and B genes (*tcdA* and *tcdB*)

and binary toxin genes (*cdtA* and *cdtB*) as per Section 2.3.4. PCR ribotyping and analysis/comparison of PCR ribotyping products were performed as per Section 2.3.4. Isolates that could not be identified after comparison with the reference library were designated with internal nomenclature, prefixed with QX.

Student's t-test was used to compare parametric prevalence data, and Mann–Whitney test for non-parametric. Fisher's exact test and χ^2 tests were used to analyse associations between categories, depending on sample size. A p value <0.05 was considered significant.

3.2.2 Prevalence in diagnostic samples

Prevalence varied across the five states (range: 41 - 75%, Table 3.2).

Table 3-1 Prevalence of Australian porcine *C. difficile* from diagnostic samples

Overall prevalence and molecular characteristics of Australian porcine *C. difficile* categorised by state, farm type and farm scouring status

	No (%) *C. difficile* isolates																	
	WA			VIC			QLD			SA			NSW			TOTAL		
Age	**Total**	**Positive**	**95% CI**	**Total**	**Positive**	**95% CI**	**Total**	**Positive**	**95% CI**	**Total**	**Positive**	**95% CI**	**Total**	**Positive**	**95% CI**	**Total**	**Positive**	**95% CI**
	305	180 (59)	53-64	66	31 (47)	35-59	34	14 (41)	26-58	12	9 (75)	47-91	6	4 (67)	30-90	423	238 (56)	52-61
≤ 7 days	274	170 (62)	56-68	43	26 (60)	46-74	31	14 (45)	29-62	12	9 (75)	47-91	6	4 (67)	30-90	366	223 (64)	58-69
> 7 days	31	10 (32)	19-50	23	5 (22)	10-42	3	0 (0)	0-56	0	0	0	0	0	0	57	15 (26)	17-39
Toxin profile																		
A-B-CDT-		0	0-2		6 (3)	1-7		0	0-2		0	0-2		0	0-2		6 (3)	1-7
A-B-CDT+		0	0-2		0	0-2		0	0-2		0	0-2		4 (2)	0.6-6		4 (2)	0.6-6
A-B+CDT-		0	0-2		0	0-2		0	0-2		0	0-2		0	0-2		0 (0)	0-2
A-B+CDT+		130 (72)	65-78		3 (2)	0.3-5		0	0-2		2 (1)	0.1-4		0	0-2		135 (75)	68-80
A+B+CDT-		0	0-2		9 (5)	2-9		14 (8)	4-13		4 (2)	0.6-6		0	0-2		27 (15)	10-21
A+B+CDT+		0	0-2		9 (5)	2-9		0	0-2		0	0-2		0	0-2		9 (5)	2-9
A-B-CDT-		1 (2)	0.04-9		0	0-6		0	0-6		0	0-6		0	0-6		1 (2)	0.04-9
A-B-CDT+		19 (33)	21-47		0	0-6		0	0-6		0	0-6		0	0-6		19 (33)	21-47
A-B+CDT-		0	0-6		1 (2)	0.04-9		0	0-6		0	0-6		0	0-6		1 (2)	0.04-9
A-B+CDT+		30 (53)	39-66		0	0-6		0	0-6		0	0-6		0	0-6		30 (53)	39-66
A+B+CDT-		0	0-6		2 (4)	0.4-12		0	0-6		3 (5)	1-15		0	0-6		5 (9)	4-19
A+B+CDT+		0	0-6		1 (2)	0.04-9		0	0-6		0	0-6		0	0-6		1 (2)	0.04-9

Table 3-2 Detailed summary of porcine *C. difficile* prevalence from diagnostic samples

Details of diagnostic samples from neonatal piglets, stratified by state, piglet age, scouring status and toxin profile

	Farm demographics				*C. difficile*		
State	**Farm ID**	**Size (No. sows)**	**Type ***	**Scouring status #**	**No. (%) isolated**	**RT**	**Toxin profile**
WA	A1	2000	FTF	SC	26/43 (60)	UK 237 (42), UK 285 (1)	A-B+CDT+
	A2				59/113 (52)	UK 237	A-B+CDT+
	A3		BU		44/54 (81)	UK 237	A-B+CDT+
	A4	1200	FTF	NS	4/28 (14)	UK 237, AU 187, AU 211	A-B+CDT+, A-B-CDT-
	B1	400	FTF	NS	19/21 (90)	UK 238	A-B-CDT+
	B2	250	FTF	SC	29/46 (63)	QX 1	A-B+CDT+
SA	C1	4000	BU	SC	7/10 (70)	UK 014	A+B+CDT-
	C2	9000	FTF	SC	2/2 (100)	UK 033	A-B+CDT+
VIC	D1	650	FTF	SC	5/19 (26)	QX 2	A-B-CDT-
	D2	1800	FTF	SC	9/18 (50)	QX 4 (1), QX 5 (8)	A-B+CDT-, A+B+CDT-
	D3	1400	FTF §	SC	8/11 (73)	QX 6 (3), QX 7 (5)	A+B+CDT-, A+B+CDT+
	D4	600	FTF	SC	5/9 (56)	QX 7 (1), QX 8 (4)	A+B+CDT+
	D5	Unknown	Unknown	SC	3/5 (60)	QX 9	A-B+CDT+
	D6	500	FTF	SC	1/1 (100)	QX 10	A-B-CDT-
	D7	1000	FTF	SC	0/3 (0)	NA	NA
QLD	E1	650	FTF	SC	0/4 (0)	NA	NA
	E2	1800	FTF	SC	15/25 (56)	QX 3	A+B+CDT-
	E3	1400	BU	SC	0/5	NA	NA
NSW	F1	1250	FTF	SC	4/6 (67)	NSW 02	ND

* FTF - Farrow-to-finish, BU - Breeder unit

\# SC - Scouring, NS - Non-scouring

§ FTF facility but swabs collected from BU

3.2.3 Piglet-level analysis

Samples were from individual piglets; there were no duplicates. Piglets ranged in age from 1 to 26 days (median = 7.1 d). *C. difficile* prevalence decreased with age of piglets, with the highest prevalence in piglets ≤7 d (range: 75–0%, Table 3.1). Prevalence in older piglets (> 7 days) was 26% (15/57).

The case definition for submission of samples was veterinary-diagnosed idiopathic scour in piglets of all ages. Fifty-nine per cent (249/423) of submitted samples fit the published case definition of porcine CDI (pasty-watery, yellow, non-haemorrhagic scour in piglets ≤7 days of age), where diagnostic tests for other pathogens in this age group were negative (rotavirus, *E. coli*, *C. perfringens*) or not clinically significant as determined by the attending veterinarian. Non-CDI case definitions included unspecified scour (*n* = 137), and scouring of all types in age groups older than 7 days (*n*

= 57). Samples from non-scouring piglets were also submitted (n = 57) and used for comparison purposes.

Overall, *C. difficile* was recovered from 56% (238/423) piglets by EC (Table 3.1) and 60% (150/249) of piglets that fit the case definition of porcine CDI. Prevalence in piglets with unspecified scour ≤7 days of age was 54% (49/91).

Scouring piglets were more likely to be colonised with *C. difficile* (n = 181) than non-scouring piglets (n = 57) (p = 0.018), irrespective of the type of scouring. This was not true at herd level; there was no association between scouring in the herd and *C. difficile* colonisation (p = 0.546), even when non-toxigenic strains were removed from the data set (p = 0.064). Asymptomatic carriage (colonised but not scouring) was detected in 52% (57/109) piglets.

More samples were submitted in winter months (n = 270) than summer months (n = 20) but *C. difficile* was isolated more frequently during summer (15/20, 75%) than winter (185/270, 69%).

3.2.4 Between-farm analysis

C. difficile was recovered from 16 of the 19 farms (84%). There was no difference in *C. difficile* prevalence between different production types (p = 0.267). *C. difficile* was as prevalent in piglets from farms with <1000 sows (7 farms, 56%, 71/127 samples) as in those with >1000 sows (10 farms, 56%, 163/290 samples) (p = 0.955). When unknown veterinary providers were excluded, the majority of samples (91%, n = 385) were submitted from three veterinary service providers. There was no difference in overall *C. difficile* prevalence between farms serviced by different veterinary providers (p = 0.062).

3.2.5 Molecular analysis: PCR ribotyping

Seventeen distinct PCR ribotypes were identified (Table 3.3). Five ribotypes (59% of isolates; n = 141) matched those previously isolated from humans in our database. These were identified by comparison to known reference profiles as RT UK 014, UK 033, UK 237, AU 211 and AU 187. One ribotype, UK 238, had previously been isolated from Australian pigs from a pilot study conducted at another farm owned by this

company in 2007 (Thomas Riley, UWA, unpublished data). The remaining 11 strain types (30%, *n* = 72) could not be assigned a ribotype based on the reference strains in our database and were assigned internal nomenclature (QX 1-10, NSW 02). When non-toxigenic strains were excluded (*n* = 2), 100% (9/9) of these matched other human unknown RT in our database, although epidemiological links could not be verified (Figures 3.1–3.3). RTs 078 and 027 were not identified.

A single RT circulated in the majority of farms, with the exception of five farms (A1, A4, D2–4). Two RTs per farm were isolated from farms A1 and D2–4. There was no link between the different RTs on each farm and scouring status of piglets, and all strains were toxigenic. RT QX 7 was present in two Victorian farms (D3, D4), separated by 100 km.

Three different RTs (UK 237, AU 211, AU 187) were recovered from non-scouring piglets in a single farrowing hut on the only outdoor farm in this study (farm A4). A single isolate of RT 285 was isolated from another farm in this group (A1); the remaining isolates from farm A1 (*n* = 42) were RT 237.

Table 3-3 Ribotype distribution of *C. difficile* isolated from diagnostic samples

Ribotypes for 238 isolates of *C. difficile* recovered from diagnostic samples submitted by veterinarians

	Toxin profile			No isolates in:						
PCR Ribotype	*tcdA*	*tcdB*	*cdtA-cdtB*	VIC	SA	QLD	WA	NSW	TOTAL	**%**
UK 237	–	+	+				129		129	54.2
QX 115	–	+	+				28		28	11.8
UK 238	–	–	+				19		19	8.0
UK 103	+	+	–			14			14	5.9
QX 158	+	+	–	8					8	3.4
UK 014	+	+	–		7				7	2.9
UK 127	+	+	+	6					6	2.5
QXP 2	–	–	–	5					5	2.1
UK 127	+	+	+	4					4	1.7
UK 281								4	4	1.7
QX 057	–	+	+	3					3	1.3
QX 158	+	+	–	3					3	1.3
UK 033	–	+	+		2				2	0.8
AU 187	–	+	+				2		2	0.8
AU 211/ QX 005	–	–	–				1		1	0.4
QXP 10	–	–	–	1					1	0.4
QX 106	–	+	–	1					1	0.4
UK 285	–	+	+				1		1	0.4
TOTAL									238	

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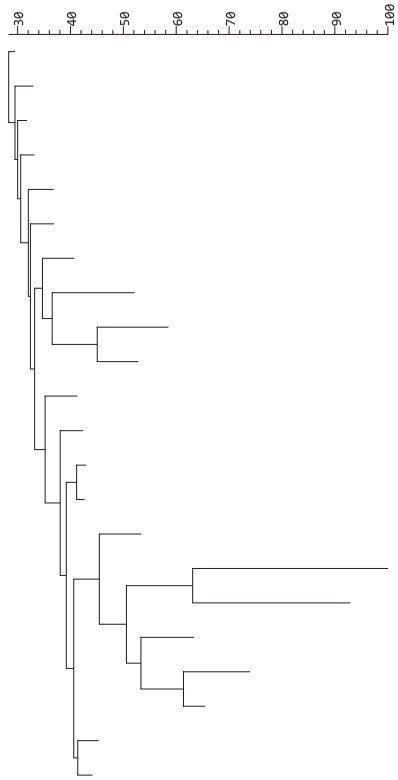
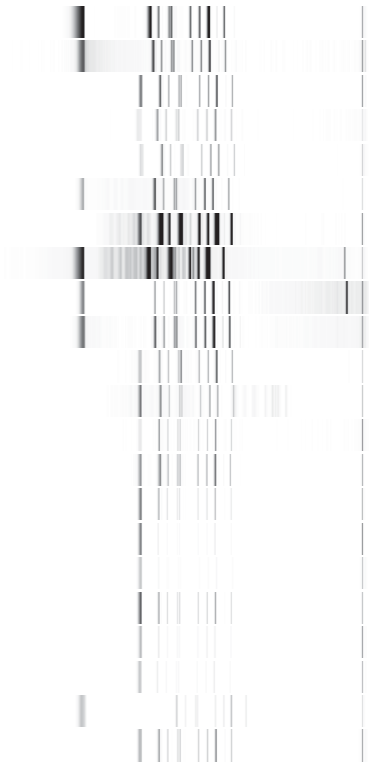
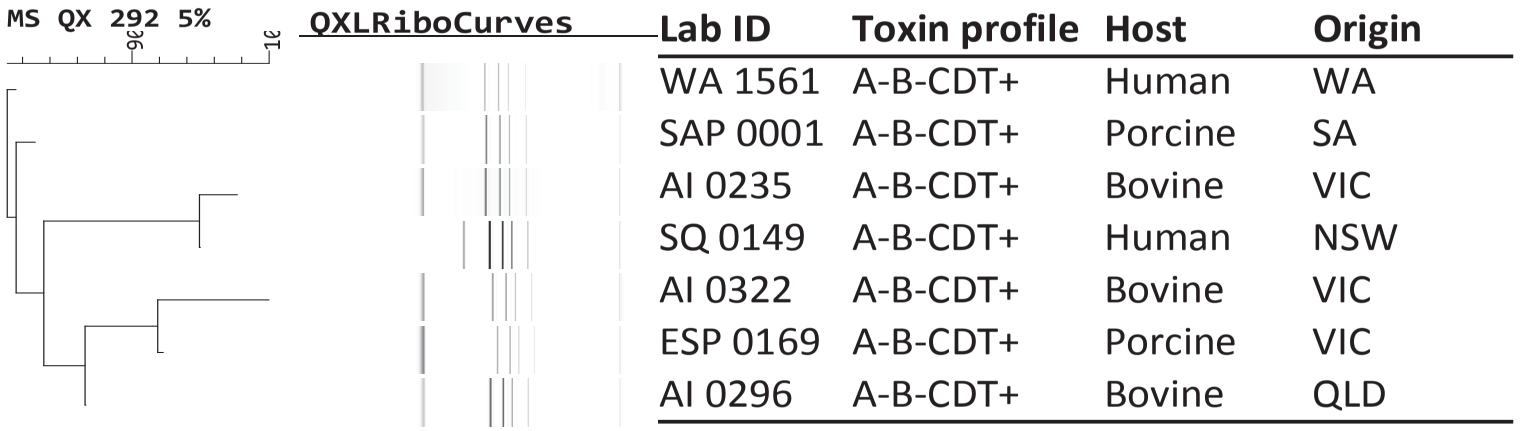




Lab ID	Toxin profile	Host	Origin
AI 0454	A+B+CDT-	Porcine	VIC
ESP 0165	A+B+CDT-	Porcine	VIC
WA 0573	A+B+CDT-	Human	WA
J 0090	A+B+CDT-	Human	Japan
CD 1106	A+B+CDT-	Chicken	Canada
AI 0446	A+B+CDT-	Equine	Unknown
H 0082	A+B+CDT-	Human	TAS
SQ 0056	A+B+CDT-	Human	NSW
VP 0014	A+B+CDT-	Porcine	VIC
VP 0025	A+B+CDT-	Porcine	VIC
STA 084	A+B+CDT-	Human	
TH CD040	A+B+CDT-	Human	Thailand
ZR 0007	A+B+CDT-	Human	
N1WA 0065	A+B+CDT-	Human	WA
ES 0486	A+B+CDT-	Human	NSW
OT 0051	A+B+CDT-	Human	Korea
OT 0171	A+B+CDT-	Human	Thailand
JIR 1109	A+B+CDT-	Human	VIC
N1QLD 137	A+B+CDT-	Human	QLD
OT 0104	A+B+CDT-	Human	Singapore
ESP 0133	A+B+CDT-	Porcine	NSW
OT 0088	A+B+CDT-	Human	Taiwan

Figure 3.1 Dendrogram of PCR ribotyping banding patterns from *C. difficile* QX 5 isolates of human and animal origin

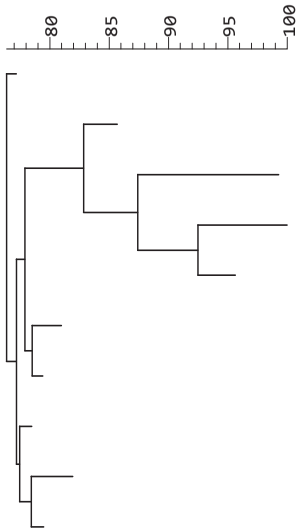
PCR ribotyping pattern analysis: neighbour joining tree, Dice coefficient (Optimisation: 1.00).

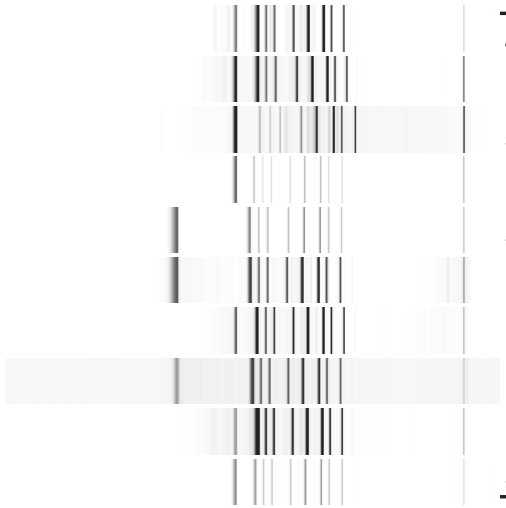


Lab ID	Toxin profile	Host	Origin
WA 1561	A-B-CDT+	Human	WA
SAP 0001	A-B-CDT+	Porcine	SA
AI 0235	A-B-CDT+	Bovine	VIC
SQ 0149	A-B-CDT+	Human	NSW
AI 0322	A-B-CDT+	Bovine	VIC
ESP 0169	A-B-CDT+	Porcine	VIC
AI 0296	A-B-CDT+	Bovine	QLD

Figure 3.2 Dendrogram of PCR ribotyping banding patterns from *C. difficile* UK 033 isolates of human and animal origin

PCR ribotyping pattern analysis: neighbour joining tree, Dice coefficient (Optimisation: 1.00).





Lab ID	Toxin profile	Host	Origin
SQ 0123	A+B+CDT-	Human	VIC
ES 0329	A+B+CDT-	Human	ACT
A.NSW 0131	A+B+CDT-	Human	NSW
Q 0022	A+B+CDT-	Human	QLD
AI 0301	A+B+CDT-	Equine	VIC
QP 0001	A+B+CDT-	Porcine	QLD
N2QLD 016	A+B+CDT-	Human	QLD
WA 0525	A+B+CDT-	Human	WA
ES 0433	A+B+CDT-	Human	NSW
AI 0475	A+B+CDT-	Bovine	VIC

Figure 3.3 Dendrogram of PCR ribotyping banding patterns from *C. difficile* QX 3 isolates of human and animal origin.

PCR ribotyping pattern analysis: neighbour joining tree, Dice coefficient (Optimisation: 1.00).

3.2.6 Molecular analysis: Toxin production genes

Overall, 87% (208/238) of isolates were toxigenic (Table 3.1) and these were associated with scouring piglets (164/208, 81%) and farms with a history of scouring (199/208, 96%) ($p < 0.0001$). There was no correlation between piglet age and toxigenicity of isolates (195/220, 89% ≤7 d of age vs 14/15, 94% ≥ 7 d, $p = 0.575$). The most common toxin production profile was A-B+CDT+ (80%, 166/208); this profile was isolated from seven farms across four states, and associated with five RTs. RT 237 accounted for the majority of A-B+CDT+ strains (78%, 129/166) and was isolated from four farms under single ownership. The other toxin profiles were A-B+CDT- (0.4%, 1/208) and A+B+CDT- (13%, 32/208). Strains producing all three toxins (A+B+CDT+) made up 4% (10/208) of the total; these were from two Victorian farms and represented two different RTs. There was a correlation between colonisation with toxigenic strains and piglets treated with antibiotics. Ninety-six percent (165/172) of treated piglets were colonised with toxigenic strains versus 30% (8/27) untreated piglets ($p < 0.0001$).

Non-toxigenic strains comprised A-B-CDT- (3%, 7/238) and A-B-CDT+ (8%, 19/238). Strains producing none of the three toxins (A-B-CDT-) were found in three farms and associated with non-scouring piglets (87%, 6/7 isolates). Strains producing only CDT (A-B-CDT+) were recovered from a single farm without a history of neonatal scour (Farm B1).

Overall, 82% (194/238) of isolates produced binary toxin (CDT+). When UK 237 was excluded, 27% (65/238) were CDT+. Nineteen of these were non-toxigenic (A-B-CDT+), from farm B1 mentioned above. The remainder were associated with toxigenic strains, producing either toxin B or both toxins A and B.

3.2.7 Association with antimicrobials

Seventeen farms provided antimicrobial usage data when submitting diagnostic samples. Figure 3.4 summarises antimicrobials used to treat scouring piglets in participating farms and the proportion of farms using each drug. One of these farms, Farm B1 (WA) reported that they did not use antimicrobials as they did not have a scouring problem. Ten antimicrobials were routinely used to treat scour in piglets. Neomycin was most commonly used (59% of farms) followed by ceftiofur and

sulfonamides (53%), then apramycin and trimethoprim (as trimethoprim-sulfamethoxazole) (35%). One farm (Farm D2, VIC) reported metaphylaxis of neonates with penicillins. Metaphylaxis with an unknown probiotic in one- to two-day-old piglets was reported in two farms (Farms D3, VIC and E2, QLD), although this had no effect on prevalence when compared with animals not receiving probiotics (p = 1.000). Multiple classes of antimicrobials were used in individual farms: five farms (2 classes), three farms (3 classes), one farm (4 classes), three farms (5 classes). Five of these farms used commercial preparations containing several classes of antimicrobials, such as co-trimoxazole (trimethoprim-sulfamethoxazole) and Scourban™ (sulfadimidine, sulfadiazine, streptomycin sulfate, neomycin sulfate).

Untreated piglets (n = 46) from scouring herds and piglets treated with antimicrobials (n = 331) were equally likely to be colonised with *C. difficile*, irrespective of the antimicrobial used (31/46, 67% vs 179/331, 54%, p = 0.089).

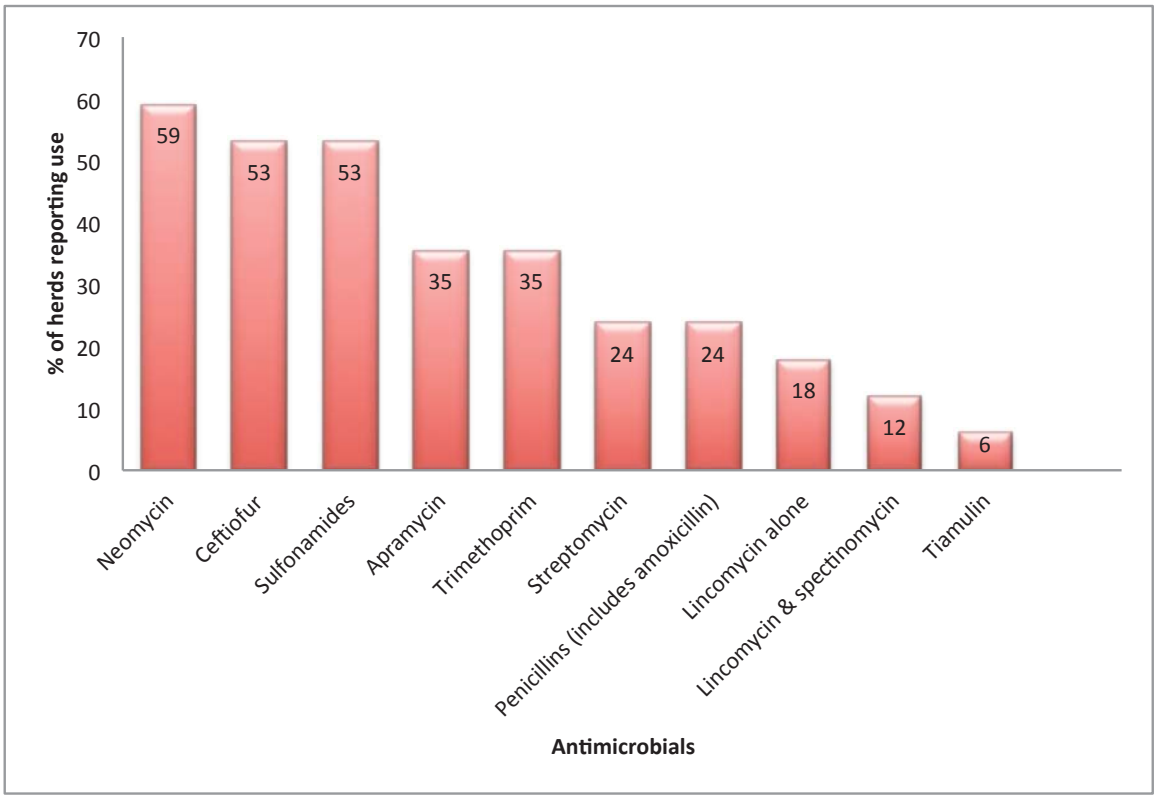


Figure 3.4 Summary of antimicrobials used in Australian neonatal pig herds and per cent of herds in this study (n = 17) that reported their use

Neomycin, ceftiofur, apramycin, streptomycin and penicillins are considered antimicrobials of high importance to human health (World Health Organisation (WHO), 2012).

3.3 Systematic period prevalence study in neonatal piglets

The aim of this study was to determine the prevalence and molecular types of gastrointestinal carriage of *C. difficile* in Australian neonatal pigs by culture of rectal swabs and characterisation of the isolates by toxin gene PCR and PCR ribotyping. Mr Daniel Knight, Research Associate, UWA, performed the laboratory work for this investigation under the supervision of Professor Thomas Riley and myself.

The University of Western Australia Animal Ethics Committee granted ethics approval for Use of Animal Tissue (FA/3/500/). Financial support was provided by APL (Project no.00462).

This study was published as: Knight DR, Squire MM, Riley TV. Nationwide surveillance study of *Clostridium difficile* in Australian neonatal pigs shows high prevalence and heterogeneity of PCR ribotypes. Appl Environ Microbiol. 2015; 81(1):119-23.

Preliminary results of this investigation were presented at the 14th Biennial Conference of the Australasian Pig Science Association, Melbourne, Australia, November 2013.

3.3.1 Experiment design

A total of 21 farms in five Australian states, New South Wales (NSW, *n* = 3), Queensland (QLD, *n* = 5), Victoria (VIC, *n* = 6), South Australia (SA, *n* = 3), and Western Australia (WA, *n* = 4), were selected to participate in the study. Farms were chosen after consultation with veterinarians, to reflect a broad geographic distribution and differences in historical scouring status. They were also selected to reflect various production types, e.g. farrow to finish, growers, and breeders, and were representative of production systems used in intensively farmed pork. Similar numbers of farms with idiopathic neonatal scour for at least six months (experimental farms, *n* = 12) and those with no history of idiopathic neonatal scour for at least six months (control farms, *n* = 9) were selected. Idiopathic scour was defined as diarrhoea of unknown aetiology that veterinarians could not attribute to *Escherichia coli*, *C. perfringens*, *Isospora suis* or rotavirus infection. Piglets (*n* = minimum of 10) were randomly selected from a minimum of four different litters at each enrolled farm. The study population was not

chosen on the basis of scouring status, although scouring status was recorded for participants (scouring, n = 181; non-scouring, n = 48).

The attending veterinarian completed a questionnaire for each participating farm to capture demographic information, including antimicrobial use and effluent re-use (Appendix 1).

Faecal samples were obtained by rectal swab from 229 neonatal piglets aged <7 days of age during the period June 2012 to March 2013. After sampling, the swabs were placed immediately in Amies transport medium and transported under ambient conditions to UWA, where they were stored at 4°C and processed within 24 h.

C. difficile was isolated and identified using the method outlined in Section 2.3.1. Samples were cultured using both DC on CA and EC. All isolates were screened by PCR for the presence of toxin A and B genes (*tcdA* and *tcdB*) and binary toxin genes (*cdtA* and *cdtB*) using the method described in Section 2.3.4. PCR ribotyping and analysis/comparison of PCR ribotyping products were performed as per Section 2.3.4. Isolates that could not be identified after comparison with the reference library were designated with an internal nomenclature, prefixed with QX.

Fisher's exact test was used to compare the prevalence of *C. difficile* in the sampled piggeries, the effect of diarrhoea and geographic distribution on the number and types of RTs identified, and correlation between scouring status and on-farm effluent re-use. A p value of <0.05 was considered significant.

3.3.2 Results

3.3.2.1 Prevalence of C. difficile *carriage*

A total of 229 piglet faecal samples were collected. *C. difficile* was isolated from 52.4% (n = 120) of the 229 samples by DC on CA and 67.2% (n = 154) by EC (p = 0.001) (Table 3.4). All CA-positive samples also were positive on EC. Compared to EC, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for CA were 77.9%, 100.0%, 100.0%, and 68.8%, respectively. The prevalence of *C. difficile* in experimental farms (71.3%) was, on average, >10% higher than that in control farms (60.5%), but this difference was not significant (p = 0.091). Similarly,

there was no significant difference between *C. difficile* prevalence in piglets with or without diarrhoea ($p = 0.141$) (Table 3.4). Prevalence varied across the five states (range, 50.9 to 82.5%). *C. difficile* was isolated more frequently in autumn/winter ($n = 121/164$, 73.8%) than summer months ($n = 33/65$, 50.8%, $p = 0.001$).

3.3.2.2 Molecular analysis: toxin production genes

Five combinations of *C. difficile* toxin genes were identified (Table 3.4). The majority (87%, 130/154) of strains were toxigenic. The most common toxin profiles were A+B+CDT- (43.5%, 67/154) and A-B+CDT+ (10%, 16/154). Non-toxigenic strains (A-B-CDT-) comprised 15.6% (24/154) of isolates. Isolates positive for all toxin genes (A+B+CDT+) were uncommon ($n = 2$). The toxin profiles of isolates recovered from the control and experimental farms and piglets were similar, except non-toxigenic strains (A-B-CDT-) were more prevalent in the control (non-scouring) farms ($p = 0.001$).

Table 3-4 Summary of *C. difficile* isolate recovery from a period prevalence study in neonatal pigs

Results are stratified by toxigenic culture, direct culture and piglet scouring status

Group and analysis method	No. of isolates in[a]: NSW	QLD	SA	VIC	WA	No./total no. (%) [95% CI][b]
Culture method						
Enrichment	23	28	33	43	27	154/229* (67.2) [60.9–73.0]
Direct	18	27	26	31	18	120/229* (52.4) [45.6–58.8]
Toxin profile						
Nondiarrheic animals ($n = 181$)						
$A^+B^+CDT^-$	14	10	9	17	0	50/181 (39.7)
$A^-B^-CDT^-$	1	14	0	2	4	21/181 (16.7)
$A^-B^+CDT^+$	0	0	0	0	16	16/181 (12.7)
$A^+B^+CDT^+$	0	0	0	1	0	1/181 (0.8)
$A^-B^-CDT^+$	5	0	17	12	4	38/181 (30.2)
Total	20	24	26	32	24	126/181 (69.6) [62.6–75.9]
Diarrheic animals ($n = 48$)						
$A^+B^+CDT^-$	0	3	4	10	0	17/48 (60.7)
$A^-B^-CDT^-$	0	1	0	0	2	3/48 (10.7)
$A^-B^+CDT^+$	0	0	0	0	0	0/48 (0.0)
$A^+B^+CDT^+$	0	0	0	1	0	1/48 (3.6)
$A^-B^-CDT^+$	3	0	3	0	1	7/48 (25.0)
Total	3	4	7	11	3	28/48 (58.3) [44.3–71.2]

[a] NSW, New South Wales; QLD, Queensland; SA, South Australia; VIC, Victoria; WA, Western Australia.
[b] CI, confidence interval. *, $P = 0.001$.

Table 3-5 Ribotype distribution for 154 isolates of *C. difficile* recovered from Australian piglets in a period prevalence study

	Toxin profile			No. of isolates in[a]:					
PCR ribotype	*tcdA*	*tcdB*	*cdtA-cdtB*	VIC	SA	QLD	WA	NSW	Total (*n* [%])
UK014	+	+	−	19	3	6		8	36 (23.4)
UK033	−	−	+	1	19				20 (13.0)
QX009	−	−	+	11				8	19 (12.3)
UK237	−	+	+				16		16 (10.4)
QX006	+	+	−			6		4	10 (6.5)
QX207	−	−	−			8			8 (5.2)
QX057	−	−	−			1	6		7 (4.5)
UK018	+	+	−		6				6 (3.9)
QX015	−	−	−			4			4 (2.6)
QX027	−	−	+				3		3 (1.9)
QX084	+	+	−			1		2	3 (1.9)
QX208	−	−	−	2				1	3 (1.9)
UK005	+	+	−		3				3 (1.9)
QX141	−	−	−			2			2 (1.3)
QX147	+	+	+	2					2 (1.3)
QX209	−	−	+		1		1		2 (1.3)
UK020	+	+	−	2					2 (1.3)
UK046	+	+	−	2					2 (1.3)
UK053	+	+	−	2					2 (1.3)
QX058	−	−	+				1		1 (0.6)
QX076	+	+	−	1					1 (0.6)
QX210	+	+	−		1				1 (0.6)
UK137	+	+	−	1					1 (0.6)
Total				43	33	28	27	23	154

[a] Distribution is given by state. VIC, Victoria; SA, South Australia; QLD, Queensland; WA, Western Australia; NSW, New South Wales.

3.3.2.3 Molecular analysis: PCR ribotypes

Twenty three RTs were identified (Table 3.5, above), nine of which were internationally recognised. Seven RTs that could be identified (UK 053, 137, 014, 020, 018, 005, 046) from comparison with our database were all from the same *C. difficile* genetic group, clade 1, but were heterogeneous in terms of sequence type (ST). The remaining RTs, UK 237 and UK 033, belonged to clade 5, ST11. No RT 078 or 027 strains were identified. The most common RT was RT 014 ($A^+B^+CDT^-$), representing 23.4% (36/154) of isolates. This RT was more prevalent in experimental farms ($p = 0.001$). RT 014 was not isolated from WA farms, but had a varied and widespread prevalence in the four other states: VIC (50% prevalence), NSW (22.2%), QLD (16.7%) and SA (8.3%). The next most prevalent RTs were RT 033 (13.0%), QX 009 (12.3%), UK 237 (10.4%) and QX 006 (6.5%). Novel RTs QX 006 and QX 009 were restricted to smaller geographic areas: QX 006 (40% NSW/60% QLD) and QX 009 (58% VIC/42% NSW). RT 033, the second most commonly identified type (13%, 20/154), was found equally between control and experimental farms and was more prevalent in non-scouring piglets, although this difference was not significant. RT 033 was found in 19/40 samples from SA and a single sample from Victoria. RT 237 was exclusively found in WA. RT 237, QX 006 and QX 009 were found only in experimental farms, but not associated with actively scouring piglets.

3.3.2.4 Piggery and sample demographics

Participating farms were either breeding or farrow-to-finish with 300–9000 sows per farm (mean = 1657). Of the 17 farms with complete questionnaire data, the mean pre-weaning mortality rate was 9.8% (range 2–14%). Pre-weaning mortality was higher in scouring farms than non-scouring farms (median rate 12 vs. 7%, respectively, $p = 0.012$). Fifteen farms provided information about effluent treatment, with 60% (9/15) using an on-site effluent treatment system; of these, six (66.0%) confirmed that treated effluent was reused within the farm, although how effluent was used was not identified. No correlation between farm scouring status and the re-use of treated effluent within the farm was found ($p = 0.559$). Antimicrobial usage data were provided by 18 of the participating 21 farms. Penicillins were most commonly used (2/3 of farms), followed by aminoglycosides, sulphonamides and cephalosporins (Table 3.6). The most common agents were amoxicillin, ceftiofur, Scourban^{TM}, and co-trimoxazole. Other antimicrobials used less routinely included tetracyclines and macrolides. Multiple

classes of antimicrobials were used in single farms; four farms (2 classes), four farms (3 classes), four farms (4 classes). Two farms in WA reported no antimicrobial use.

Table 3-6 Summary of antimicrobial use in Australian piggeries

Antimicrobial use was reported by veterinarians for farms participating in a period prevalence study of *C. difficile* in neonates (*n* = 18)

Farm ID No.	Scouring	Non-scouring	Penicillins	Sulfonamides	Aminoglycosides	Cephalosporins	Tetracyclines	Macrolides	Pleuromutilins	Quinolones
WA01	•									
WA02		•	•	•	•					
WA03	•									
QLD01		•								
QLD02	•			•	•	•				
QLD03	•		•	•		•				
QLD04		•	•	•	•	•				
QLD05	•		•		•		•	•		
VIC01		•	•							
VIC02		•	•	•						
VIC03		•	•							
VIC04	•		•	•	•					
VIC05	•									
VIC06	•		•		•					
NSW03	•			•				•		
SA01	•		•			•				
SA02	•		•	•	•	•				
SA03		•	•				•	•	•	
Total (n)	**11**	**7**	**12**	**8**	**7**	**5**	**2**	**3**	**1**	**0**
Total (%)	**61.1**	**38.9**	**66.7**	**30.8**	**38.9**	**27.8**	**11.1**	**16.7**	**5.6**	**0.0**

3.4 Discussion – prevalence studies

3.4.1 *C. difficile* prevalence

C. difficile emerged in the USA as an enteric pathogen of swine in the early 2000s and has since been recognised worldwide as such (Rodriguez-Palacios, Borgmann et al., 2013; Songer, 2004). Porcine CDI presents as typhlocolitis characterised by profuse non-haemorrhagic yellow pasty-to-watery diarrhoea ('scouring'). Sequelae include high pre-weaning mortality rates, poor growth rates and reduced weight at weaning (Songer and Uzal, 2005).

Although frequently diagnosed outside Australia, there is scant awareness of *C. difficile* as a pathogen of piglets in this country. To understand the scope of this issue locally two studies were conducted to evaluate the prevalence and molecular characteristics of *C. difficile* in the Australian pig population:

- a retrospective analysis of diagnostic samples submitted for *C. difficile* culture and typing
- a systematic Australia-wide period prevalence study in neonatal piglets (≤7 days of age).

3.4.2 *C. difficile* prevalence is widespread in Australian neonatal piglets at rates higher than major pig-producing countries

Although the retrospective study (study A) had some limitations due to the nature of convenience samples, this is the first data reporting *C. difficile* colonisation of neonatal piglets in Australia. Overall prevalence by enrichment culture was 56% (all samples) and 60% in neonates with symptoms that fit the case-definition of porcine CDI. This is higher than prevalence rates reported in retrospective analyses of diagnostic samples from the Netherlands (25%, enrichment culture) (Koene, Mevius et al., 2012) and Canada (48%, EIA + culture + histopathology) (Chan, Farzan et al., 2013), and farrowing-unit study samples from the USA (34%, enrichment culture) (Susick, Putnam et al., 2012). Results for the diagnostic prevalence study were confirmed by the period-prevalence study (study B) that demonstrated a 67% prevalence rate in neonatal herds from across Australia (Knight, Squire et al., 2015). Both studies demonstrate that *C. difficile* prevalence in Australian piglets is higher than in the major swine-producing regions of the world (the Netherlands, USA and Canada) as well as lesser producers

such as Slovenia (50.9%) (Avbersek, Janezic et al., 2009) and the Czech Republic (56.7%) (Goldova, Malinova et al., 2012), and is similar to recent reports from Sweden (67.2%) (Noren, Johansson et al., 2014) and Germany (73%) (Schneeberg, Neubauer et al., 2013).

Without a standardised protocol for laboratory isolation of porcine *C. difficile*, comparator studies in this section were chosen if they used an enrichment method. Several studies have shown that an enrichment step reduces false negative results, enhancing reliability of prevalence statistics (Blanco, Alvarez-Perez et al., 2013; Gould and Limbago, 2010).

Prevalence was higher in pigs ≤ 7 days of age (54% and 67%, studies A and B) versus older pigs (26%, study B only), consistent with colonisation frequency declining with age (Weese, Wakeford et al., 2010). Age-dependent susceptibility is also true for humans (McFarland, Brandmarker et al., 2000) and other animals (Baverud, Gustafsson et al., 2003; Knight and Riley, 2013; Rodriguez-Palacios, Koohmaraie et al., 2011; Zidaric, Zemljic et al., 2008).

Farm type and size, veterinary provider, and state of origin did not have a significant impact on *C. difficile* prevalence, although molecular diversity was higher in the single outdoor farm included in the study, possibly via increased exposure of piglets to environmental strains of *C. difficile*. There may be a seasonal prevalence pattern as significantly more samples were submitted in winter months than summer months in both studies, although *C. difficile* isolation peaked in summer. Several studies have identified this seasonal phenomenon in animals (Norman, Harvey et al., 2009; Rodriguez-Palacios, Barman et al., 2014). Neonatal piglets are unable to regulate their body temperature, rendering them exquisitely sensitive to cold stress, with subsequent reduction in innate immunity (Cheng, Morrow-Tesch et al., 1990). Compounding this, doors and windows in the farrowing facility may be closed to maintain heat, thereby increasing spore load.

3.4.3 Asymptomatic carriers confound diagnosis of CDI in scouring herds but are consistent with pathobiology of enteropathogenic organisms in piglets

Determining an association between *C. difficile* and CDI was outside the scope of the prevalence investigations, whose focus was to ascertain the presence or absence of the

organism in herds rather than specific diagnosis of *C. difficile* enteritis in piglets. Clinical aspects of *C. difficile* are discussed in Chapter 5. Nevertheless, the fact that 423 diagnostic samples were submitted in a two-year period to determine a cause for idiopathic scour cannot be ignored. Also relevant is the finding that farms with a history of neonatal scour had a higher pre-weaning mortality rate than those without (12 vs. 7%).

Asymptomatic carriage/colonisation is a common feature of individual animals in swine herds with CDI (Alvarez-Perez, Blanco et al., 2009). This was true in our investigation: *C. difficile* was isolated from 52% (57/109) non-scouring piglets in study A and 70% (126/181) in study B. Toxigenic *C. difficile* was distributed equally between scouring and non-scouring piglets in study B, but was associated more often with scouring piglets in study A, likely due to bias inherent in diagnostic samples.

Asymptomatic carriage of enteropathogens in piglets is not unique to CDI; asymptomatic animals have been reported in herds affected with ETEC (2012) and STEC (Cornick, Jelacic et al., 2002) colibacillosis, and salmonellosis (Gray, Fedorka-Cray et al., 1996). Despite this, the issue of asymptomatically colonised animals in CDI is a contentious one in Australia, and continues to confound diagnostic efforts. Asymptomatic colonisation may represent subclinical, early or mild infection, transient carriage or transient colonisation. Without repeat sampling of the prevalence cohorts transience is impossible to rule out. Correspondingly, without longitudinal evidence the possibility that sampling occurred during the disease incubation period is also impossible to ascertain.

There are several plausible explanations for high asymptomatic carriage rates of *C. difficile* in our studies. Herds in this study were colonised with non-toxigenic strains of *C. difficile* (Study A: 26/238 isolates, 11%; Study B: 24/254 isolates, 15.6%), which contributed to prevalence numbers but were associated with non-scouring piglets. Non-toxigenic strains are less common in pigs where RT 078 predominates, but they have been reported at low prevalence (7.7%) in one study (Janezic, Ocepek et al., 2012). The higher prevalence reported in our studies probably reflects greater genotypic diversity in general in our piglet strains. This phenomenon occurred in a Japanese study characterised by higher strain diversity than North American and European studies (Usui, Nanbu et al., 2014), where non-toxigenic strains comprise 20% of the total.

Secondly, the well-established link between humoral immunity and protection against symptomatic CDI may be involved (Kyne, Warny et al., 2001; Leav, Blair et al., 2010). Piglets are immunologically naïve and rely completely on passive immune transfer via maternal lactogenic antibodies (Rooke and Bland, 2002). Colostrum intake is also essential to provide energy for thermoregulation and gut development (Devillers, Le Dividich et al., 2011). Factors such dam parity (gilt versus sow) (Miller, Collins et al., 2008), teat positioning and access (De Passille and Rushen, 1989) and birth weight variability (Algers, Madej et al., 1991) may influence the quality and volume of protective maternally-derived lactogenic antibodies ingested by the naïve piglet, with concomitant differing clinical outcomes in response to pathogen challenge. This theory is supported by the fact that scouring resolves when piglets are cross-fostered to a sow with non-scouring progeny, suggestive of increased access to, or higher quality of, lactogenic antibodies. The presence or absence of symptoms may reflect a delicate interplay between protective antibodies and infectious dose, where immune response cannot compensate for repeated oral challenge by environmental spores.

Asymptomatic animals in the farrowing shed environment may also be important in the infectious cycle. *C. difficile* spores and vegetative cells are shed into the immediate environment in the faeces of both scouring and non-scouring pigs (Hopman, Keessen et al., 2011).

3.4.4 *C. difficile* RT in Australian piggeries are unique and genotypically diverse

C. difficile RT 078 is an A+B+CDT+ strain with similar virulence attributes to RT 027 (Goorhuis, Bakker et al., 2008; Walker, Eyre et al., 2013). It is the predominant RT isolated from swine in Canada (Keel, Brazier et al., 2007), the USA (Norman, Harvey et al., 2009), and the Netherlands (Debast, van Leengoed et al., 2009a). Our results showed that, unlike major swine-producing regions, RT 078 was not found in Australian neonatal pigs. Instead, a tremendous diversity of *C. difficile* strains circulates in Australian neonatal pigs.

Failure to isolate RT 078 was expected, since it has not been found in any Australian livestock (Knight and Riley, 2013; Knight, Thean et al., 2013) and is not endemic in human populations in Australia (Foster, Collins et al., 2014). Other strains often associated with pigs worldwide (RT 027, 150, 002, 045 and 081 (Janezic, Zidaric et al., 2014)) were also not found.

Molecular typing in study A revealed 17 different RTs, 12 (71%) of which are described for the first time in this study. Similarly, there were 23 RTs, including 14 novel (61%), in study B. Recent epidemiological studies in humans in Queensland (QLD) and Western Australia (WA) have reported 27% (Huber, Hall et al., 2014) and 16% (Foster, Collins et al., 2014) unique RTs, respectively. There was no cross-over when unique strains were compared between these human studies and the current piglet study. Further surveillance and typing of piglet strains should be conducted as our work represents only 5% (7/149) of QLD farms and 8% (4/48) of WA farms. Predominant human strain types circulating in major pig-producing states Victoria and New South Wales were not available for comparison. All other unknown RTs identified in pigs have subsequently been isolated from humans and other animal species (chicken, cattle, horses), both in Australia and South-East Asia. There was no epidemiological link between animal–human strains in our database. This requires further analysis; disseminated transmission of the same genotype is possible but only broad trends can be identified using the lower discriminatory power of ribotyping. WGS may show that substantial genetic diversity exists, even within isolates of the same RT, and these studies are currently underway with several animal–human spillover strains. Indeed, preliminary results suggest that the RT 014 strains isolated from piglets are a different sequence type to RT 014 strains isolated from humans (Knight and Riley, UWA, unpublished).

Clonal outbreaks occurred at individual sites, and RTs rarely crossed state borders. Australian *C. difficile* strains were expected to differ from strains isolated in the rest of the world because of the stringent import restrictions on live animals into Australia. Clonality and restricted distribution of RTs could be attributable to isolation of farms and vertical integration of Australian pork producers. Diversity of strains may reflect environmental strains present at individual farm sites. An inciting event (illness with another pathogen and antimicrobial treatment, for example) may cause an initial outbreak, with the strain clonally expanding in the breeding herd via environmental spore contamination.

The most common RT in study A was RT 237 (a representative of which was strain AI 35), comprising 54% of isolates; however, this is likely an artefact of larger sample submissions from farms at which this strain circulated. Prevalence of 10.4% for this RT in study B is more feasible. As the first RT isolated from Australian pigs it has

undergone further characterisation (Chapter 5) and was used in challenge studies in a piglet model of infection (Chapter 5). Phylogenetic analysis of MLST data indicates it belongs to ST11 and clade 5, as does RT 078 (Stabler, Dawson et al., 2012). RT 033, another clade 5 ST11 strain, was isolated from two samples in study A and was the second most prevalent strain in study B. The characteristics that underpin the success of clade 5 strains in pigs are not known. Gene-based tropisms related to breed may be a factor, with Yorkshire and Duroc the most common breeds in the USA and Canada where RT 078 dominates. The most numerous Dutch breed is Landrace x Large White (van Asten and Buis, 1977), the same as Australia, but the proximity of Dutch pig farms to each other may better explain the initial spread and establishment of RT 078 to the exclusion of other strains. The genetic basis for this dominance is unknown, but could feasibly involve a clade-specific PaLoc variation (Dingle, Griffiths et al., 2011; Elliott, Dingle et al., 2014),

RT 014 (A+B+CDT-, MLST clade 1) was the most prevalent RT in study B, comprising 23.4% of isolates. RT 014 is the most common RT infecting humans in Australia (Foster, Collins et al., 2014). It has been isolated rarely from animals outside Australia, and never from pigs (Koene, Mevius et al., 2012; Rodriguez-Palacios, Borgmann et al., 2013). Initial WGS results suggest that human and pig RT 014 strains are genetically different (Knight and Riley, unpublished). RTs 237 and 033 have also been isolated from humans, albeit in low numbers (Foster, Collins et al., 2014). Many other clinically important RTs circulating in humans in Australia, including emergent RT 251, 126, 127, 078, 244 (Foster, Collins et al., 2014; Huber, Hall et al., 2014), were not isolated from piglets in this study.

Three different RTs (UK 237, AU 211, AU 187) were recovered from a single farrowing hut on the only outdoor farm in this study (farm A4). This farm was owned by the same company as another three farms (A1–3) at which RT 237 dominated. There was no movement of pigs between the outdoor farm and the other three herds, but they did share staff. Only one strain, QX 7, was isolated from more than one farm. There was no movement of animals between these farms but they shared the same veterinarian. Contamination of samples can be ruled out, as these strains were isolated on separate occasions and multiple replicates. This highlights the highly infectious and hardy nature of *C. difficile* spores, which have been found on farm workers' boots (Noren, Johansson

et al., 2014) and healthcare staff's clothing (Perry, Marshall et al., 2001) and hands (Landelle, Verachten et al., 2014).

3.4.5 *C. difficile* strains in Australian piglets are mostly toxigenic and genotypically different to the rest of the world

The symptoms of CDI result from production of toxins tcdA and tcdB in the gut of affected animals. Some strains produce an additional binary actin-ADP-ribosylating toxin (CDT), the role of which is not well elucidated, although it is postulated to assist with colonisation. CDT-producing strains are strongly associated with animals; 20%–100% of animal strains are CDT+, versus <10% of human isolates (prior to the BI/NAP1/027 outbreak) (Rupnik, 2007). Strains that produce only CDT and not tcdA or B (A-B-CDT+) colonise the gut but do not cause symptomatic disease in hamsters (Geric, Carman et al., 2006); however, a strain with this toxin profile has been recovered from a bacteraemic patient in Australia (Elliott, Reed et al., 2009).

Toxigenic strains, producing tcdB and/or tcdA, were abundant (87%) in both studies, with genotypic heterogeneity between studies. This is less than the >99% prevalence of toxigenic strains reported in other studies where RT 078 predominated (Avbersek, Janezic et al., 2009; Hopman, Keessen et al., 2011), and higher than the 58% reported in a study with greater RT diversity (Thakur, Putnam et al., 2010). These findings support human studies that show non-toxigenic *C. difficile* comprise 20%–25% of the total *C. difficile* population in humans (Schmidt and Gilligan, 2009).

The most common toxin profile identified in Study A was A-B+CDT+ (80%, 166/208). This toxin type was overrepresented, as 78% (129/166) of these were RT 237. It is still clinically relevant, however, as A-B+CDT+ strains were isolated from seven farms across four states, representing five different RTs. A-B+CDT+ strains were much less prevalent in study B (10%, 16/154), presumably because the study sample better represented the population. Clinically relevant A-B+ strains are well described in humans (Elliott, Squire et al., 2011) but rare in pigs outside Australia (Janezic, Zidaric et al., 2014; Thakur, Putnam et al., 2010). To date, Australian A-B+ strains (human and piglet) appear genotypically different from those in the rest of the world (Elliott, Squire et al., 2011). Toxin A production is abolished via a large deletion in the PaLoc removing the entire *tcdA* gene. This contrasts with four other identified A-B+ variants

which possess PaLoc mutations involving smaller insertions and deletions (Rupnik, Kato et al., 2003).

The toxin profile A+B+CDT- (43.5%, 67/154) dominated in study B, comprising nine RTs, three of which were novel. This profile was the second most prevalent in study A (13%, 32/208). A+B+CDT- strains were widespread, found in four farms across three states and encompassing four RTs (three novel). This finding is unusual as animal strains outside Australia are generally positive for CDT. CDT positive isolates made up only 41% (63/154) of pig isolates and 54.5% (55/101) of the top five prevalent RTs in study B. These strains made up 82% (194/238) of isolates in study A, but when UK 237 isolates are removed (n = 129), only 27% of strains were CDT+ (including 19 non-toxigenic A-B-CDT+ strains). These prevalence figures are significantly less than reported elsewhere (Rupnik, 2007) further supporting the unique molecular epidemiology of *C. difficile* in Australian piglets.

Although there was a significant association between the presence of toxigenic strains and scouring piglets and farms with a history of scouring in study A, this likely reflects diagnostic sample submission, which was biased towards scouring piglets. Study B showed no association between *C. difficile* colonisation and scouring, consistent with all other piglet studies worldwide. Significantly more non-toxigenic strains (A-B-CDT-) were isolated from non-scouring farms in both studies. It is tempting to conclude that competitive exclusion of toxigenic strains in these herds was responsible for this relationship. This hypothesis is supported by a human study where targeted probiotics in the form of orally administered spores of non-toxigenic *C. difficile* strain VP 20621 successfully prevented CDI in patients with disrupted gut flora (Villano, Seiberling et al., 2012). Similarly, mortality decreased, weaning weight increased and there was less *C. difficile* toxin in the gut of piglets administered oral spores of a non-toxigenic *C. difficile* strain (Songer, Jones et al., 2007). There is a caveat: in vitro evidence demonstrates that non-toxigenic strains can acquire the PaLoC from toxigenic strains (Brouwer, Roberts et al., 2013).

3.4.6 There was no association between *C. difficile* in neonatal piglets and antimicrobial use but reliance on antimicrobials of high and critical importance in Australian piggeries

More than 90% of CDIs occur in conjunction with antimicrobial therapy, making this the most important risk factor for development of CDI in humans. This is primarily through perturbation of gut flora but also because *C. difficile* is resistant to multiple antibiotics, allowing it to colonise during treatment (Rupnik, Wilcox et al., 2009). There is evidence that antibiotic administration does not contribute to colonisation of neonates with immature gut flora but may contribute to higher rates of colonisation and CDI in three- to ten-week-old nursery animals (Arruda, Madson et al., 2013; Susick, Putnam et al., 2012). Similarly, there is no association between antimicrobial treatment and *C. difficile* colonisation. Administration of antimicrobials to older piglets in the farrowing shed could constitute an inciting event, contaminating the environment with spores and resulting in a *C. difficile* outbreak in naïve piglets. In particular, the use of ceftiofur, a third-generation veterinary cephalosporin to which *C. difficile* is intrinsically resistant, is likely a high-risk practice, especially if administered to neonates as protective gut flora is developing.

Antimicrobial resistance (AMR) arising from antimicrobial use in the livestock industry is another consideration. Veterinary antimicrobials are rated according to their importance for human use (World Health Organisation (WHO), 2012). The use of agents of high or critical importance in veterinary medicine attracts most risk as there are no alternative treatments for animals or humans should AMR arise. Risk assessment is limited by a lack of Australian data on AMR in bacteria of importance, and of detailed information about antimicrobials used in different sectors of the livestock industry. There is no systematic surveillance of AMR in Australian pigs and only one published study on antimicrobial use (Jordan, Chin et al., 2009), although a draft discussion paper was jointly released by the Departments of Agriculture and Health to address this issue using a ‘one health’ approach (Australian Government Department of Agriculture and Australian Government Department of Health, 2014).

The small amount of available data reveals some worrying trends. Jordan et al. revealed widespread use of antimicrobials of low importance to human health in Australian pigs, but reported ceftiofur use in 25% of herds. Multi-resistant *E. coli* (Barton, Pratt et al.,

2003) and MRSA ST398 (Groves, O'Sullivan et al., 2014) have been reported in local swine. Resistance genes, such as *ermB* conferring macrolide-lincosamide-streptogramin B (MLSB) resistance and *tetW* conferring tetracycline resistance, have recently been discovered in a *C. difficile* UK 014 strain isolated from Australian pigs. Human UK 014 strains isolated during the same time period do not possess these genes (Dan Knight, UWA, unpublished data).

Similar to Jordan et al. there was a wide assortment of antimicrobials used in neonatal pigs in our study, and usage was widespread, with 16 of the 19 farms in study A using these agents in neonatal herds. Unlike the Jordan et al. study, there was a reliance on drugs of high-critical importance in Study A. These included neomycin (59% of neonatal herds), ceftiofur (53%), apramycin (35%), streptomycin (24%) and penicillins (24%). Study B showed penicillins (66.7%) and aminoglycosides (38.9%) were most commonly used, both classes comprising agents of critical importance. Although APL maintains that antimicrobials are not used for growth promotion purposes (Australian Pork Australian Pork Limited, 2013), metaphylaxis of neonates with penicillin was reported in one herd in study A, although whether the dosage was therapeutic or subclinical was not disclosed.

The Australian pork industry's own (unpublished) research indicates 'that resistance in broad spectrum cephalosporins such as ceftiofur is currently at negligible levels within the pig industry i.e. there is widespread reliance on other drugs, rated to be of low importance in the context of human health'. Despite this, the Pork Co-operative Research Centre has taken a responsible position and committed $AUD7 million under their Herd Health Management program to reduce antimicrobial usage in the industry by 50% over five years (Australian Pork Australian Pork Limited, 2013).

3.4.7 Limitations

Convenience sampling has a number of well-described limitations, primarily lack of representation of the study population and non-random selection of subjects. However, it is a useful and cost-effective method to obtain preliminary data to inform future probability-based studies. Limitations relating to convenience sampling are identified throughout this chapter where appropriate.

The major limitation of the prevalence studies is the small sample size, representing only 5% of the total pig operations in Australia (Table 3.7). The prevalence findings were consistent between both studies, however, lending support to the hypothesis that the situation reported here is similar throughout the industry. Clearly, additional confirmatory data is required, along with continued Australia-wide surveillance, to identify epidemiological trends.

Table 3-7 Farms sampled in Australian *C. difficile* prevalence studies

Source: (Australian Pork Limited, 2012-2013)

	Prevalence study samples		
State	**No farms sampled (*n*)**	**Total farms (*N*)***	**% sampled (*n/N*)**
WA	4	48	8
SA	3	93	3
VIC	11	108	10
QLD	7	149	5
NSW	2	161	1
TAS	0	16	0
NT	-	0	-
ACT	-	0	-
TOTAL	27	575	5

Note: * Number of operations by pig farms by state

There are several additional limitations applicable to these prevalence studies. In a true retrospective cohort study the population would be compared in terms of disease development in exposed versus non-exposed individuals. Because samples were being submitted for diagnostic purposes there is a selection bias towards all exposed individuals; hence, controls (non-exposed individuals in the two non-scouring farms) were not matched to the exposed population, either in frequency or risk factors for disease development, as they were at separate farms. To minimise this, the systematic cohort study was designed to ascertain if *C. difficile* was more prevalent in exposed versus non-exposed populations.

There is also an information bias. Due to the emergent nature of this organism, potential for disease misclassification is high. Piglets were chosen based on the probability they had *C. difficile*, which depended on the veterinarians' knowledge of CDI, and/or samples were not sent until all other pathogens had been ruled out. Veterinarians therefore sent samples from scouring pigs that did not necessarily match the age/clinical

definition of CDI. The denominator (total number of samples) in study A was reduced to include only cases that matched CDI on submission (from clinical notes sent with the samples).

Comparison to international studies is also problematic as isolation methods differ between studies. To minimise this, EC data was used as the basis of comparisons.

Temporal variation in prevalence is also a factor. Isolation of *C. difficile* may indicate not colonisation but the transient passage of ingested spores, especially where the farrowing shed is highly contaminated. To control for this a longitudinal study with a dose-response component based on environmental contamination is currently under way.

3.4.8 Conclusion

Despite study limitations, it is clear that *C. difficile* is commonly found in the faeces of neonatal piglets in Australia. These strains are heterogeneous and genotypically distinct, and the majority are toxigenic. The use of antimicrobial agents of high-critical importance in neonatal herds is worrying from a *C. difficile* and AMR perspective.

Chapter 4 Environmental contamination with *C. difficile* spores

4.1 Introduction

Contamination of the environment with *C. difficile* spores plays a critical role in transmission in the human hospital setting. Little is known about the epidemiology of *C. difficile* spore contamination in the piggery environment. To understand the natural history of *C. difficile* in piggeries, a nine-month prospective study was designed to determine the proportion of pens contaminated with *C. difficile* spores in a new farrowing facility before and after occupation with pigs, and the density of spore contamination (Section 4.2).

Several studies have characterised the prevalence of pathogens in piggery effluent in Australia, but spore-forming organisms were not included (Chinivasagam and Blackall, 2005; Chinivasagam, Corney et al., 2007; Chinivasagam, Thomas et al., 2004). Given the hardy nature of spores, thorough risk assessment should include *C. difficile* to determine properly the transmission risk within and external to the piggery.

4.2 Prospective evaluation of *C. difficile* contamination in a farrowing facility

4.2.1 Experiment design

The study was conducted in a new farrowing shed on an established 2000-sow piggery in WA. This piggery previously had a severe neonatal scour problem; approximately 80% of neonates experienced idiopathic scour in 2009/10. Sixty-two per cent (124/200) of faecal samples from neonatal piglets sent to our laboratory from this facility between June 2009 and February 2010 were positive for *C. difficile*. All but one isolate was RT UK 237. At the time of this study neonatal scouring on the farm had reduced to negligible levels (personal communication, farm manager).

The farrowing shed design was conventional, with 225 pens and adjoining creep areas (each 2 x 0.6 m) housed within a 75 x 17 m steel and concrete building on a new concrete slab. Pens had plastic slatted floors and creep areas were heated using heat

mats to 16–20^{0}C above air temperature. During the study period organic material was removed from pens by high pressure hosing, followed by disinfection with FarmFluid S™ (a non-sporicidal agent containing phenolic compounds and tar oils).

Pens were sampled using the method described in Section 2.3.2 during the period October 2010 to August 2011. The number of sampling points was calculated to ensure sufficient power (95% confidence, Section 2.3.2.1). The orange heat mat in every second pen and the corners of the farrowing shed were sampled on each visit with the exception of the first visit, where every fourth pen was chosen as significant contamination was not expected. Pens did not need to be occupied with pigs at the time of sampling to be included in the study. Samples were taken at the completion of construction and prior to occupation with pigs ('background sampling'), then at one, six and nine months post-occupation. Sampling utilised both Transwabs (all sample sets, N = 398) and Polywipe sponges (in addition to Transwabs for a subset of the final sample set, n = 50). All Transwab samples were cultured by DC and EC methods on TCCFA and compared with CA. Polywipe sponge samples were cultured by DC on both TCCFA and CA using the method outlined in Section 2.3.2.2.

ANOVA was used to compare parametric recovery data using different methods on different media, with Dunnett's multiple comparisons correction applied. The $\chi 2$ test for trend was used for longitudinal analyses. Student's t-test was used to assess relationship between scouring piglets and spore load. A p value <0.05 was considered significant.

4.2.2 *C. difficile* prevalence and impact of sampling/isolation methods

During the nine-month surveillance period a total of 448 environmental samples were collected. Overall, 20% (89/448, 95% CI 16-24%) were positive for *C. difficile* by DC on TCCFA, 28% (127/448, 95% CI 24-33%) by EC on TCCFA, and 48% (213/448, 95% CI 43-52%) by DC on CA (Table 4.1).

C. difficile recovery on EC on TCCFA versus DC on TCCFA was not stastically different (p = 0.0872). Analysis of identical *C. difficile* positive samples collected with Transwab and processed on all three media types (n = 293) showed that DC on CA (166/293, 56%) performed numerically better than than culture on TCCFA by either

direct (60/293, 20%, $p = 0.322$) or enrichment (67/293, 23%, $p = 0.308$) methods, although this was not statistically significant.

Samples collected with Polywipe sponges and plated directly onto TCCFA were equally likely to yield *C. difficile* (9/50, 18%) when compared with DC of Transwabs on TCCFA (8/50, 16%), but less likely when compared with EC of Transwab samples (22/50, 44%). Comparison of DC on CA was not performed, but when Polywipe samples were cultured on CA for spore enumeration purposes, recovery of *C. difficile* increased to 97% (47/50) (Table 4.1).

A random subset of samples ($n = 8$) was characterised by PCR ribotyping (method, Section 2.3.4.2) to assess the relatedness between environmental and clinical samples from the farm. A single RT, UK 237, was identified (data not shown).

4.2.3 Longitudinal analysis of *C. difficile* prevalence

The proportions of environmental contamination were compared at different sampling times (before occupation with pigs then and at one, six and nine months). Mean prevalence, calculated using all Transwab culture methods for each time period, was 0%, 40%, 45% and 37% respectively (Range: 0–82%). There was a significant longitudinal increase in *C. difficile* prevalence ($p = 0.0323$), particularly one month after the shed was occupied with pigs (Table 4.1). If results from CA direct culture of Transwab samples are assumed to be the most sensitive, the prevalence is higher at 0%, 61% and 82% (results for the final sample set are not included as this was performed with Polywipes cultured on CA only).

Table 4-1 Summary of *C. difficile* isolation from the farrowing unit environment

C. difficile was isolated by different sample and culture methods before and at 1, 6 and 9 months' post-occupation of a new farrowing unit with pigs

Sampling time points				Isolation of *C. difficile*						
Date	Occupation status	Total samples (*N*)	Method	No (%) positive for *C. difficile* (*n*)					Spore count (spores/cm^2)	
				DC (TCCFA)	EC (TCCFA)	DC (CA)	Total	$\tilde{x}$	$\tilde{x}$	Range
Oct-10	Background sampling	46	NRS Transwab	0 (0)	0 (0)	0 (0)	0	0	0	0
Dec-10	Occupied for 1 month	116	NRS Transwab	33 (29)	34 (29)	71 (61)	138	34	2	0-25
May-11	Occupied for 6 months	116	NRS Transwab	27 (23)	33 (29)	95 (82)	155	33	1	0-20
Aug-11	Occupied for 9 months	120	NRS Transwab	29 (24)	60 (50)	ND	89	45	ND	ND
Aug-11	Occupied for 9 months	50[§]	Polywipe sponge		ND	47 (94)			34	0-174
Total (all) [95%CI*]		398		89 (20) [16-24]	127 (28) [24-33]	213 (48) [43-52]				

Note: * = Confidence interval; § = subset of Transwab sites also sampled with Polywipes

Table 4-2 Quantitative analysis of *C. difficile* spore-contaminated pens

Results are stratified by spore density ≥4 spores/cm^2 and 17-29 spores/cm^2 before and 1, 6 and 9 months post-occupation with pigs

Sampling time points				No. *C. difficile* spore-contaminated crates					
Date	**Occupation status**	**Total crates sampled (*N*)**	**Method**	**≥4 spores/ cm^2 (*n*)**	**% (*n/N*)**	**95% CI***	**17-29 spores/ cm^2 (*n*)**	**% (*n/N*)**	**95% CI***
Oct-10	Background sampling	46	NRS Transwab	0	0	0	0	0	0
Dec-10	Occupied for 1 month	116	NRS Transwab	21	18	12-26	4	3	1-9
May-11	Occupied for 6 months	116	NRS Transwab	11	9	5-16	3	3	1-7
Aug-11	Occupied for 9 months	50[§]	Polywipe sponge	35	70	56-80	21	42	29-56
Total		278		67	24	19-29	28	10	7-14

Note: * = Confidence interval; § = Transwab counts not performed

4.2.4 *C. difficile* spore loads

Spore counts were performed for all time periods using the method outlined in Sections 2.3.2.2/3. Transwab samples cultured on CA were used for spore counts for all except the last time period, where Polywipe sponge samples cultured on CA were used. Table 4.1 shows the overall frequency of *C. difficile* isolation and the corresponding spore loads. There was a wide variation in the number of spores detected, ranging from 0–174 spores/cm^2. Overall, the mean concentration of viable spores for each time period was 0, 1, 2 and 34-spores/cm^2 (the final sample set was collected with Polywipes cultured on CA). This equates to 0, 1.2×10^4, 2.4×10^4, and 4.08×10^5 spores per pen, respectively (range: 0–2.088×10^6 per pen).

Recent reports suggest that environmental contamination with 4 spores/cm^2 of toxigenic *C. difficile* (Strain 630, A+B+CDT-) is sufficient to infect 50% of immunocompetent mice during 1 hour of exposure (Lawley and Young, 2013). 'Heavy contamination' is defined as 17–29 spores/cm^2 (Lawley, Clare et al., 2010). If we assume these findings can be extrapolated to immune-naive piglets, Table 4.2 (above) shows the number and proportion of spore-contaminated pens at each sampling period that meet these criteria.

Comparison between spore numbers in pens containing scouring piglets and their near environment (≤ 3 pens either side) and remaining pens showed no correlation between scouring piglets and spore load ($p = 0.538$).

A review of product literature and MSDS of disinfectants used in the farrowing environment reveals that the commonly used agents Microtech 7000, Farm Fluid S and Virkon S are not sporicidal (Table 4.3). Disinfecting agents were not tested against *C. difficile* spores.

Table 4-3 Summary of disinfectants commonly used in Australian piggeries

Disinfectants are listed according to their active ingredients and sporicidal capability as determined by manufacturer's literature and MSDS

Trade Name	Manufacturer	Class	Active ingredient/s	Sporicidal	Concentration	Application time	Effective in presence of organic material	Safety/ compatibility
Microtech 7000 (also known as Vantocil FHC)	Artech Technologies Pty Ltd	Biocide:polymeric biguanide + quaternary ammonium compound (QAC)	Polyhexamethylene biguanide HCl Benzalkonium chloride	No Sporistatic only	1:500	5 minutes then rinse	✗	
Farm Fluid S	BioSentry/ Antec	Phenolics + tar oils	419.2g/L high boiling tar acids 324.9g/L acetic acid 52.4g/L cresylic acid	No[@]	1-2%	20-30 mins then rinse	✓[§]	Corrosive to plastics & rubber Severe skin and eye irritant
Virkon S	DuPont	Peroxygen producing formulation	potassium peroxymonosulfate	No	1%	10 min[*]	✓[#]	

@ - Product literature says yes but refers to *A. niger* fungal spores

*- Sporicidal after 2 hours (Hernndez A, Martro E, *et al*. Assessment of *in-vitro* efficacy of 1% Virkon against bacteria, fungi, viruses and spores by means of AFNOR guidelines. J Hosp Infect. 2000 Nov;46(3):203-9)

§ - requires cleaning first for use at this concentration

- reference provided by company disputes this (Amass SF, Ragland D, Spicer P. Evaluation of the efficacy of a peroxygen compound, Virkon S, as a boot bath disinfectant. Swine Health Prod. 2001;9(3):121-3.)

4.3 *C. difficile* prevalence in farrowing unit effluent: a pilot study

We hypothesised that *C. difficile* would survive in effluent throughout the treatment process due to the resistant nature of its spores, thereby contributing to contamination of the farrowing shed and broader piggery environment.

To test this we conducted a pilot study to ascertain the presence, number and molecular type of *C. difficile* at all stages of farrowing unit influent, effluent treatment, and storage in the two-stage treatment system located on same large farrow-to-finish facility investigated in Section 4.2. Two-stage treatment involves biological inactivation of pathogens via an interconnected primary anaerobic pond followed by a facultative pond (Figure 4.1). This farm also used a final evaporative (aerobic) pond for storage and evaporative disposal of treated effluent. No additional tertiary disinfection treatments were performed at the site. Sedimented solids from the anaerobic pond were stockpiled on site or spread to land. Treated liquid effluent from the ponding system was recycled to storage tanks associated with each farrowing shed and used for flushing under-pen gutters and high-pressure cleaning of the sheds (Figure 4.1). Ponds were maintained in accordance with the National Environmental Guidelines for Piggeries (2nd edition) 2011 (Australian Pork Limited, 2011). Data relating to pond functioning/stability were not available.

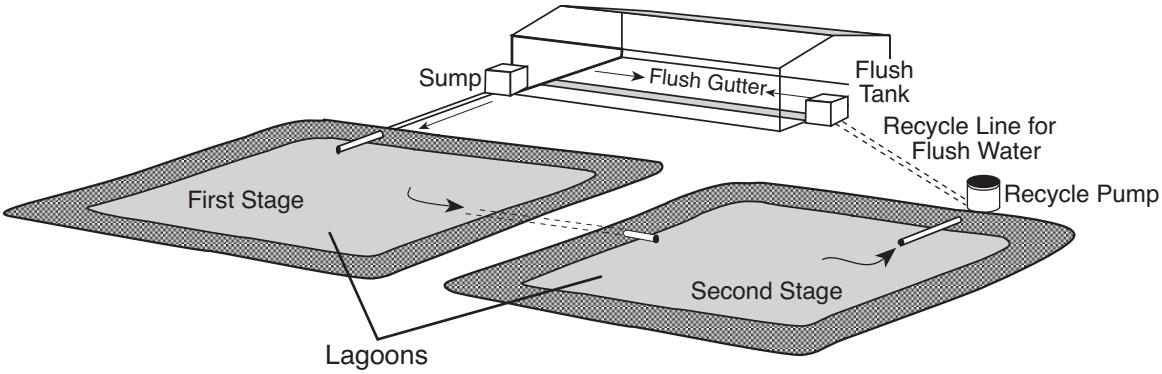


Figure 4.1 Two-stage treatment of piggery effluent

Biological inactivation of effluent occurs in the primary anaerobic pond ('first stage lagoon') followed by the 'second stage' facultative lagoon. Treated effluent is transported back to the flush tanks where it is stored prior to use to flush under-pen gutters in the farrowing sheds. An aerobic bulk storage pond can also be utilized as an optional third stage prior to flush tank.

Source: http://www.epa.gov/agriculture/ag101/porkmanure.html

Ms Su Chen Lim (Masters of Infectious Disease, UWA) performed the laboratory work for this investigation in April 2011 with my assistance and supervision.

This study was published as: Squire MM, Lim SC, Foster NF, Riley TV. Detection of *Clostridium difficile* after treatment in a two-stage pond system. van Barneveld RJ, editor. Adelaide, Australia: APSA Biennial Conference, Australasian Pig Science Association; 2011. 215.

4.3.1 Experiment design

One-litre samples (n = 10) of effluent were collected from sampling points representing pre-treatment, each treatment step and storage repositories (Figure 4.2). Samples were collected during a single sampling trip in April 2011, as per the method described in Section 2.3.2.4. Samples were taken using purpose-designed equipment at a depth of at least 30 cm except for storage tank samples, which were taken from the surface of filled tanks. Samples were transported by car under ordinary environmental conditions and stored at ambient conditions until analysis within seven days.

One mL of effluent from each sampling point was cultured by the direct spread plate method on CA (Section 2.3.1.3). Black colonies typical of *C. difficile* on this medium were counted and a selection of colonies from each plate was subcultured onto BA and confirmed as *C. difficile* by their colony morphology and characteristic odour. Molecular characterisation was performed by PCR ribotyping and PCR detection of toxin genes (Section 2.3.4).

Figure 4.2 Onsite effluent treatment system at the piggery under investigation. Sampling points are marked

4.3.2 Enumeration of *C. difficile* at effluent treatment stages

C. difficile was isolated from 100% (10/10) of samples, representing influent and all stages of effluent treatment. Numbers of organisms at each stage of the effluent treatment system are shown in Table 4.4. Spore numbers ranged from 35 cfu/mL (storage tanks, post treatment) to 230 cfu/mL (facultative pond stage). Overall spore numbers declined by 44% throughout the treatment system (80 cfu/mL to 35 cfu/mL), although spores increased in number in the anaerobic and facultative treatment phases (130 and 230 cfu/mL, respectively).

Fourteen *C. difficile* isolates were subcultured and examined by PCR ribotyping and toxin gene PCR. All isolates were PCR ribotype UK 237, the toxigenic strain that predominated in piglets at this farm. Toxin profiling revealed that, like UK 237, all isolates were toxin A-B+CDT+ (data not shown).

Table 4-4 Quantitative analysis of *C. difficile* spores from farrowing shed effluent at all stages of influent and effluent treatment

Treatment stage	Sampling point	No. samples	*C. difficile* positive	Spores (cfu/ mL)
Pre-treatment	Sump from farrowing sheds	1	1	80
Anaerobic pond	Entrance and exit from pond	2	2	130
Facultative pond	Middle of pond	1	1	230
Evaporative pond	Middle of pond	1	1	93
Storage tanks	Surface of tanks	5	5	35

4.4 Discussion – environmental contamination

4.5 Farrowing unit contamination study

Clostridium difficile is different to most other piggery pathogens because it produces highly resilient spores. During CDI infectious spores are excreted into the environment by infected individuals and spread by direct contact (Samore, Venkataraman et al., 1996). Contamination of the environment with *C. difficile* spores plays a critical role in transmission in the human hospital environment (Gerding, 2009). Spores can persist for long periods of time as they are resistant to UV, heat, desiccation and commonly used disinfectants, including alcohol-based hand washes (Gerding, Muto et al., 2008).

Neonatal pigs acquire infection through contamination of the farrowing shed with spores (Hopman, Keessen et al., 2011). Both scouring and non-scouring piglets colonised with *C. difficile* shed spores into the environment (Alvarez-Perez, Blanco et al., 2009), and disinfectants commonly used in Australian piggeries are not sporicidal. Spore contamination of sites within veterinary facilities that housed CDI-afflicted animals has been reported as well. (Weese, Staempfli et al., 2000a).

We hypothesised that spore contamination would be significant in the farrowing shed environment because of scouring piglets, augmented by lack of sporicidal disinfection. The aim of this study was to quantitatively and qualitatively determine the development of spore contamination prospectively in a new farrowing facility. This is the first study to quantitatively investigate spore contamination of piggery facilities contemporaneously with its development.

4.5.1 Prevalence of *C. difficile* spore contamination in the farrowing shed

4.5.1.1 C. difficile *spore recovery from environmental samples is superior on a specific C. difficile chromogenic agar (CA) to TCCFA*

There is no reference method for environmental sampling in piggeries, so samples were collected with rayon-tipped swabs and cultured by DC and EC on TCCFA, and DC on CA. A subset of samples in the final sample set was taken with Polywipes to compare performance with swabs.

We showed that compared to EC and DC on TCCFA, DC on CA performed better at detecting *C. difficile* when using rayon-tipped swabs for sample collection. Of the 293

samples collected in this study, 57% (166/293) were positive for *C. difficile* by DC on CA versus 20% (60/293) for DC on TCCFA and 23% for EC on TCCFA (67/293). This is lower than the 72% reported in farrowing pen contamination in the only other environmental study in piggeries (Hopman, Keessen et al., 2011). The difference in recovery may be partly due to the large surface area (540 cm^2) sampled with dry electrostatic cloths in the Dutch study versus 100 cm^2 in our study. The cloths were also subject to spore enrichment for seven days before culture.

The use of rayon swabs to sample environmental surfaces has been tested in several studies. Rayon swabs were the least effective of several sampling methods in a hospital-environmental *C. difficile* sampling study (Claro, Daniels et al., 2014). An investigation of *Bacillus anthracis* spore recovery from a non-porous surface (Rose, Jensen et al., 2004) found inferior recovery for rayon swabs because of their inability to loosen spores from the surface. Spore recovery using swabs was also less precise with high standard deviations relative to the mean recovery efficiency, possibly based on collection error (angle and pressure of sampling) as well as spore quantity, surface composition and non-homogeneous spore distribution. Brown et al. (Brown, Betty et al., 2007) showed that positive samples obtained using swabs were indicative of high spore concentration, at least 1 cfu/cm^2.

We found no difference in *C. difficile* isolation rates using swabs (16%) versus commercially available sponges pre-moistened with peptone-saline (Polywipe) (18%). Collection error was minimised as the same operators collected samples on each occasion and sample sites were identical throughout the study. Although recovery efficiency was similar, sponges had significant advantages: they were easier to use in the field, more convenient when sampling a large surface area that was difficult to access, and did not require enrichment when used with CA medium. Culture medium likely played a more significant role in spore recovery than sample collection methodology. We found 97% (47/50) of pens were positive for *C. difficile* when 100cm^2 samples were taken with Polywipes and cultured on CA. This is consistent with findings from several studies that *C. difficile* recovery from clinical specimens is superior on CA than TCCFA, alone or with enrichment (Carson, Boseiwaqa et al., 2013; Knight, Squire et al., 2014); and is also true for quantitative recovery of *C. difficile* from human faecal specimens (Boseiwaqa, Foster et al., 2013).

4.5.1.2 Environmental prevalence increased significantly with piglet occupation but could not be explained by scouring piglets alone

We found a significant, increasing trend in the proportion of *C. difficile* in farrowing shed pens across the nine-month sampling period with 0, 61, and 82% prevalence, respectively, at three sampling times. Remarkably, more than half the pens were contaminated with *C. difficile* spores only one month after occupation when spore contamination was below detectable levels at baseline. At the one-month sampling point only four piglets (all < 7 days) of a possible ~2000 had visible scour, suggesting that actively scouring piglets were likely only partially implicated in spore contamination. Sows farrow at weekly intervals, so litters that farrowed earlier in the month may have had self-limiting scour that contributed to environmental contamination. Regardless, infectious dose and subsequent scouring would have been minimal given that spores could not be identified in any pen at background sampling, and sows are colonised at very low rates (Weese, Wakeford et al., 2010). Spore degradation is improbable during this time period as spore numbers shed in excreted faeces remain constant for 30 days (Lawley, Clare et al., 2009). There was no correlation between spore load in pens containing scouring piglets and near environment (≤ 3 adjacent pens) compared to all other pens in the farrowing shed, lending support to the hypothesis that other factors contributed to the high environmental prevalence of *C. difficile* spores.

4.5.1.3 Spore density was high but its significance to infection dynamics in piglets is unknown

Quantitative analysis revealed spore counts of 0, 1, 2 and 34-spores/cm^2 equating to 0, 1.2×10^4, 2.4×10^4, and 4.08×10^5 spores per pen, respectively (range: 0–2.088×10^6 per pen). This is substantially higher than spore contamination of hospital floors of patients infected with *C. difficile* (Mean: 0.1/cm^2) (Mutters, Nonnenmacher et al., 2009), likely due to specific sporicidal cleaning in this sector and obvious differences in toileting practice. Spore density increased at each sampling point despite cleaning and disinfection of pens vacated by weaned piglets and their sow, suggesting that sporicidal cleaning was ineffectual.

Environmental prevalence is linked to increased risk of CDI in humans (Fawley and Wilcox, 2001). The converse is also true: reduction in environmental spores decreases CDI incidence (Mayfield, Leet et al., 2000; McMullen, Zack et al., 2007; Wilcox, Fawley et al., 2003); the threshold number of environmental spores required to initiate

or increase infection remains unknown, however. Lawley et al. (Lawley, Croucher et al., 2009) determined the dose required to infect 50% of mice (ID50) in a murine transmission model was 5 spores/cm^2 for 1 hour of exposure. A spore density of 30–40 spores/cm^2 infected 100% mice within one hour, and 17–29 spores/cm^2 was regarded as heavily contaminated. If this scenario were true for piglets, one quarter of pens sampled in this study, on average, contained sufficient spores to infect piglets. Whether this model is predictive of the piglet response is unknown, but it provides a useful basis for future hypotheses that can be tested in a piglet, rather than murine, model.

Transmission dynamics between piglets and their environment were impossible to determine in this study. Consistent with our previous findings, a clonal strain, RT UK 237 in this instance, predominated in the facility. Even with highly discriminatory WGS it would be difficult to determine transmission events over a short time frame with a single clone.

Neither our study nor the Lawley et al. investigation took into account vegetative cells. Vegetative cells survive long enough to be infectious on pen flooring where piglets root. On dry surfaces, vegetative *C. difficile* cells die rapidly, but they remain viable for six to seven hours on moist surfaces in room air (Jump, Pultz et al., 2007; Lawley, Croucher et al., 2009). The environmental samples were not maintained in an anaerobic environment prior to processing, and were processed 24 hours after sampling, so any vegetative cells would have desiccated prior to culture; hence the total density of *C. difficile* (vegetative cells plus spores) in the farrowing shed environment is likely to be under-represented in our study.

4.5.2 *C. difficile* spore eradication in the farrowing shed is largely ignored by the pork industry

Environmental contamination is a key component of *C. difficile* transmission, so any treatment regimen must focus on interrupting the spore transmission cycle. Spores can survive in the environment for several months (Barbut and Petit, 2001) as they are resistant to heat, UV, and most commonly used detergents and disinfectants including alcohol (Dubberke, Gerding et al., 2008). Sub-inhibitory levels of sporicidal disinfectants or exposure to non-chlorine based cleaning agents can promote sporulation (Wilcox and Fawley, 2000).

Proper environmental cleaning and disinfection can reduce CDI in a human healthcare setting (McMullen, Zack et al., 2007; Wilcox, Fawley et al., 2003) and veterinary environment (Weese and Armstrong, 2003). Current human healthcare guidelines for sporicidal cleaning and disinfection of the environment specify disinfection with household bleach diluted 1:10 with water (prepared fresh daily) after adequate cleaning (Cohen, Gerding et al., 2010). Alternatively, an Environmental Protection Agency (EPA)-approved sporicidal product can be used instead of bleach. EPA approvals are stringent and testing must meet ASTM International standards (ASTM E2197) using spores of an approved *C. difficile* strain (ATCC 43598) (U.S. Environmental Protection Agency, 2014).

Although producers often clean and disinfect farrowing sheds after each production round there is no requirement for them to do so. A literature search of three principal pork management guidelines (Australian Pork Limited, 2011) (Primary Industries Standing Committee, 2008) (Animal Health Australia, 2013) found cleaning and disinfection of pens was not mentioned. Australian Pork Industry Quality Assurance Program (APIQ) standard operating procedure (General Operations, cleaning and maintenance) (Animal Health Australia, 2013) specifies, ‘pens and feeders are cleaned between batches of pigs, ensuring that manure build-up is minimised’, but there are no detailed protocols to provide clarity to producers, nor is disinfection mentioned. The finding that disinfectants in common use in farrowing units (Microtech 7000, Farm Fluid S, Virkon S) are not effective against *C. difficile* spores is therefore not surprising; such agents readily inactivate vegetative bacteria, viruses and fungal spores, but not bacterial spores.

APL has responsibly taken a position to ‘minimise the use of antibiotics through vaccines and better management of animals’ (Australian Pork Limited, 2013). Funded research into appropriate infection control measures to manage CDI in piggeries, and efficacy testing of these measures, is the next logical step. The industry-led quality assurance program, APIQ, also requires more than minimum standards to prevent local and broader environmental spread of *C. difficile* spores.

4.6 Fate of *C. difficile* in treated effluent from farrowing sheds

4.6.1 *C. difficile* survives effluent treatment in a two-stage pond system

The spore load in the farrowing pens developed rapidly in the absence of a CDI outbreak, suggesting an extrinsic source of spores. Discussions with farm management revealed that liquid byproduct from farrowing shed effluent, after treatment in an on-site ponding system, was recycled to clean the farrowing units. It was also used to flush under-pen gutters. It was assumed that *C. difficile* spores would survive effluent treatment, based on the resistant nature of its spores. It was also assumed that recycling of this product through the farrowing facility would increase spore density in pens.

Despite the limitations inherent in the pilot nature of this study, the finding that spores of the same molecular type that colonised piglets on this farm survived effluent treatment was expected. It is consistent with the finding that *C. difficile* could be isolated from 96% of anaerobically digested sludge from human waste plants (Xu, Weese et al., 2014), in some cases increasing in number during the process. Overall, *C. difficile* counts decreased by 44% in this study but proliferated throughout the anaerobic/facultative phases of treatment, which mimics the laboratory spore enrichment process. This may be an artefact of small sample size. Decreased spore counts overall may also be explained by sampling procedures. Samples that included pond or tank sediment (the most anaerobic fraction) would likely increase isolation rates. Spore settling in storage tanks might also occur. Storage tank samples were taken from the surface of tanks and from a depth of 30 cm in ponds (which were 2–5 m deep) because of sampling logistics. Pre-enrichment in the laboratory was not required to isolate *C. difficile*, however, implying that substantial numbers of spores survived treatment in the pond system. Although pond stability and functioning data were not available, increased spore numbers during the anaerobic phases of treatment indicate that sufficient anaerobiosis was established to allow *C. difficile*, a fastidious anaerobe, to proliferate.

Robust risk analysis is precluded by the small sample size and pilot nature of this study. Despite this, *C. difficile* resists pond-based effluent treatment and is likely disseminated into the environment. Although the final counts were low, constant incursion of small numbers of organisms into the sheds, in the absence of sporicidal cleaning, would increase spore burden in the pens. The addition of high pressure hosing and

amplification reservoirs such as scouring piglets would further contribute to spore burden. It is likely that lagoons servicing farrowing sites contain larger numbers of spores than grower-finisher or breeder unit treatment plants due to *C. difficile*'s preference for neonatal gastrointestinal tracts.

Safe re-use of animal wastes to capture energy and nutrients through anaerobic digestion processes is desirable. Biogas capture and utilisation incentives recently introduced to reduce methane emissions make pond treatment and effluent re-use particularly attractive to producers. The intensification of pork production combined with use of manure and pond sludge for fertilizer and compost, both on-farm and commercially, increases the risks of dissemination when pathogens remain viable in treated effluent. In Australia, a formal agreement known as the Emergency Animal Disease Response Agreement (EADRA) binds state and territory governments and livestock industries together to deal with emergency animal disease matters. Their published guidelines, the *National Farm Biosecurity Manual for Pork Production* (Animal Health Australia, 2013), operationalise biosecurity standards to manage risk associated with pathogens. Specifically the standards seek to 'reduce spread of diseases among pigs already contaminated with a disease agent and minimise spread of microorganisms of public health significance' and, as Standard 3.1 states, 'To ensure that water used in pig sheds for drinking, cooling and cleaning is of a standard suitable for livestock and does not introduce pathogens to the pigs'. APL recognises that thorough assessment of risk to animals and humans associated with re-use of piggery effluent should include spore-forming organisms, and has funded further research to evaluate *C. difficile* in piggery effluent treated in anaerobic ponds and prevalence in biosolid byproducts, including land application and compost (Project No. 2012/1032). This investigation is currently in progress.

4.6.2 Effluent re-use outside the piggery: are humans at risk?

Recent evidence suggests that CA-CDI incidence is increasing. A population-based US study demonstrated a four-fold increase in CA-CDI from 1991–2005 (Khanna, Pardi et al., 2012b). In Australia, CA-CDI rates doubled during 2011 and increased by 24% between 2011 and 2012 (Slimings, Armstrong et al., 2014). The assumption that hospitals are the primary source of CDI has been challenged by the findings of a four-year WGS study in England of isolates from 1250 patients with CDI at hospitals and in

the community (Eyre, Cule et al., 2013). This study concluded that 45% of the 1223 isolates successfully sequenced were genetically diverse and distinct from all previous cases and that 65% could not be linked to a case of CDI in the same hospital. This suggests a reservoir of *C. difficile* outside healthcare centres.

There is abundant evidence that *C. difficile* is found in treated biosolids and effluent (Romano, Pasquale et al., 2012; Viau and Peccia, 2009; Xu, Weese et al., 2014). *C. difficile* could feasibly be disseminated to human populations by land application of contaminated effluent, by contamination of waterways with run-off, particularly during flooding events, or by water bodies that receive wastewater treatment plant effluents. There is no evidence regarding the fate of *C. difficile* spores in compost derived from pond sludge, in the longitudinal effects of land application of either raw or treated liquid effluent or biosolids, in effluent treated in storage pits, or in composted piglet carcasses.

Data showing relationships between pig and human strains of *C. difficile* in Australia may offer some insights. There was no evidence of the emergent clinically important human RTs 251, 126, 127, 078 and 244 in pigs in the current study; however, RT014, the most common human strain in Australia, was also the predominant strain in piglets in a period prevalence study. In prevalence studies 71% (12/17) and 61% (14/23) of RTs isolated from piglets had not been previously described in Australia or elsewhere. Recent epidemiological studies in humans in Australia have reported 27% (Huber, Hall et al., 2014) and 16% (Foster, Collins et al., 2014) novel RTs, respectively, but there was no similarity with the piglet strains identified in our prevalence studies. These data are encouraging but moderated somewhat by the limited coverage of Australian pig studies to date.

Farm workers in direct contact with contaminated waste may be at particular risk. Farmers and pigs in the Netherlands share identical strains of *C. difficile*, suggesting interspecies transfer is possible either directly or through a common source of environmental organisms (Knetsch, Connor et al., 2014). One study suggested that non-swine workers were equally likely to shed *C. difficile* in their wastewater as swine workers, but disease rates were not investigated (Norman, Scott et al., 2011). Occupational exposure of otherwise healthy individuals to *C. difficile* rarely results in CDI unless predisposing antibiotics are administered (Arfons, Ray et al., 2005; Friedman, Pollard et al., 2013), although one study found that 47% of workers at a

healthcare campus received antimicrobials in the preceding year (Carmeli, Venkataraman et al., 1998). Immunocompromised workers such as the elderly or pregnant women, or those with comorbid conditions, may also be at risk for CDI acquisition when working in a highly contaminated environment (Loo, Bourgault et al., 2011).

4.6.2.1 Aerial dissemination of C. difficile *spores*

Aerial dissemination of *C. difficile* spores is another potential hazard that warrants further investigation in the piggery environment. This phenomenon has been reported in hospitals (Best, Fawley et al., 2010; Roberts, Smith et al., 2008), predominantly associated with personnel activity and toilet flushing (Best, Sandoe et al., 2012). Molecular characterisation was used to confirm the epidemiological link between CDI cases, airborne dispersal and environmental contamination (Best, Fawley et al., 2010).

Airborne *C. difficile* in farrowing units has been reported (Keessen, Donswijk et al., 2011), with *C. difficile* of the same molecular type detected 20 m from the farm. Enumeration studies show counts increased with personnel activity. This is supported by other studies of personnel exposure to microbe-containing dust in farrowing units, peaking during activities such as weaning (O'Shaughnessy, Donham et al., 2010) and high pressure hosing (O'Shaughnessy, Peters et al., 2012). The Dutch farm in the Keessen et al. study was artificially ventilated with fans driving air directly into the

environment; similar tunnel ventilation systems are used in Australian piggeries (Figure 4.3).

Figure 4.3 Example of a tunnel ventilated conventional piggery shed

Source: Australian Pork Ltd http://australianpork.com.au/wp-content/uploads/2013/09/FACT-SHEET-Identifying-Energy-Use-Activities-Dec-14.pdf

Pumping of raw and treated effluent in open channels (Figure 4.4), flushing of under-pen gutters with treated effluent, high pressure hosing of pens before restocking, and sweeping conventional shed laneways to reduce the amount of cleaning water required also pose a risk of aerosolising spores. Chinivasagam and Blackall (Chinivasagam and Blackall, 2005) found flushing of under-pen gutters did not significantly increase the numbers of *E. coli* in piggery shed air. These findings cannot be extrapolated to *C. difficile* spores as their physical behaviour in air due to size difference, aggregative propensity and stage of maturation is likely to be different from vegetative bacteria (Wilcox, Bennett et al., 2010). Manure storage facilities, compost bunds or treatment

lagoons also provide the potential for bioaerosols containing *C. difficile* to disseminate in high winds (Figure 4.4).

Figure 4.4 Open effluent drainage sump at a Western Australian piggery.

Raw effluent is collected via a series of interconnected open channels into this sump before being directed to ponding system for biological inactivation. Raw effluent is exposed to prevailing winds and is a possible source of environmental contamination via bioaerosols

4.7 Conclusion

This study into different culture-based methods for the recovery of *C. difficile* from farrowing pens and the development of spore contamination in a farrowing facility is the first of its kind and a useful baseline for future studies to ascertain spore burden in piggery operations, especially in assessing efficacy of cleaning and disinfection regimens. It is likely that an exogenous *C. difficile* source contributed to contamination of the farrowing shed. *C. difficile* was resistant to effluent treatment in a two-stage pond system. This added to the spore burden in the piggery environment and was a source for colonisation in piglets. More research is needed to ascertain risks to human health, if any.

Chapter 5 Clinical aspects and diagnosis of *C. difficile*

5.1 Introduction

C. difficile is not currently recognised in Australia as a pathogen of neonatal piglets despite widespread reports of idiopathic scour, high *C. difficile* prevalence in domestic piglets, and mounting evidence internationally of its association with neonatal scour (Knight, Squire et al., 2015; Squire and Riley, 2012); consequently, bacteriological and histopathological investigations for *C. difficile* are not routinely performed in Australian animal health laboratories. Due to Australia's geographical isolation and strict quarantine laws on the importation of livestock it was hypothesised that Australian strains of *C. difficile* would be different to strains from the rest of the world and that *C. difficile* disease presentation might be different also.

Three experiments were conducted to study systematically the clinical, virulence and diagnostic aspects of Australian *C. difficile* strains to assist veterinarians and laboratories to recognise disease and detect this organism in piglets:

- experiment 1: isolation of the novel strain AI 35 (RT UK 237) from neonatal piglets with genetic characterisation, evaluation of toxin production and in vivo virulence in a mouse model of infection
- experiment 2: evaluation of commercially available methods for diagnosis of *C. difficile* in Australian piglet clinical samples
- experiment 3: clinical and histopathological evaluation of CDI in piglets using Australian piglet-derived *C. difficile* strains.

5.2 Experiment 1: Isolation of the novel porcine strain AI 35 and evaluation of toxin production and in-vivo virulence

The aim of this study was to determine *C. difficile* prevalence in herds of scouring neonatal piglets and evaluate *C. difficile* isolated from these piglets using toxin gene PCR, PCR ribotyping, toxin cytotoxicity testing, animal infection analysis and whole

genome sequencing. Prior to this *C. difficile* in Australian piglets had not been systematically investigated.

The UWA Animal Ethics Committee granted ethics approval for Use of Animal Tissue (FA/3/500/). The Monash University School of Biomedical Sciences Animal Ethics Committee approved mice infection model experiments. Financial support was provided by the Australian Research Council (grant no. DP1093891) and the National Health and Medical Research Council (grant no. 545858).

This study was published as: Squire MM, Carter GP, Mackin KE, Chakravorty A, Noren T, Elliott B, et al. 'Novel molecular type of *Clostridium difficile* in neonatal pigs, Western Australia', *Emerging Infectious Diseases* 2013; 19(5).

Preliminary results of this investigation were presented at the 3rd International *C. difficile* Symposium, Bled, Slovenia, 22–24 September 2010.

5.2.1 Experiment design

Faecal samples from scouring and non-scouring neonatal piglets were submitted for *C. difficile* diagnosis during 2009. Animals were from three farms at two geographical locations (20 km apart) owned by a commercial farrow-to-finish operation in Western Australia. At the time of this study 50%–80% of litters were experiencing scours that could not be attributed to the usual neonatal enteric pathogens. Clinical presentation was early onset non-haemorrhagic, yellow, pasty-to-watery scour. Disease course without treatment was ill-thrift, anorexia, dehydration and death. All piglets were treated prophylactically at 1–3 days of age with beta-lactam antibiotics (amoxicillin, penicillin). Ceftiofur was also used to treat scouring piglets (personal communication, farm veterinarian).

One hundred and thirty-one faecal samples were taken from two herds with the most severe scouring problem at the same geographical location. An additional 54 samples were from a high biosecurity herd with a variable scouring problem, at a separate location. Eleven of these 54 were asymptomatic during sampling. Samples were cultured as described in Section 2.3.1 using both DC and EC methods on TCCFA. Putative *C. difficile* colonies were subcultured onto pre-reduced blood agar and

identified by Gram stain, characteristic colony morphology and smell, and toxin gene PCR.

Toxin profiling was by PCR detection of toxin A (*tcdA*), toxin B (*tcdB*), and binary toxin (*cdt*) genes (see Section 2.3.4). Isolates were PCR ribotyped and compared with Australian human reference strains and international RTs from the Anaerobe Reference Laboratory (Public Health Laboratory Service, Cardiff, UK). Strain AI 35 was confirmed as toxigenic by Vero cell cytotoxicity testing (see Section 2.3.4).

Sample DNA preparation, genome shotgun sequencing, assembly and annotation of strain AI 35 were performed at the Australian Genome Research Facility (AGRF) (see Section 2.3.9).

Virulence of AI 35 in mice was established by infecting C57/B6 mice via oral gavage with 10^7 *C. difficile* spores. *TcdB* production was quantitated in a Vero cell cytotoxicity assay (see Section 2.3.8). Specialist staff at Monash University, Melbourne, performed these experiments.

Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla CA, USA). Student's t-test was used to compare quantitative toxin production in different strains, and the log-rank (Mantel Cox) test for survival analyses. Weight loss resulting from toxigenic strain challenge in mice was assessed using ANOVA. A p value <0.05 was considered significant.

5.2.2 Results

5.2.2.1 C. difficile *isolation and genetic analyses*

C. difficile was isolated from 114/185 piglets (62%) overall. Individual isolation rates were: herds with severe scours: 70/131 (53%), herd with variable scours: 33/43 (77%), and asymptomatic animals: 11/11 (100%). Isolates were clonal, consisting of the same novel strain AI 35 (RT237), not previously described in animals in Australia or elsewhere. All isolates were *tcd*A- *tcd*B+ *cdt*A+ *cdt*B+ by PCR analysis.

The Illumina HiSeq2000 platform was used to generate paired-end reads totalling 31,085,914 bp. These were assembled into 117 contigs using the Velvet software suite (Zerbino and Birney, 2008). Comparison against the previously sequenced genome *C.*

difficile reference strain CD630 (RT 012, ST54, A+B+CDT-) showed a PaLoc structure unique to strain AI 35 (Figure 5.1). A large deletion had removed the *tcdA* and *tcdC* genes and a portion of the adjacent *cdd1* gene located outside the PaLoc region. The AI 35 CdtLoc was complete and contained an intact copy of *cdtR* unlike RT 078 isolates that encode a *cdtR* with a premature stop codon. Strain AI 35 also encoded a variant *tcdE*.

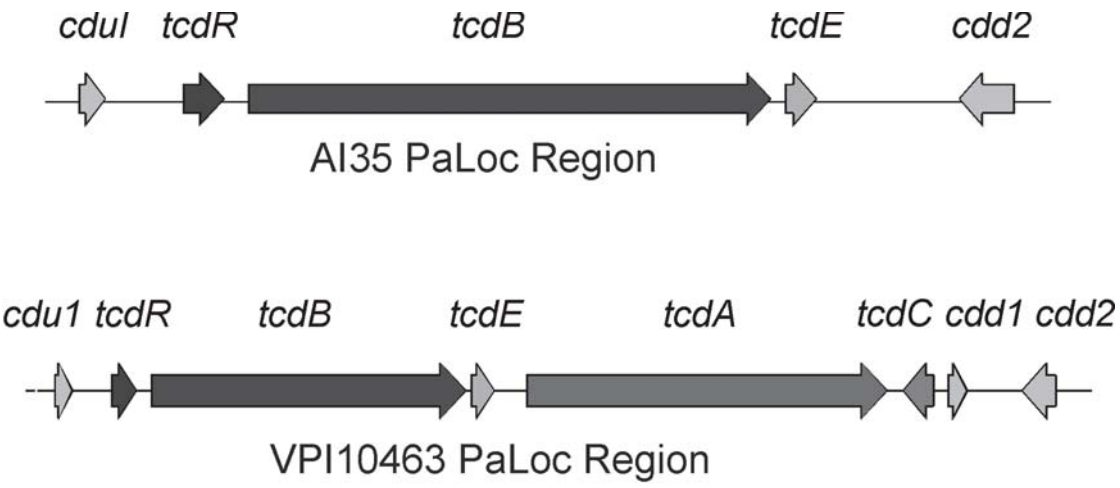


Figure 5.1 Structure of PaLoc and flanking regions in *C. difficile* strains AI 35 and VPI 10463.

Boxes indicate open reading frames; arrows indicate direction of transcription. Encoded genes are indicated above the arrows. Figure not drawn to scale.

5.2.2.2 Analysis of mobile genetic elements: phages

Two intact phages were predicted from AI 35 genome sequence by PHAST analysis (Zhou, Liang et al., 2011) (Figure 5.2). Phage ΦC2, a 43.8 Kb element, was identified at position 1722137-1765969 with 100% nucleotide sequence identity to the *C. difficile* ΦC2 genome (NCBI reference sequence: NC_009231.1). Phage CDMH1, a 40.2 Kb element at position 4096062-4136294, had a 95.3% identity to *C. difficile* phage CDMH1 genome (NCBI reference sequence: NC_024144.1) at the nucleotide level.

Total : 11 prophage regions have been identified, of which 3 regions are intact, 7 regions are incomplete, 1 regions are questionable.

REGION	REGION_LENGTH	COMPLETENESS	SCORE	#CDS	REGION_POSITION	POSSIBLE PHAGE	GC_PERCENTAGE	DETAIL
1	31.1Kb	incomplete	40	14	35017-66187	PHAGE_Clostr_phiMMP02_NC_019421, ...	27.20%	Detail
2	19.9Kb	incomplete	40	22	708853-728801	PHAGE_Clostr_phiCD27_NC_011398, ...	24.21%	Detail
3	27.2Kb	incomplete	60	30	1231449-1258691	PHAGE_Clostr_phi_CD119_NC_007917, ...	27.93%	Detail
4	43.8Kb	intact	100	43	1722137-1765969	PHAGE_Clostr_phiC2_NC_009231, ...	28.77%	Detail
5	14.9Kb	incomplete	60	23	1854950-1869946	PHAGE_Clostr_phiMMP02_NC_019421, ...	28.25%	Detail
6	28.6Kb	questionable	80	36	2123195-2151820	PHAGE_Clostr_phiC2_NC_009231, ...	27.44%	Detail
7	53.5Kb	intact	150	60	2592878-2646430	PHAGE_Clostr_phiC2_NC_009231, ...	28.93%	Detail
8	19.2Kb	incomplete	40	25	2812055-2831263	PHAGE_Clostr_phiC2_NC_009231, ...	29.62%	Detail
9	17.6Kb	incomplete	20	17	3385274-3402963	PHAGE_Prochl_P_SSM2_NC_006883, ...	29.78%	Detail
10	7.7Kb	incomplete	60	8	3863249-3870958	PHAGE_Clostr_CDMH1_NC_024144, ...	30.12%	Detail
11	40.2Kb	intact	150	64	4096062-4136294	PHAGE_Clostr_CDMH1_NC_024144, ...	27.84%	Detail

Figure 5.2 Analysis of the whole genome of *C. difficile* strain AI 35 by the Phage Search Tool (PHAST)

In silico analysis by PHAST predicted intact phages ΦC2 and CDMH1 (red) in the AI 35 genome. Source: (Zhou, Liang et al., 2011)

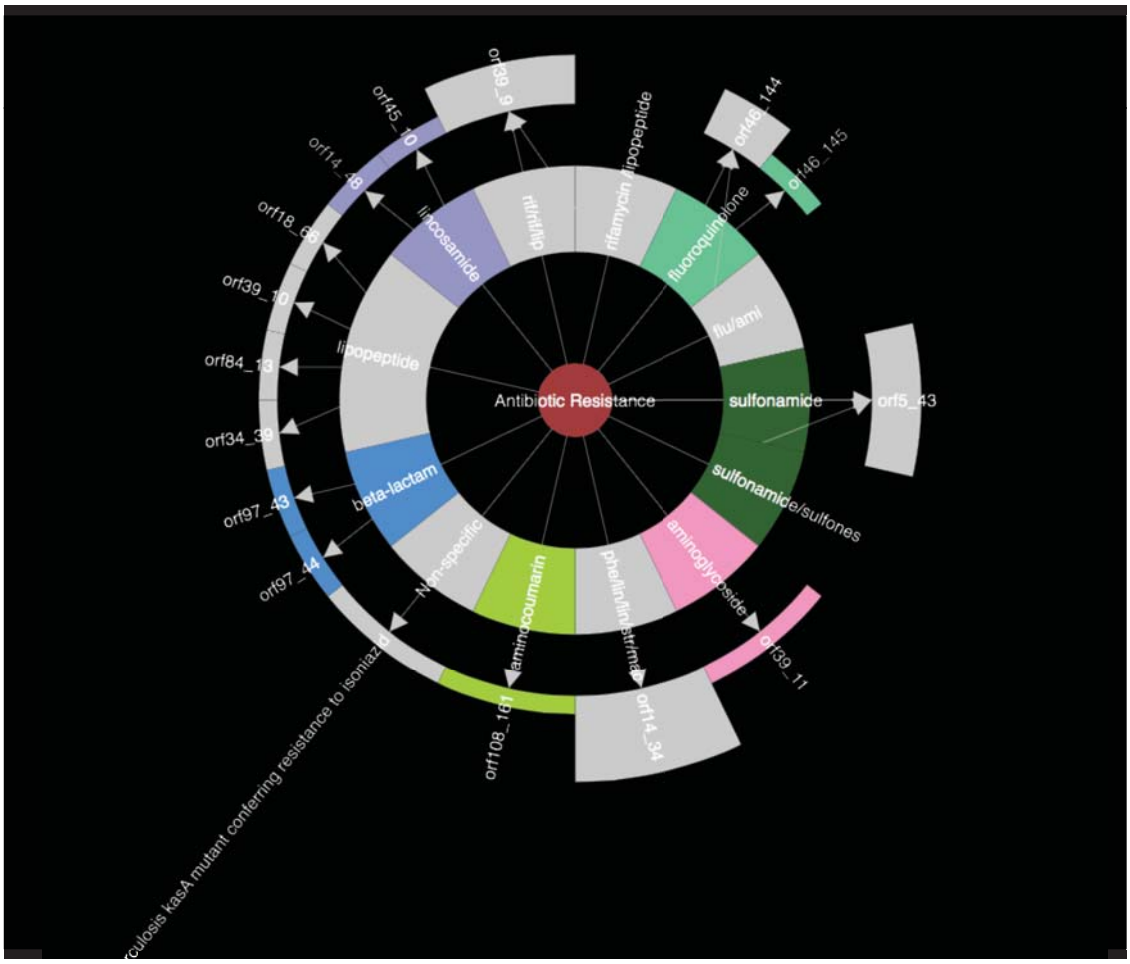


Figure 5.3 Analysis of the whole genome of *C. difficile* strain AI 35 by the Resistance Gene Identifier (RGI)

Analysis presented as a resistance wheel, predicting resistance to a range of antibiotic classes. Source: RGI web-based analyser: (McArthur, Waglechner et al., 2013)

5.2.2.3 Resistance gene analyses and antibiogram phenotype

In silico analysis of acquired resistance genes using ResFinder 2.1 did not identify any genes conferring resistance (identity threshold: 98%).

The Comprehensive Antibiotic Resistance Database (CARD) (McArthur, Waglechner et al., 2013) predicted several genes potentially involved in resistance including β-lactamases (*BlaR1, MecA, B, C*), lincosamide ABC-type transport systems, *rpsL* streptomycin-resistance, *gyrA/B* fluoroquinolone resistance, and *tlyA* aminoglycoside resistance, all at a low level of identity (<60%). High identity sulphonamide resistance via *folP* (dihydropteroate synthase) mutation was predicted (98.86% identity) (Figure 5.3).

Antibiogram results revealed that AI 35 was susceptible to vancomycin, metronidazole, clindamycin, erythromycin, amoxicillin-clavulanate, ceftriaxone, meropenem, moxifloxacin, tetracycline, and pipericillin-tazobactam, using EUCAST and CLSI breakpoints for *C. difficile*. Breakpoints are not available for aminoglycosides or trimethoprim but MICs were 32 mg/L for tobramycin, gentamicin and trimethoprim, and 128 g/L for spectinomycin.

5.2.2.4 Toxin B quantitation

Strain AI 35 produced approximately 25-fold less tcdB than RT 027 ($p = 0.0354$) and RT 078 ($p = 0.0074$) but similar levels to strain 630, a known low toxin-producing strain.

AI 35-mediated CPE on Vero cells (Figure 5.4a) was similar to that reported for the lethal toxin (tcsL) of *C. sordellii* and *C. difficile* tcdB-variant strains 8864 and 1470 (Figure 5.4c).

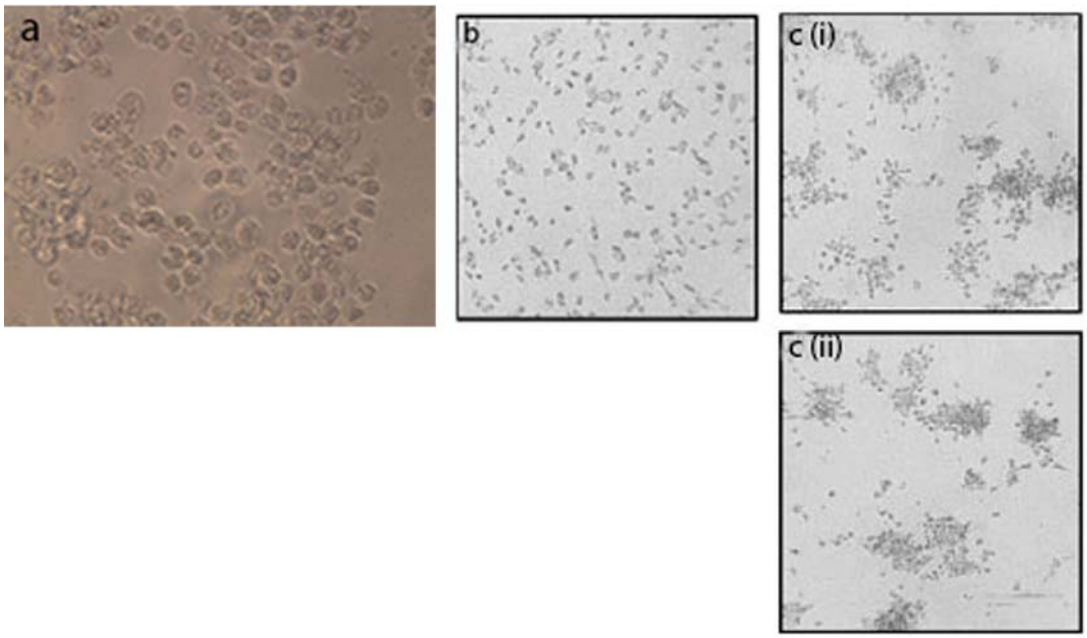


Figure 5.4 Cytopathic effect (CPE) on Vero cells of *C. difficile* toxin

(a) *C. difficile* AI 35 variant toxin; (b) CPE on Vero cells of *C. difficile* toxinotype I strain displaying typical actinomorphic morphology; (c) CPE on Vero cells of *C. difficile* strain 1470 (i), and *C. sordellii* lethal toxin (ii). Vero cells were incubated for 48 h in culture filtrates of *C. difficile* AI35. Cells show rounding rather than the characteristic actinomorphic morphology induced by other strains of *C. difficile* where contraction of the cytoplasm leaves long projections radiating away from the rounded cell body. See figures (b) and (c) for comparison. Source: (Blake, 2004)

5.2.2.5 Virulence in mice

Strain M7404 (RT 027) was significantly more virulent than both AI 35 ($p = 0.0001$) and JGS6133 (RT 078) ($p = 0.0002$), with all mice infected with this strain succumbing to fatal infection (Figure 5.5a). By comparison, mice infected with AI 35 and RT 078 all survived until the end of the trial (Figure 5.5a).

Despite low toxin production, AI 35 caused significantly greater weight loss than the RT 078 strain ($p = 0.0011$) (Figure 5.5b).

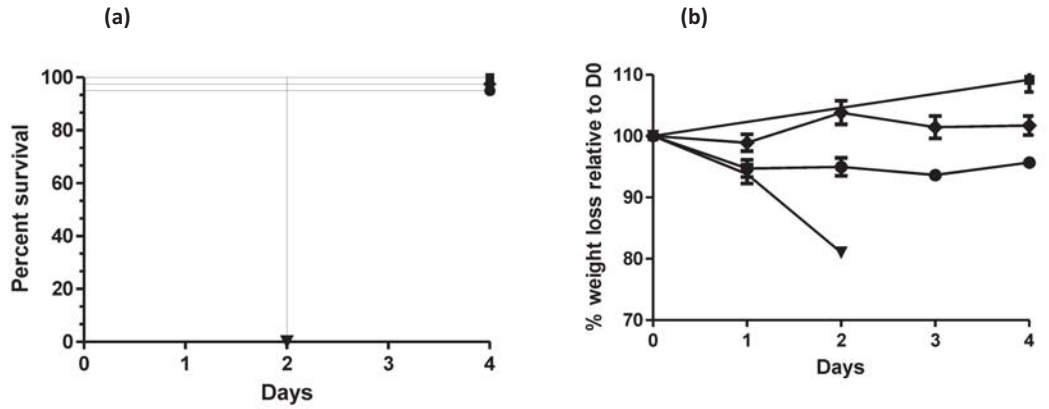


Figure 5.5 (a) Survival and (b) percentage of weight lost in mice over 4 days after infection with *C. difficile*

Male C57/B6 mice were infected with *C. difficile* spores for strains M7404 (RT 027) (triangles), JGS6133 (RT 078) (diamonds), or AI 35 (circles); phosphate buffered saline (squares) was used as control. Error bars in panel (b) indicate SEM.

5.3 Laboratory diagnosis of *C. difficile* in neonatal pigs

Whilst many commercially available assays for the detection of *C. difficile* have been systematically evaluated for use in humans, their performance with stool samples of animal origin has not been validated. Currently no guidelines are available for diagnosing CDI or detecting *C. difficile* in animals.

Unlike the northern hemisphere where RT 078 predominates in swine herds, in Australia there are many different RT circulating among livestock (sheep, cattle and pigs), including RT 033, 126, 127, and 237 (Knight, Squire et al., 2015; Knight, Thean et al., 2013; Squire, Carter et al., 2013). To understand the role of *C. difficile* in piglet disease in Australia, it is essential that veterinary diagnostic laboratories are able to detect the organism reliably and in a cost-effective manner.

The aim of this investigation was to evaluate the suitability of four commercially available assays for detecting *C. difficile* in specimens of piglet faeces.

This study was published as: Knight DR, Squire MM, Riley TV, 'Laboratory detection of *Clostridium difficile* in piglets in Australia'. J Clin Microbiol. 2014; 52(11):3856-62.

Financial support was provided by the Co-operative Research Centre for High Integrity Australian Pork (Pork CRC, Willaston, South Australia), Project 2A-104.

5.3.1 Experiment design

A total of 157 rectal swabs were obtained by veterinarians from piglets aged <14 days (31%, 49/157 actively scouring) from June 2012 to March 2013. Samples were from 16 farms (piggeries) across five Australian states: NSW, *n* = 2; QLD, *n* = 6; VIC, *n* = 4; SA, *n* = 1; and WA, *n* = 3. Farms varied in facility type (e.g., farrow to finish, growers, or breeders) and were geographically distinct. Samples were transported under ambient conditions in Amies transport medium (Thermo Fisher Scientific, Waltham, MA, USA) to UWA. The mean transport time from the farm to the laboratory was eight days (range: 2–20 days).

Samples were stored at 4^0C and prepared for analysis within 24 h using the methods described in Section 2.3.7. Putative *C. difficile* isolates were identified as per Section 2.3.1.4.

We evaluated the performance of four commercial assays to detect *C. difficile.* Assays were performed according to manufacturers' instructions and compared against toxigenic culture (TEC) as a 'gold standard'. The ability of *C. difficile* to produce tcdA and tcdB was determined by toxin gene PCR (see Section 2.3.4).

Assays included two commercially available PCR methods for the detection of toxin A and B genes; (illumigene® *C. difficile* loop-mediated isothermal amplification PCR for *tcdA* (LMIA-PCR, Meridian Bioscience Inc., Cincinnati, OH, USA) and BD GeneOhmTM Cdiff Assay for *tcdB* (real-time PCR, BD Diagnostics, La Jolla, CA, USA), an enzyme immunoassay (EIA) for toxins A and B (EIA-tcdA/tcdB, C. diff Quik Chek Complete, Alere, North America, Inc., Orlando, FL, USA) and culture on the chromogenic agar *C. difficile* ChromID™ (CA, bioMérieux, Marcy l'Etoile, France). Isolates were characterised by PCR ribotyping and PCR detection of toxin genes *tcdA* (toxin A), *tcdB* (toxin B) and *cdt* (binary toxin) using the methods outlined in Section 2.3.4.

The sensitivity and specificity were calculated for each test against the gold standard assay (EC/TEC). Sensitivity and specificity data were used to calculate the positive (PPVs) and negative predictive values (NPVs). Fisher's exact test was used to compare the recovery of *C. difficile* in the test systems with its recovery by the EC/TEC. For an assay detecting organisms (DC and EIA-GDH), a result was considered a true positive if positive by EC. For an assay detecting toxin or toxin genes (EIA-tcdA/tcdB, real-time PCR, and LMIA-PCR), a result was considered a true positive if positive by TEC. Discrepant results (false positives and false negatives) with respect to EC/TEC were repeated, as were any equivocal or unresolved results. The percentage of concordance with EC/TEC was calculated for each assay.

5.3.2 Results

5.3.2.1 C. diffic*ile isolation*

Table 5-1 Detection of *C. difficile* in Australian piglet faeces (*n* = 157) using commercial assays

		C. difficile assay results:			Assay concordance (no. [%])	
Target	Assay[a]	No. (%) positive	No. (%) negative	*P*	With EC	With TEC
C. difficile	EC	62 (39.5)	95 (60.5)			
	DC	57 (36.3)	100 (63.7)	0.56	152/157 (96.8)	
	EIA-GDH	33 (21.0)	124 (79.0)	<0.001	114/157 (72.6)	
Toxin/toxin gene	TEC	36 (22.9)	121 (77.1)			
	EIA-TcdA/TcdB	3 (1.9%)	154 (98.1)	<0.001		122/157 (77.7)
	Real-time PCR	19 (12.1)	138 (87.9)	0.01		132/156 (84.1)[b]
	LMIA-PCR	14 (8.9)	143 (91.1)	<0.001		125/157 (79.6)

C. difficile was isolated by EC from 39.5% (*n* = 62/157) of samples and by TEC from 22.9% (*n* = 36/157) of samples (Table 5.1). The recoveries from piggeries in different states ranged from 26.0% to 54.5%. The recoveries of *C. difficile* isolates from piglets with (36.7%) and without (40.7%) diarrhea were not significantly different (*p* = 0.141). *C. difficile* was detected in 36.3% (*n* = 57/157) of samples by DC, 21.0% (*n* = 33/157) of samples by EIA-GDH, 1.9% (*n* = 3/157) of samples by EIA-tcdA/tcdB, 12.1% (*n* = 19/156) of samples by real-time PCR, and 8.9% (*n* = 14/157) of samples by LMIA-PCR (Table 5.1).

5.3.2.2 C. difficile *genotyping*

PCRs revealed that 58.1% (*n* = 36) of isolates were positive for at least one toxin gene (*tcdA*/*tcdB*). Overall, five toxin profiles were observed, the most common being A positive, B positive, CDT negative (A+B+CDT-) (*n* - 33, 53.2%). Two isolates (3.2%) were A+B+CDT+, one (1.6%) was A-B+CDT-, and five (8.1%) had the uncommon genotype of A-B-CDT+. The remainder (*n* = 21, 33.9%) were negative for any toxin genes. Multiple RTs were identified (Figure 5.6). Of the 62 isolates obtained from neonatal pigs, 32.3% (*n* = 20) were assigned one of eight internationally recognised RTs. The remaining isolates were assigned the prefix QX and given an internal number. No RT 027 or RT 078 was identified. QX 006 (A+B+CDT-) was the most common RT found overall, representing 16.1% (10/62) of isolates. The next four most prevalent RT were QX 207 (12.9%), QX 057 (11.3%), UK 014 (11.3%), and QX 020 (8.1%).

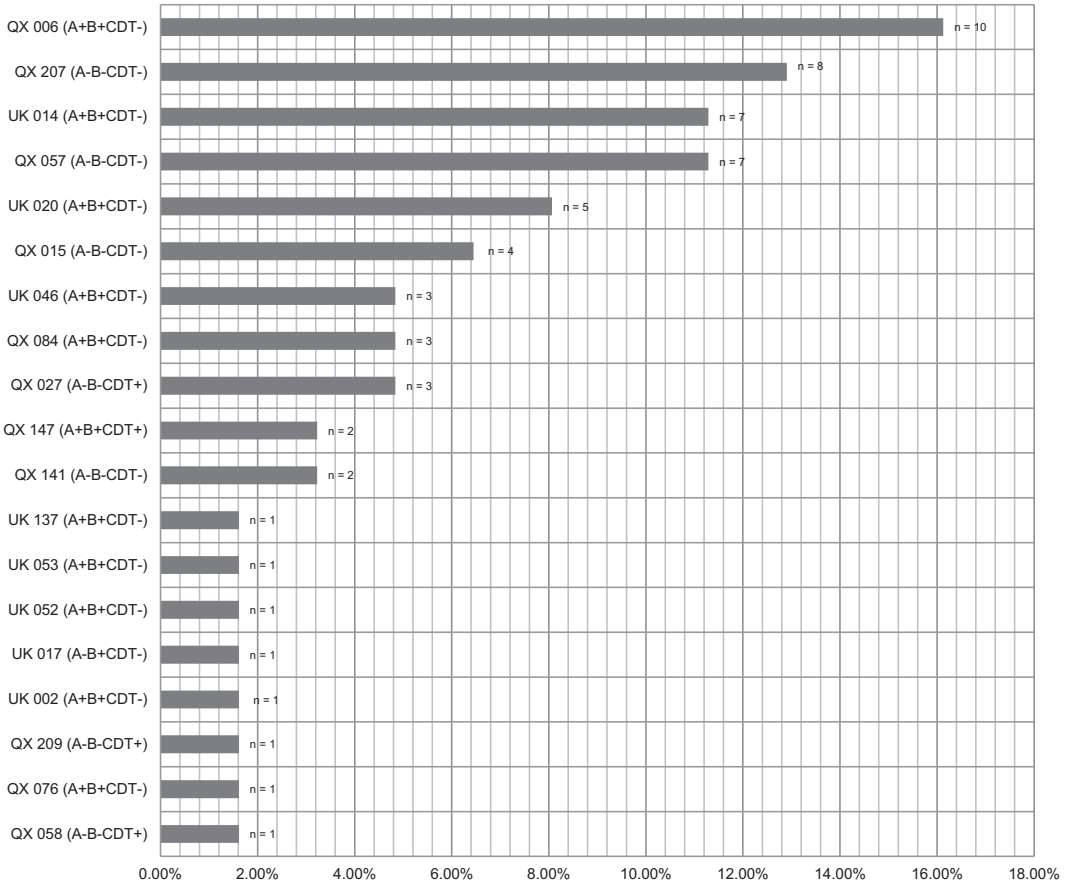


Figure 5.6 Summary of PCR ribotypes and toxin gene profiles of *C. difficile* recovered from piglet faeces (*n* = 62)

5.3.2.3 Concordant and discordant results

DC and EIA-GDH concordances with EC were 96.8% (152/157) and 72.6% (114/157), respectively. The combined concordance for both assays with EC was 77% (121/157) (Table 5.1). Real-time PCR, EIA-tcdA/tcdB, and LMIA-PCR concordances with TEC were 84.1% (132/157), 77.7% (122/157), and 79.6% (125/157) respectively. The combined concordance for all three assays with TEC was 73.9% (116/157) (Table 5.1). There was a high number of discordant results, principally false negatives but also a few false positives (data not shown). There was a single equivocal result for real-time PCR that could not be resolved after repeat testing, resulting in a reduced total of samples for this assay (n = 156).

5.3.2.4 Sensitivities, specificities, PPVs and NPVs of all assays compared to EC/TEC

The prevalence of nontoxigenic (A-B-) strains of *C. difficile* in this study was high (42%). This observation raised the possibility of a population bias favouring strain types that do not have the targets (toxin or toxin genes) that the non-culture toxin-based methods (EIA-tcdA/tcdB, real-time PCR, and LMIA-PCR) are designed to detect. To assess these three assays fairly, they were evaluated against TEC, whilst assays to detect organisms (DC and EIA-GDH) were evaluated against EC (Table 5.2). Of all the comparator assays, DC had the highest sensitivity and specificity (91.9% and 100.0%, respectively). The sensitivity of EIA-GDH was 41.9% (Table 5.2). For the other three assays (EIA- tcdA/tcdB, real-time PCR, and LMIA-PCR), sensitivities were low, ranging from 5.6 to 42.9%, and predictive values for all assays varied widely (PPV range, 64.3 to 100.0%; NPV range, 71.0 to 95.0%) (Table 5.2).

Table 5-2 Performance of DC and EIA-GDH and EIA-tcdA/tcdB, LMIA-PCR and real-time PCR, compared to EC and TEC

		Performance (95% confidence interval)				
Comparator test	Parameter[b]	DC	EIA-GDH	EIA-TcdA/TcdB	RT-PCR	LMIA-PCR
EC	% sensitivity	91.9 (82.2–97.3)	41.9 (29.5–55.2)			
	% specificity	100.0 (96.2–100.0)	92.6 (85.4–97.0)			
	% PPV	100.0 (93.7–100.0)	78.8 (61.1–91.0)			
	% NPV	95.0 (88.7–98.3)	71.0 (62.1–78.8)			
TEC	% sensitivity			5.6 (0.8–18.7)	42.9 (26.3–60.6)	25.0 (12.2–42.2)
	% specificity			99.2 (95.5–99.9)	96.7 (91.7–99.1)	95.9 (90.6–98.6)
	% PPV			66.7 (11.6–94.5)	78.9 (54.4–93.8)	64.3 (35.2–87.1)
	% NPV			77.9 (70.5–84.2)	85.4 (78.4–90.9)	81.1 (73.7–87.2)

[a] DC, direct culture (*C. difficile* chromID agar; bioMérieux); EIA-TcdA/TcdB, enzyme immunoassay (EIA) for TcdA and TcdB (C. diff Quik Chek; Alere); EIA-GDH, EIA for *C. difficile* glutamate dehydrogenase (GDH) (C. diff Quik Chek; Alere); real-time PCR, real-time PCR for *tcdB* (GeneOhm Cdiff; Becton Dickinson); LMIA-PCR, loop-mediated isothermal amplification-PCR for *tcdA* (illumigene *C. difficile*; Meridian); EC, enrichment culture; TEC, enrichment culture with PCR for toxin genes. *b* There was a single unresolved result for real-time PCR.

[b] PPV, positive predictive value; NPV, negative predictive value.

5.4 Experiment 3: Clinical and histopathological evaluation of CDI in piglets using Australian piglet-derived *C. difficile* strains

In this study newborn piglets were challenged with Australian porcine field strains of *C. difficile*. Glenn Songer, Research Professor, Department of Veterinary Microbiology, Iowa State University (ISU) (now retired) supervised the experiment. The study was conducted under the auspices of Emeritus Professor D.L. 'Hank' Harris at Harris Vaccines Iowa because it offered specialised facilities. Specialist animal handling staff were provided by ISU.

Ethics approval was granted by the Harris Vaccines Institutional Animal Care and Use Committee (102011-11-IACUC-HV).

The aim of this study was to determine if features of porcine CDI could be experimentally induced when test piglets were challenged with toxigenic *C. difficile* strains isolated from scouring Australian neonatal piglets.

5.4.1 Experiment design

Twenty-nine conventional newborn piglets (Landrace x Large White) were snatch farrowed from a 2500 sow farm in Iowa, USA. Farrowing sows were randomly selected, their vulvas disinfected immediately prior to parturition (Clorox Disinfecting

Wipes, The Clorox Company, Oakland, CA, USA) and piglets delivered directly onto sterile surgical drapes (Figure 5.7). Piglets were placed into disinfected plastic crates and ear tagged for identification. Each piglet was aseptically administered a 10 mL intragastric aliquot of pooled colostrum obtained after disinfecting the teats of farrowing sows. Baseline rectal swabs were taken for *C. difficile* culture.

Figure 5.7 Snatch farrowing of piglets for *C. difficile* challenge study

Piglets were delivered into sterile drapes after cleaning the sow's vulva with hypochlorite disinfecting wipes (Clorox Disinfecting Wipes, The Clorox Company, Oakland, CA, USA)

Animals, including controls, were housed in disinfected 0.5 m^3 plastic crates in one large room at a facility (BSL-2 level) maintained by Harris Vaccines (Figure 5.8). Environmental temperature was maintained at approximately 35^0C using central heating combined with heat lamps over each crate. Piglets were supplied 150 mL of puppy milk replacer in a bowl in each crate (Esbilac Milk Replacer Liquid, PetAg, Hampshire, IL, USA) twice daily at 6am and 6pm. Animals, feed and equipment were handled to prevent cross-contamination.

Figure 5.8 Piglet housing for the *C. difficile* challenge study.

Piglets were housed in Clorox-disinfected 0.5m^3 plastic crates in one large room at a BSL-2 level facility. Temperature was maintained at 35^{0}C using central heating and heat lamps over each crate

Four hours after birth, test piglets (*n* = 18) were divided into three groups and challenged intragastrically with 1.5mL of spores of one of three strains of toxigenic Australian field isolates of *C. difficile* (AI 35, VP 27, QP 6, Table 5.3). Challenge strains are unique Australian strains originally isolated from scouring 2–7-day-old piglets. Challenge inocula were prepared and administered according to Section 2.3.6.1, except that the pre-germination stage was conducted aerobically. Control piglets (*n* = 11) were divided into two groups and given either a sham inoculum of 1.5 mL of PBS/BHIB prepared under the same conditions as challenge inocula (*n* = 6) or JGS 753, a non-toxigenic porcine strain of *C. difficile* kindly provided by Professor Songer (*n* = 5).

Table 5-3 *C. difficile* strain and dosage details for the piglet challenge experiment.

	C. difficile challenge inocula			
	Group	**Toxin profile**	**Dose (spores/ 1.5 mL)**	**RT**
Test subjects (*n* = 18)	AI 35 (*n* = 6)	A-B+CDT+	1.43×10^4	UK 237
	QP 6 (*n* = 6)	A+B+CDT-	1.19×10^4	QX 3
	VP 27 (*n* = 6)	A+B+CDT+	8.5×10^3	QX 8
Controls (*n* = 11)	JGS 753 (*n* = 5)	A-B-CDT-	6.6×10^3	JGS 753
	Sham control (*n* = 6)	NA	0	NA

Piglets were monitored for signs of disease and rectal swabs taken for *C. difficile* culture at 24 and 48 h post-inoculation.

After 72 h the piglets were humanely euthanised by an intravenous overdose of phenobarbital and necropsied by ISU veterinary staff. At necropsy each piglet was blind scored for gross intestinal and systemic lesions (Tables 2-4/5, Section 2.3.6.6). Tissue samples and gastrointestinal contents were collected and processed as described in Sections 2.3.6.5 and 6 for histopathology, *C. difficile* toxin ELISA, and bacteriology including culture for *C. difficile*, *Salmonella* spp., *E. coli* and *C. perfringens*. PCR ribotyping of *C. difficile* isolates was performed as per Section 2.3.4.

Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla CA, USA). Student's t-test was used to compare parametric data, and Mann–Whitney test for non-parametric. Fisher's exact test and χ^2 tests were used to analyse contingency data. Temporal analysis *C. difficile* isolation was assessed using χ^2 test for trend. A p value <0.05 was considered significant.

5.4.2 Results

5.4.2.1 Bacteriology and typing

Results are summarised in Tables 5.4 and 5.5. Pre-inoculation baseline swabs were negative for *C. difficile* except one piglet in the AI 35 inoculum group that was positive for a *C. difficile* RT 078 strain, likely from environmental spores at the piggery or experiment facility. There was a significant increase in *C. difficile* isolation over time ($p < 0.0001$). Six test piglets were culture positive for toxigenic strains within 24 h of inoculation, and remained culture positive at necropsy. *C. difficile* could not be isolated from any control animals at 24 h including the non-toxigenic controls. All but one animal positive for *C. difficile* at 48 h (15/16) were also positive at 72 h. At 72 h, 94% (17/18) of test piglets were culture positive for *C. difficile* versus 64% (7/11) controls, or a total of 24/29 (84%) piglets. Controls were colonised with a range of RTs consisting of inoculating strains and the RT 078 contaminating strain.

Table 5-4 Summary of *C. difficile* faecal culture strains isolated from piglets at 0, 24, 48, 72 hours post inoculation.

		Inoculum group (N = 29)																			
		AI 35				QP 6				VP 27				JGS 753				Uninoculated			
hpi[§]		0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72
Faecal culture strain	**Toxin profile**																				
NG^		5	2	1		6	5	3	1	6	5	2		5	5	3	1	6	6	4	3
AI 35	A-B+CDT+		3	4	6			1	1												
QP 6	A+B+CDT-							1	1												
VP 27	A+B+CDT+										1	2	2								
JGS 753	A-B-CDT-															1	1				
RT 078	A+B+CDT+	1	1	1			1	1	3			2	4			1	3			2	3

[§] hours post inoculation

^ No Growth

RT 078 predominated, accounting for 48% (22/46) of isolates overall, followed by AI 35 with 33% (15/46), and VP 27 with 11% (5/46) isolates. QP 6 and the non-toxigenic strain JGS 753 both produced 4% (2/46) isolates. Different strains predominated at different time points (Table 5.5). *C. difficile* positive piglets at 72 h did not always carry the inoculating strain, and results were strain-dependent. 100% (6/6) of piglets inoculated with strain AI 35 were positive for this strain at necropsy whereas only 33% (2/6) of VP 27, 17% (1/6) QP 6 and 20% (1/5) JGS 753 inoculated piglets were positive for those strains at 72 h. Fifty per cent (3/6) of uninoculated piglets and 60% (3/5) non-toxigenic control piglets were positive for RT 078 at 72 h.

Salmonella spp. (Serogroup B) was isolated by tetrathionate broth enrichment from eight test piglets and seven controls. *E. coli* was isolated from five animals but toxin and fimbrial adhesin genotyping was negative in all cases. One piglet was positive for *C. perfringens*.

Table 5-5 Summary of *C. difficile* faecal culture strains isolated from piglets at 0, 24, 48, 72 h post inoculation.

Results are presented as the total (%) for all piglets at each time point. There was a significant increase in isolation of *C. difficile* throughout the experiment ($p < 0.0001$)

		Piglets faecal culture postive for *C. difficile* (*N* = 29)							
hpi[§]		0		24		48		72	
		n	***% (n/N)***	***n***	***% (n/N)***	***n***	***% (n/N)***	***n***	***% (n/N)***
Faecal culture strain	**Toxin profile**								
NG^		28	97	23	79	13	45	5	17
AI 35	A-B+CDT+	0	-	3	10	5	17	7	24
QP 6	A+B+CDT-	0	-	0	-	1	3	1	3
VP 27	A+B+CDT+	0	-	1	3	2	7	2	7
JGS 753	A-B-CDT-	0	-	0	-	1	3	1	3
RT 078	A+B+CDT+	1	3	2	7	7	24	13	45
TOTAL *C. difficile* isolated (%)		1 (3)		6 (21)		16 (55)		24 (83)	

[§] hours post inoculation

^ No Growth

5.4.2.2 Histopathology

Results are summarised in Table 5.6. Classic microscopic lesions of porcine CDI (caecal and colonic lesions and mesentritis) were observed in 70% (15/23) of piglets positive for toxigenic *C. difficile* at 72 h and 50% (3/6) of animals either not shedding *C. difficile* or shedding a non-toxigenic strain.

The mean lesion score for CDI lesions was significantly higher in culture positive animals (5.9, 149/24) vs. culture negative (2.6, 13/5) ($p = 0.008$). The CDI microscopic lesion score in *C. difficile* positive piglets was not significantly different between toxigenic strains with more than 1 score/strain (RT 078: 8, AI 35: 3, VP27: 5, $p = 0.344$).

Small intestinal microscopic lesions were uncommon in culture positive piglets. Two (8%) animals presented with microscopic small intestinal mucosal lesions that were

scored as ‘rare, ≤4 erosions’. Neutrophilic infiltration of the small intestinal lamina propria was more common, found in 8/24 (33%) culture positive piglets.

Table 5-6 Summary of microscopic findings at necropsy 72 h post-inoculation with *C. difficile*

				Lesions of porcine CDI															*Small intestine lesions*			
				Colon						**Caecum**												
	Faecal culture group	**Toxin profile**	***n***	Goblet cell loss		PMNs		Mucosal alterations		Goblet cell loss		PMNs		Mucosal alterations		Mesentritis		**Total CDI lesions ($\bar{\chi}$)**	PMNs		Mucosal alterations	
				Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean		Range	Mean	Range	Mean
C. difficile positive (*n* = 24)	AI 35	A-B+CDT+	7	0-3	0.6	0-3	0.7	0-3	0.6	0-2	0.4	0-2	0.6	0-2	0.4	0-1	0.1	**24 (3.4)**	0-1	0.3	0	0
	QP 6[+]	A+B+CDT-	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**	1	1	1	1
	VP 27	A+B+CDT+	2	0	0	1	1	1	1	0-3	1.5	0-2	1	0-1	0.5	0-1	0.5	**9 (4.5)**	1-2	1	0	0
	RT 078	A+B+CDT+	13	0-3	1.3	0-3	1.4	0-3	1	0-3	1.4	0-3	1.4	0-3	1.1	0-3	0.7	**107 (8.2)**	0-2	0.3	0-1	0.1
	JGS 753[^]	A-B-CDT-	1	1		1		0		0		0		0		0		**2 (2)**	0		0	
	Overall mean																	**5.9**				
C. difficile negative (*n* = 5)			5	0-2	0.4	0-2	0.6	0-3	0.6	0-1	0.2	0	0	0	0	0-3	0.8	**13 (2.6)**	0-1		0	0
	Overall mean																	**2.6**				

Table 5-7 Summary of clinical symptoms and gross findings in piglets at necropsy 72 h post-inoculation with *C. difficile*

Scouring severity at 72 h is indicated by perineal staining score

				Clinical signs scored at necropsy at 72 h post-inoculation																
	Faecal culture group	**Toxin profile**	***n***	**Body Condition**		**Hydration**		**Perineal staining**		**Mesocolonic oedema**		**Gut contents**		**SI/ LI lesions**		**Stomach contents**		**Toxin**		**TOTAL ($\bar{\chi}$)**
				Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	
	AI 35	A-B+CDT+	7	0-2	0.7	0-1	0.4	0-1	0.3	2-3	0.7	0-3	1.7	0-1	0.1	1	1	0.002-2.43	0.6	**35 (5)**
	QP 6[+]	A+B+CDT-	1	1	1	1	1	0	0	0	0	2	2	0	0	0	0	0.001	-	**4 (4)**
C. difficile positive (*n* = 24)	VP 27	A+B+CDT+	2	0	0	1	1	0	0	0	0	0-3	1.5	0	0	1	1	0.031-1.7	0.9	**7 (3.5)**
	RT 078	A+B+CDT+	13	0-3	1.4	0-3	1.5	0-3	1.1	0-3	0.8	0-3	1.9	0	0	0-1	0.8	0.006-3.5	1.5	**88 (6.8)**
	JGS 753[^]	A-B-CDT-	1	2	2	2	2	1	1	0	0	2	2	0	0	1	1	0.099	-	**8 (8)**
	Overall mean																			**5.9**
C. difficile negative (*n* = 5)			5	1-2	1.2	0-3	1.4	0-3	1.6	0	-	3	3	0	0	0-2	1	0.002-0.03	0	**41**
	Overall mean																			**8.2**

Five animals culture positive for AI 35 and RT 078 strains presented with colonic mucosal ulceration ('volcano lesions') with concomitant goblet cell loss and neutrophilic infiltration into the lamina propria. The majority of these (4/5) had mesocolonic oedema. Four of these animals were culture positive; the remaining piglet was the only piglet culture positive at 48 h but negative at 72 h. Sixty per cent (3/5) of the animals with volcano lesions were toxin positive by EIA.

5.4.2.3 Clinical symptoms and gross findings at necropsy

Results are summarised in Table 5.7. Disease course was comparable in all groups, including test and control animals. Most piglets (27/29, 94%) presented with signs of ill thrift (dehydration and/or weight loss) but survived until necropsy at 72 h. The mean clinical signs score (including scouring status determined by perineal staining) was greater for culture negative animals ($\bar{\chi}$ = 8.2) than culture positive ($\bar{\chi}$ = 5.9) but this was not significant (p = 0.088). The mean lesion score in *C. difficile* positive piglets was not significantly different between toxigenic strains with more than 1 score/strain. (RT 078: 6.8, AI 35: 5, VP27: 4, p = 0.281).

Seven test piglets (7/18, 39%) and two controls (2/11, 18%) developed mild-moderate non-haemorrhagic pasty yellow diarrhoea by 48 h post-challenge; 86% (6/7) of the test piglets were also culture positive at 48 h, although the non-toxigenic strain JGS 753 was isolated from one of these piglets. At 72 h diarrhoea had resolved in five of the original scouring piglets, but 10 additional test piglets were scouring, a total of 14 piglets overall (14/29, 48%). *C. difficile* was isolated from 10 (10/14, 71%) of these. By 72 h post-inoculation scouring had progressed from pasty to watery yellow diarrhoea. Of ten piglets that did not develop diarrhoea throughout the course of the experiment, nine were culture positive. When all animals positive for *C. difficile* at 72 h were considered (n = 24), 42% (10/24) were scouring versus 80% (4/5) controls. Two of these animals were positive for *Salmonella* spp.

Mesocolonic oedema was identified in seven piglets (Figure 5.9); all were culture positive at 72 h. Only one animal (inoculated with test strain AI 35) presented with obvious necrotic lesion/s of the small and large intestine, and these were scored as mild. The number and exact location of the lesion/s were not identified.

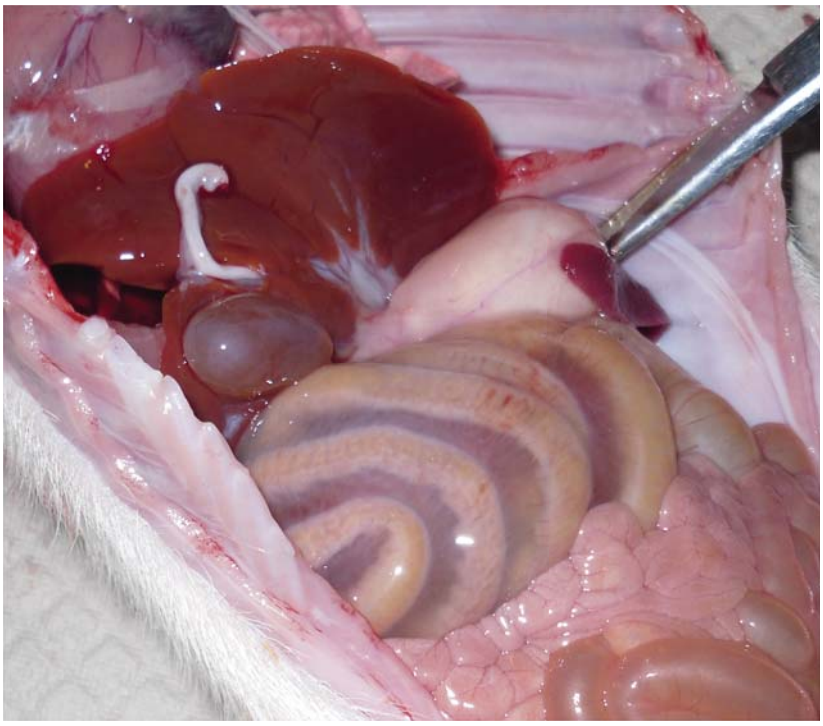


Figure 5.9 Severe oedema of the mesocolon (≥ 3 mm between loops) observed in piglets at necropsy.

Mesocolonic oedema is a unique porcine lesion that is not pathognomonic for *C. difficile* but correlates strongly with production of *C. difficile* toxins in the large intestine (Yaeger, Kinyon et al., 2007)

5.4.2.4 Toxin production

Toxin results are summarised in Table 5.7. All isolates were positive by PCR for their respective toxin genes.

When faecal culture positive animals (n = 24) were considered, 67% (16/24) had toxin in their gut contents versus 0% (0/5) of culture negative animals. Associations could not be determined between the presence of toxin and mesocolonic oedema (p = 0.3955), or CDI lesions (p = 0.5907) in culture positive piglets.

Using the EIA-tcdA/B method both toxigenic profiles A+B+ and A-B+ were equally likely to be detected (A+B+: 11/16, 69%; A-B+: 5/7, 71%). When compared to DC on CA the sensitivity, specificity, PPV and NPV were 70.8, 100, 46.1 and 100% respectively.

5.5 Discussion

5.5.1 Isolation and characterisation of *C. difficile* strain AI 35 (RT 237)

Despite its recognition in other pork-producing countries as an enteric pathogen of piglets, *C. difficile* in Australian piglets was not systematically investigated prior to 2009. Specialist pig veterinarians and pork producers Australia-wide reported herds with long-standing, high-morbidity, idiopathic scour in neonates, presenting with clinical features of CDI. A diagnosis of CDI could not be confirmed in these cases due to the lack of diagnostic capacity in Australia, including a lack of awareness of *C. difficile* as a pathogen in this age group.

The aim of this study was to determine if *C. difficile* was present in herds of neonatal piglets from the same producer, but at different locations and with different scouring histories. A novel strain of *C. difficile* isolated from these piglets was evaluated using multiple identification methods.

5.5.1.1 A novel C. difficile *RT, UK 237, was prevalent in scouring Western Australian piglets*

This is the first published finding of *C. difficile* in Australian piglets. Despite differences in scouring history, mean prevalence across all animals in the study was 77%. This is consistent with high prevalence in subsequent Australian studies (this thesis) and lower than prevalence reported in diagnostic studies from major swine-producing countries (USA, Canada and the Netherlands) (Chan, Farzan et al., 2013; Koene, Mevius et al., 2012; Susick, Putnam et al., 2012). Variation in scouring severity in piglets despite high levels *of C. difficile* colonisation may mimic human disease. Human CDI presents with a spectrum of symptoms ranging from asymptomatic carriage to fulminant CDI with PMC. The spectrum of CDI severity may be explained by strain-dependent variation in expression of virulence factors along with differences in host immunity. CDI in piglets is likely a multifactorial issue involving strain aspects, host aspects (weight, thrift etc.), quality of passive immunity, and infectious dose.

Asymptomatic animals were considered as a separate group to ascertain if high asymptomatic carriage rates seen in other studies (Alvarez-Perez, Blanco et al., 2009) (Yaeger, Kinyon et al., 2007) were occurring here. The finding of high carriage rates in

apparently healthy animals was in accord with other studies, although the 100% prevalence figure was probably an artefact of the small sample size (n = 11).

It was considered possible that *C. difficile* strains in Australian piglets would differ from international strains because of Australia's geographic isolation and strict quarantine laws regarding importation of livestock. It was also assumed that there would be little commonality between piglet and human strains because of Australia's large land mass and relatively low human and pig densities, offering little opportunity for spillover of strains. Australia has, on average 2,600,000 pigs housed in a land area of 7,686, 850 km^2, of which only 6.5% (499,645 km^2) is arable. This equates to approximately five pigs/km^2 and three humans/km^2. Denmark, by comparison, has a stocking density of 5, 536 pigs and 127 human;/km^2 (Australian Bureau of Statistics, 2007; Australian Government Department of Agriculture, 2015; Statistics Denmark, 2009a; Statistics Denmark, 2009b); hence, the finding that strain AI 35, later shown to be RT UK 237, had not previously been described in humans or animals, was expected. The toxin profile (A-B+CDT+) was also unusual as A-B+ strains are rarely isolated from pigs (Janezic, Zidaric et al., 2014; Thakur, Putnam et al., 2010).

5.5.1.2 Analysis of C. difficile *strain AI 35 revealed unique genotypic features*

5.5.1.2.1 Strain AI 35 has a unique PaLoc and CdtLoc structure

Genome sequencing of strain AI 35 showed a novel PaLoc structure. A large deletion had removed the *tcdA* gene, consistent with other Australian A-B+ strains which differ from international A-B+ strains (Elliott, Squire et al., 2011). The *tcdC* gene was also deleted. A 10,366 bp deletion encompassing *tcdA* and *tcdC* was confirmed recently in a WGS PaLoc study where AI 35 was mapped to the reference strain 630 (Elliott, Dingle et al., 2014).

The *tcdC* deletion suggested that AI 35 may be a more proficient toxin producer, similar to the outbreak strains RTs 027 and 078. This is controversial, however: the role of tcdC as an anti-sigma factor in negatively regulating toxin expression has recently been disputed. Advances in precise genetic manipulation techniques have demonstrated that toxin production in *C. difficileΔtcdC* strains does not differ significantly from wild-type strains (Bakker, Smits et al., 2012; Cartman, Kelly et al., 2012). Toxin potency studies indicated that the same was true for AI 35; lack of *tcdC* did not equate to enhanced

toxin production in a Vero cell cytotoxicity model (Section 6.1.2.2.). Strain AI 35 produced approximately 25-fold less toxin B than RT 027 and RT 078, but similar levels to strain 630, a low toxin-producing strain. This did not correlate perfectly with clinical virulence in our mouse model, however; AI 35 produced more weight loss than an RT 078 strain, suggesting that toxinA/B quantity is not associated with clinical outcome and possibly hinting at a role for binary toxin.

A portion of the upstream PaLoc flanking gene *cdd1* was also deleted, leaving a fragment that was confirmed by more recent investigation (Elliott, Dingle et al., 2014). The PaLoc integration site is located between *cdd1* and *cdu1* genes on the PaLoc borders (Braun, Hundsberger et al., 1996). These flanking sequences are implicated in exchange of the entire PaLoc (hence, clade specificity) via homologous recombination, allowing loss and gain of toxigenicity (Dingle, Elliott et al., 2014). For example, the absence of a perfect PaLoc integration site in clade C-I has been suggested to explain its non-toxigenic status. The *cdd1* allele in clade 5 A-B+ strains (of which AI 35 is a member) varies from its clade 5 A+B+ counterparts whose *cdd1* is more homologous to *cdd1* in clades 1-4. This indicates that A-B+ clade 5 strains likely acquired their PaLoc in a separate acquisition event to A+B+ strains in the same clade. This event has been dated phylogenetically to about 1,300 years ago (Elliott, Dingle et al., 2014), making it older than RT 078 clones that emerged more recently (Goorhuis, Debast et al., 2008; Knetsch, Connor et al., 2014).

The AI 35 CdtLoc was complete and contained an intact copy of *cdtR*, unlike RT 078 isolates, which encode a *cdtR* with a premature stop codon (Metcalf and Weese, 2011). This suggested that AI 35 was a more proficient binary toxin producer than RT 078 strains (Carter, Lyras et al., 2007), but this has not been confirmed experimentally.

Despite these variations, MLST showed that strain AI 35 belonged to the same clade 5 and ST11 as RT 078 strains from the USA and Europe (strain AI 15 in the report by Stabler et al. is the same RT as AI 35 (Stabler, Dawson et al., 2012)). Clade 5 strains like RT 078 are highly genetically divergent from those in other clades (He, Sebaihia et al., 2010). All other A-B+ strains fall into clade 4, clustering primarily in South-East Asia. Despite clade 5 MLST loci divergence, homology between the glucosyltransferase domains of *tcdB* in clades 4 and 5 indicates that recombination events occurred between

the two, suggesting geographic proximity. One group proposed that clade 5 strains may have originated in Australia (Elliott, Dingle et al., 2014).

5.5.1.2.2 Intact phages associated with putative virulence factors were predicted from the genome sequence

Rapid expansion of *C. difficile* outbreak strains in the last decade may be explained by MGEs, especially given that the core *C. difficile* genome mutates at an estimated single SNP per genome per year (Didelot, Eyre et al., 2012). MGEs constitute 11% of the *C. difficile* genome (Sebaihia, Wren et al., 2006) comprising mainly conjugative transposons and bacteriophages integrated into the genome (prophages). Strain R20291, an epidemic strain of RT 027 from 2006, had acquired an additional 234 genes when its genome was compared with an historic RT 027 from 1985. These genes are theorised to contribute to its virulent phenotype (Stabler, He et al., 2009).

Two intact phages were predicted from AI 35 genome sequence: ΦC2 and CDMH1. The role these phages play in AI 35 virulence is unclear. CDMH1 has previously been identified in a clade 2 RT 251 human *C. difficile* strain from the USA (personal communication, Daniel Knight, UWA) and encodes a putative quorum sensing (QS) homologue of the *agr* pathway (Hargreaves, Kropinski et al., 2014).

Phage CDMH1 is not ubiquitous in Australian human *C. difficile* strains, and has not been identified in animals to our knowledge. CDMH1 is a *C. difficile* myovirus and closely related to ΦCD119 and ΦC2 except that it houses a predicted QS *agr* operon homologous to the *agr* bacterial genome locus (Hargreaves, Kropinski et al., 2014). Transcriptional profiling of RNA from a *C. difficile* RT 027 strain with insertional activation of the genomic *agrA* gene showed that the *agr* regulon involves genes coding for flagellar assembly and function and tcdA expression (tcdB expression was not differentially regulated in the mutant but was not as well expressed as tcdA in the wild-type, which may have contributed to this finding), and other regulatory genes. The *agr* mutant showed reduced colonisation in mice, presumably due to the flagellar defect (Martin, Clare et al., 2013). The relationship between phage-encoded and genomic encoded *agr* loci has not been investigated but it is feasible that there is a functional relationship because of the cellular cost of maintaining two similar regulons in the same bacterial cell. It is conceivable that phage CDMH1 also plays a role in toxin regulation, but this requires experimental confirmation.

Phage ΦC2 is a well-characterised and ubiquitous *C. difficile* phage that mediates transfer of erythromycin resistance via transduction of the *ermB*-containing *Tn*6215 between *C. difficile* strains (Goh, Hussain et al., 2013). Genomic *ermB* and *Tn*6215 (or other MGEs of significant similarity) were not identified in AI 35, but it is feasible that phage ΦC2 may mediate transfer of other resistance or virulence genes as its involvement in the transduction of other genes has not been widely analysed. The ability of ΦC2 to transfer *Tn*6215 between different species has also not been tested. Likewise, ΦC2 may be associated with other transposons. This is an area for future research.

A target for future research is the putative *C. difficile* holin protein, tcdE, which has phage origin. TcdE is structurally and functionally similar to other phage holin proteins that lyse bacterial host cells to release progeny phages, so that it has a predicted role in extracellular toxin release in *C. difficile* (Tan, Wee et al., 2001). AI 35 had a novel *tcdE* but was a low-toxin producer. It is possible that there is a temporal relationship between TcdE and toxin release, which would be easy to investigate as *tcdE* mRNA is presumably transcribed at the same time as that of *tcdB*.

5.5.1.2.3 AI 35 resistance gene (ARG) profile and antibiogram phenotype

In silico analysis predicted a range of putative resistance genes with only sulfonamide resistance predicted at high identity. Comparison of antibiogram data with low-identity resistance gene predictions confirmed that that these were not clinically relevant.

Clinically relevant sulfonamide resistance has not been reported in *C. difficile*. Although sulfonamide susceptibility was not tested experimentally it correlated with reported sulphonamide use for treating neonatal scour on the same farm that AI 35 was isolated from (personal communication, farm veterinarian).

A sequence-based approach has recently been used to identify *ermB* and the uncommon *tetW* ARG in another porcine *C. difficile* strain, UK 014 (Dan Knight, UWA, unpublished data). Future work will be undertaken to identify ARGs in other porcine strains as they are sequenced, with a focus on identifying adjacent markers of horizontal gene transfer, indicators of host bacterium of origin and correlation with antimicrobial use on-farm.

5.5.1.3 Virulence and disease severity

5.5.1.3.1 Strain AI 35 produced low levels of a variant tcdB that is as potent in vitro as high levels of toxin

In vitro testing using Vero cells showed AI 35 was a low toxin producer, with ~25-fold less toxin B than outbreak strains RT 027 and RT 078, and similar titres to the low toxin-producing strain 630. Regardless of its low-level toxin production, strain 630 is still a virulent and multidrug (clindamycin-chloramphenicol-erythromycin-rifampicin-tetracycline) -resistant strain responsible for an outbreak of severe infection with PMC in humans (Wust, Sullivan et al., 1982).

AI 35 produced atypical CPE in Vero cells, consistent with cytotoxicity produced by *C. difficile* strains 8864 (ST 122) and 1470, which in turn resembles that of *Clostridium sordellii* lethal toxin (tcsL), another member of the large clostridial toxin family. Chaves-Olarte et al. demonstrated that strain 8864 tcdB was a novel cytotoxin equating to a functional hybrid between tcdB from reference strain VPI 10463 and *C. sordellii* tcsL (Chaves-Olarte, Low et al., 1999). AI 35-*tcdB* has 91% and 95% identity at the nucleotide level to strains 8864-*tcdB* and 1470-*tcdB* respectively.

Strains 8864 and 1470 are A-B+ human strains with mutations in the toxin B N-terminal catalytic domain that affect glucosylation substrate specificity (Soehn, Wagenknecht-Wiesner et al., 1998). Because of this, cells intoxicated with variant strains produce only a rounding effect (Chaves-Olarte, Low et al., 1999; Torres, 1991) rather than the characteristic actinomorphic morphology induced by other strains of *C. difficile* where contraction of the cytoplasm leaves long projections radiating away from the rounded cell body (Thelestam and Florin, 1984). In vitro, *tcdB*-variant strains have the same cytotoxic potency as *C. difficile* strain VPI 10463, which produces high levels of tcdA and tcdB (but is CDT-) (Chaves-Olarte, Low et al., 1999). Although similar morphologically and genetically to variant-tcdB-producing strains, comparison of the toxigenic potential of AI 35 with strains 8864 and 1470 in Vero cells is needed to confirm this finding. The presence of a novel *tcdE* at the 3’ end of *tcdB* in AI 35 (this study) with 75% identity at amino acid level to *tcsE* from *C. sordellii* strain VPI 9048 (Elliott, Dingle et al., 2014) and 85% identity at nucleotide level (this study) in concert with the *tcdB* findings may be further evidence of interspecies recombination.

Vero cell cytotoxicity, but not morphology, may also be attributable to CDT production in AI 35. Toxin supernatants contained both tcdB and CDT. Purified CDT is toxic to Vero cells at concentrations of 50ng CDTa and 250ng CDTb (combined) (Sundriyal, Roberts et al., 2010). Strain 1470 does not produce CDT but CPE in Vero cells was morphologically similar to other variant tcdB strains, suggesting that tcdB alone is responsible for variant morphological effects. Experimental confirmation of this with purified tcdB and CDT from AI 35 is required.

5.5.1.3.2 Strain AI 35 caused more weight loss in mice than a RT 078 strain

Experiments were conducted in mice to determine if strain AI 35 caused disease in a mouse model and comparing its virulence with known outbreak strains RT 027 and RT 078.

Strain M 7404 (RT 027) was significantly more virulent than strains AI 35 and JGS 6133 (RT 078). All mice infected with strain RT 027 died, but mice infected with strains AI 35 and RT 078 survived. Despite low toxin production, AI 35 caused significantly greater weight loss in mice than did the RT 078 strain, suggesting greater disease severity. This difference may be the result of production of a variant tcdB; similar toxins were eight-fold more toxic to mice than tcdB produced by strain VPI 10463 (Lyerly, Barroso et al., 1992). It is unlikely that lack of tcdA alone accounted for decreased virulence. Mutant *C. difficile* strains expressing A-B+CDT+ were equally as cytopathic as wild-type (A+B+CDT+) strains in Vero cells, and were more virulent in a hamster model than the wild-type strain (mean time from infection to end point, 3.0 and 3.7 days respectively) (Kuehne, Collery et al., 2014). This is corroborated by human clinical studies suggesting that A-B+ strains cause more severe disease than A+B+ strains (Kyne, Warny et al., 2001).

Interpreting animal model findings and extrapolating them to another host should be approached with caution. For example, CDT had no adverse effects when purified and injected into mice (Popoff, Rubin et al., 1988), but A-B-CDT+ strains cause disease in pigs and humans (Elliott, Dingle et al., 2014; Elliott, Squire et al., 2011; Rupnik, Kato et al., 2003). For this reason, a challenge experiment was conducted in neonatal piglets to determine if we could reproduce CDI by inoculating animals with spores of several strains of *C. difficile* including AI 35.

5.5.1.4 Conclusion

Our results show that a toxigenic *C. difficile* strain in Australian piglets from a farm in Western Australia is of a different RT, UK237, than that commonly found in other parts of the world and the remainder of Australia. This strain contained a unique PaLoc and produced more weight loss in mice than did the more common RT 078 animal strain. Identifying this strain is the first step in detecting and responding to this emerging disease in piglets in Australia.

5.5.2 Laboratory diagnosis of porcine *C. difficile* infection in Australia

In this study 157 specimens of piglet faeces were assessed for the presence of *C. difficile* or its toxins by EC/TEC, two toxin gene-based PCR methods (real-time PCR and LMIA-PCR), DC using chromogenic agar (DC on CA), and an EIA for GDH and toxins A and B. This was the first evaluation of commercially available diagnostic assays for detection of *C. difficile* or its toxins in a diverse range of *C. difficile* strains from Australian neonatal pigs.

5.5.2.1 Confirmation of high prevalence and genotypic heterogeneity of C. difficile in Australian piglets

Of the 157 samples collected in this study, 22.9% and 39.5% were positive for *C. difficile* by TEC and EC, respectively, confirming that *C. difficile* is prevalent in Australian piglets. This is lower than the prevalence reported in our previous studies (60% EC on TCCFA and 67.2%, EC on CA), likely due to the lower proportion of scouring piglets in this study (31% vs. 59% and 42%); it is similar to other major pig-producing countries (Chan, Farzan et al., 2013; Koene, Mevius et al., 2012; Susick, Putnam et al., 2012).

Numerous PCR RTs were identified, some of which were internationally recognised strains, predominantly RTs associated with carriage and disease in humans. The most prevalent RT was QX 006 (16.1%), followed by QX 207 (12.9%), UK 014 (11.3%), QX 057 (11.3%), and UK 020 (8.1%). These top five RTs made up 60% of the isolates recovered by TEC. RT 014 and RT 020 are often grouped together due to their very similar RT fingerprint. RT 014/020 is the most common RT in many countries, including the Netherlands (Bauer, Notermans et al., 2011) and Australia (B. Elliott and T.V. Riley, unpublished data). RT 014 is well established in nosocomial cases of CDI

and is a leading cause of disease in the community (Bauer, Notermans et al., 2011); it has been found in a small number of livestock (Koene, Mevius et al., 2012). RT 046 made up approximately 5% of isolates and has recently been described in piglet and human populations in Sweden (Noren, Johansson et al., 2014). As in our recent prevalence studies (Chapter 3), RT distribution was clonal with many RTs unique to individual farms and states.

Overall, 58% (*n* = 36) of isolates were positive for *tcdA* or *tcdB* or both. Of the remaining isolates, 34% (*n* = 21), including about half of the isolates constituting the top five RTs, were nontoxigenic (A-B-CDT-) and 8.1% (*n* = 5) of isolates were positive only for binary toxin (CDT+). These data indicate heterogeneity in the test population and are consistent with our prevalence investigation findings.

5.5.2.2 DC on CA was the best method for detection of C. difficile

Other studies have evaluated different GDH- and toxin-based detection assays in animals, including piglets (Alvarez-Perez, Alba et al., 2009; Anderson and Songer, 2008; Chouicha and Marks, 2006; Keessen, Hopman et al., 2011; Medina-Torres, Weese et al., 2010; Post, Jost et al., 2002), with varied success. The diverse strain population, broad geographic distribution of sampling sites, and sample transport logistics involved in our study provide a unique scenario for assessing the local performance of these assays for detecting CDI in piglets.

To date, few studies have evaluated DC (Boseiwaqa, Foster et al., 2013; Carson, Boseiwaqa et al., 2013; Eckert, Burghoffer et al., 2013) and only one included samples of animal origin; and these were *C. difficile* isolates, not faecal samples (Boseiwaqa, Foster et al., 2013). This study presents the first data worldwide on the performance of a chromogenic medium for recovery of *C. difficile* from animal faecal samples.

DC performed the best of all the comparator assays and had a high sensitivity (91.9%) and specificity (100.0%). The overall recovery of *C. difficile* by DC was high (36.3%) and comparable to that by EC (39.5%) (96.8% concordance). This is consistent with studies performed on human faeces (Boseiwaqa, Foster et al., 2013; Eckert, Burghoffer et al., 2013).

5.5.2.3 Molecular and toxin based assays performed poorly in C. difficile detection

Toxin- and molecular-based assays (EIA-tcdA/tcdB, real-time PCR, and LMIA-PCR) performed poorly in detecting toxigenic and non-toxigenic *C. difficile* in porcine faeces. Concordances of these assays with TEC were lower than expected (EIA-tcdA/tcdB, 77.7%; real-time PCR, 84.1%; and LMIA-PCR, 79.6%), and sensitivities ranged from 5.6 to 42.9%. Surprisingly, given the high prevalence of *C. difficile* in the population, the PPV and NPV for the molecular-based assays (real-time PCR and LMIA-PCR) were unacceptably low (PPV, 78.9% and 64.3% respectively; NPV, 85.4% and 81.1% respectively). This concurs with other studies that found the performance of molecular-based assays to detect *C. difficile* in faecal samples of pigs, horses, and dogs was less than in human faecal samples (Anderson and Songer, 2008; Chouicha and Marks, 2006; Keessen, Hopman et al., 2011).

The poor performance of all assays except DC was primarily due to the high number of discordant results, principally false negatives. Several environmental and host factors are thought to influence the performance of human diagnostic assays and may be relevant in animal studies. Lyerly et al. suggested that low specificity in enzyme immunoassays was attributable to toxin degradation due to multiple freeze-thaw cycles (Lyerly, 1992). This is unlikely to account for discordant results in our study as samples were thawed only once, according to manufacturer's recommendations. Nonspecific binding of host faecal proteins to toxin in the gastrointestinal tract may result in low levels of free unbound toxin in the sample (affecting enzyme-linked immunosorbent assays [ELISA] and cytotoxic assays) (Jure, Morse et al., 1988). Inhibitory substances or inactivating enzymes in animal faeces may result in low specificity in commercial assays (Anderson and Songer, 2008; Lyerly, 1992), although there is limited data in the literature to support this hypothesis. It is conceivable, however, that inherent differences in faecal composition between animals and humans influence the binding of primers or EIA antigens.

Faecal proteases may also degrade toxin in the stool (Gumerlock, Tang et al., 1991). It is possible that the long transit time of samples (mean transport time of eight days) had a detrimental effect on toxin levels in the faeces, reducing them below the level of detection of EIA-based assays, even though they were processed within 24 h of receipt into the laboratory. Toxin levels are known to be detectable in equine faecal samples

after 30 days in anaerobic storage at 4^0C (Weese, Staempfli et al., 2000b), but they decrease significantly in human samples after two days' aerobic storage at 25^0C (Bowman and Riley, 1986). This could explain the difference between the poor results presented here for EIA (for toxigenic samples) and those reported by Keessen and colleagues (80% to 90%) (Keessen, Hopman et al., 2011). In that study, porcine faeces were collected in April (European spring) and transported under refrigeration from farms within the relatively small geographic area of the Netherlands. In our study samples were transported over large distances (mean distance from farm to laboratory of ~3,600 km) under ambient storage conditions; this reflects the circumstances under which samples are routinely transported from the site of collection to the veterinary laboratory. The fact that DC worked so well under these conditions underscores its suitability as a diagnostic test for *C. difficile* in Australia.

5.5.2.4 DC on CA performance was unaffected by RT

It is important that diagnostic tests perform well, independent of the strain types present in the test population. For example, the sensitivity of EIA methods is affected by different RT (Tenover, Novak-Weekley et al., 2010), illustrating the importance of using the correct diagnostic test. This is important to diagnose emerging RTs, not just the current circulating strains.

DC on CA performed consistently well across all 19 RTs, toxigenic and non-toxigenic. Similarly, Eckert et al. (Eckert, Burghoffer et al., 2013) found no relationship between RT and isolate recovery using chromogenic agar. Conversely, all non-culture methods evaluated in this study performed consistently poorly across all 12 toxigenic RTs.

The results of this study contrast with those of Keessen et al. (Keessen, Hopman et al., 2011) who reported significantly higher sensitivities for a real-time PCR assay (91.6%) and a range of EIA platforms. In that study, 99% (70/71) of isolates recovered from samples of porcine faeces were RT 078, the predominant RT circulating in animals in Europe. *C. difficile* strains circulating in Australian piglets are genotypically different from those in the rest of the world. It is feasible that differences in antigenic features, toxin expression, or PaLoc primer binding sites may occur in Australian strains, resulting in poor performance of molecular- and toxin-based assays. This is compounded by lack of homogeneity in strain types.

5.5.2.5 DC is cost-effective, rapid, reliable and simple to use

The performance of any assay is ultimately influenced by the choice of reference method. EC/TEC have not only high sensitivities and specificities for *C. difficile* but also the benefit of recovery of the isolate, which can be used for further epidemiological typing and antimicrobial susceptibility testing. EC/TEC are, however, slow and laborious, often taking up to five days for completion, and unlikely to be adopted by veterinary laboratories as a standard practice for *C. difficile* testing.

Culture on CA provides a viable method for presumptive detection of *C. difficile* in the veterinary laboratory. DC on CA outperformed the molecular methods assessed in this study as well as EC on TCCFA by negating the need for pre-reduction of media, enrichment, alcohol shock, or the 48 hours of incubation usually required for recovery of *C. difficile*. Strict anaerobiosis is still required, but this medium performs well when used in anaerobic jars, negating the requirement for an expensive anaerobic chamber. The CA plates are highly selective, limiting growth of endogenous flora to enable easy identification of *C. difficile* black colonies after 24 hours (Figure 5.10). Another benefit of CA is its relatively low cost (plates are ~AU$3 each). This is important as CDI is typically diagnosed in several piglets from a scouring herd, not an individual piglet. The high sensitivity and NPV of DC also negates the need for repeat testing, especially when multiple samples are submitted initially.

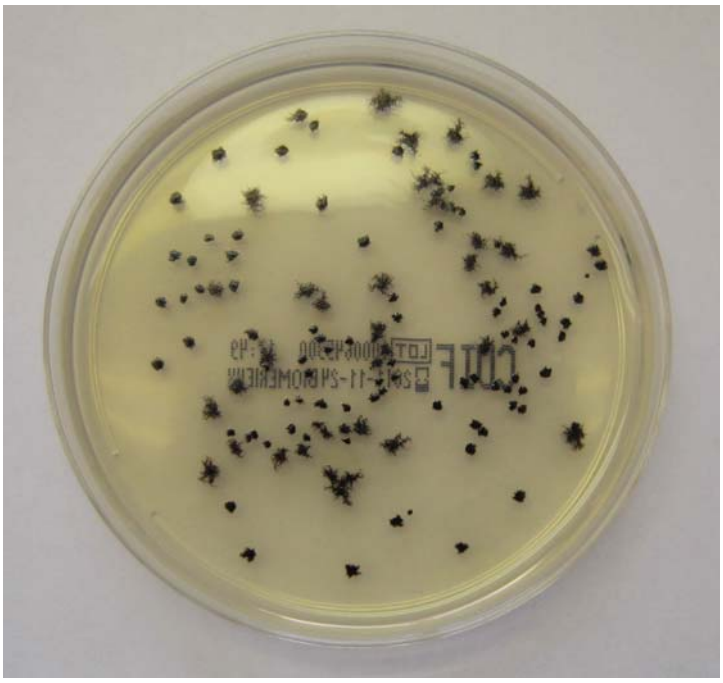


Figure 5.10 Colonies of *C. difficile* AI 35 (RT 237) on CA after 24 h incubation

There is the additional advantage of recovering isolates for genotyping for surveillance purposes, toxin gene analysis, and antimicrobial susceptibility testing. Reliable diagnosis of toxigenic strains of *C. difficile* in piglets is especially vital to the veterinarian, given the abundance of other pathogens in this population. The presence of toxin genes correlates well with toxin production (Kato, Ou et al., 1991; Rupnik, Brazier et al., 2001; Stubbs, Rupnik et al., 2000) and is a simple and robust PCR-based method.

5.5.2.6 Conclusion

This study confirms the high prevalence and unique strain types of *C. difficile* present in Australian neonatal pig populations. Due to its fastidious requirements for anaerobiosis and growth, *C. difficile* has been difficult to isolate in the veterinary laboratory, which may have impaired previous estimates of its prevalence. Despite the poor performance of commercially available non-culture-based diagnostic assays, our data suggest that DC on CA represents a reliable, cost-effective option for veterinary laboratory detection of *C. difficile* in piglets.

5.5.3 Infection study

Outside Australia, the incidence of CDI and recognition of disease in piglets has increased in veterinary medicine over the past decade. An abundance of experimental and field evidence indicates that porcine CDI presents as mild to severe, pasty, non-haemorrhagic scouring with well described clinical and histopathological features. Clinical signs include severe loss of condition and dehydration, mesocolonic oedema, and gross necrotic lesions of the colonic mucosa without small intestinal involvement. Microscopically, loss of goblet cells, infiltrating neutrophilia of the lamina propria, and inflammatory lesions of the colon and caecum, particularly the distinctive 'volcano' lesions, are considered pathognomonic for CDI (Arruda, Madson et al., 2013; Keel and Songer, 2006; Lizer, Madson et al., 2013; Steele, Feng et al., 2010).

Whether Australian strains that differ genotypically from the rest of the world could cause enteric disease in piglets has not previously been investigated. The objective of this research was to provide a clinical and histological evaluation of CDI in neonatal piglets inoculated with strains isolated from scouring piglets in Australia, in particular an unusual new RT 237 strain (AI 35) that was highly prevalent in a Western Australian piggery.

5.5.3.1 Enteric disease that mimics porcine CDI was reproduced in piglets

Features of CDI were reproduced in neonatal piglets orally challenged with toxigenic Australian field isolates of *C. difficile*. This study supports previous work from the USA (Arruda, Madson et al., 2013; Lizer, Madson et al., 2013; Steele, Feng et al., 2010) that demonstrate *C. difficile* and its toxins can be isolated from piglets exhibiting clinical and histopathological features of CDI.

5.5.3.1.1 C. difficile was isolated from the majority of test piglets, but was not always the inoculating strain

Seventeen of 18 test piglets were positive for *C. difficile* by DC on CA at 72 h versus 64% (7/11) controls; the single culture-negative piglet at 72 h was culture positive at 48 h. The most plausible explanations for this finding in a single piglet are non-homogeneity of spores in the faecal sample or spore numbers below the detectable limit; however, intermittent colonisation, transient passage of spores through the gut, intermittent shedding and reinfection from strains shed by other piglets cannot be ruled

out. Clearly the re-acquisition propensity was present as different strains were isolated from the same piglet at different time points. As the piglets displayed clinical and histopathological signs of CDI and 42% overall at 72 h still had the same strain isolated that they were inoculated with, these alternative explanations are less likely.

Interestingly, 100% (6/6) of piglets inoculated with strain AI 35 were positive for this strain at necropsy whereas only 33% (2/6) of VP 27, 17% (1/6) QP 6 and 20% (1/5) JGS 753 inoculated piglets were positive for those strains at 72 h. This suggests that AI 35-specific colonisation factors warrant further investigation.

Isolation of an RT 078 strain from piglets throughout the study highlights the tenacity of *C. difficile* environmental spores and underscores the difficulty in eradicating them completely. Every effort was made to ensure there was no contamination of either the facility or piglets prior to the experiment. Sows were closely monitored prior to farrowing to avoid contact between the newborn piglets and the floor or sow. Sow vulvas were cleaned with hypochlorite and piglets delivered into sterile cloths. Sow teats were also disinfected with Clorox wipes prior to collecting colostrum. The experiment facility was decontaminated with hypochlorite prior to the study, as were the tubs housing the piglets, and food bowls. Tubs and bowls were purchased specifically for this study. A strict protocol of handling, cleaning and feeding was followed to prevent cross-contamination.

A single piglet was positive for RT 078 by rectal swab prior to inoculation. Keessen et al. showed that piglets could be positive for *C. difficile* within one hour of birth (Hopman, Keessen et al., 2011). Piglets were housed at the piggery for two hours during farrowing and at the BSL-2 facility for four hours prior to inoculation, so it is impossible to ascertain where this piglet ingested RT 078 spores. It is also difficult to establish whether other piglets ingested RT 078 spores at the same time, or ingested spores shed from piglets in the experiment facility. The increase in RT 078-positive piglets throughout the course of the experiment likely indicates the latter.

5.5.3.1.2 Clinical signs

When all animals positive for *C. difficile* at 72 h were combined, 67% had toxin in their gut contents and 30% had mesocolonic oedema. Toxin and mesocolonic oedema were not identified in any culture negative animal.

Scouring was not a good indicator of CDI, with 80% (4/5) of culture negative animals scouring at 72 hours compared with 42% of culture positive animals. Although this concurs with findings in both field (Yaeger, Kinyon et al., 2007) and experimental (Lizer, Madson et al., 2013) conditions, the most severe scouring occurred in piglets that were *C. difficile* and *Salmonella* spp. negative. Ten piglets did not develop diarrhoea throughout the course of the experiment; nine of them were *C. difficile* culture positive. These data suggested a non-bacterial contribution to scouring. Oliveira *et al.* (Oliveira, Galina et al., 2003) described 80%–90% mortality in piglets fed only Esbilac puppy milk replacer in the first week of a *Haemophilus parasuis* infection model, attributable to severe diarrhoea. Scouring in control piglets was reported in the Lizer et al. study (Lizer, Madson et al., 2013) but alpha and beta2 toxin-producing *C. perfringens* was isolated from these piglets. It is possible that ad libitum feeding with Esbilac contributed to scouring in this study, potentially masking scouring resulting from CDI and making it difficult to draw conclusions about the relationship between *C. difficile* and scouring. This also resulted in difficulty interpreting clinical signs scores, as scouring and its sequelae (dehydration and weight loss) were included in this category.

Severe clinical signs seen in other studies (Arruda, Madson et al., 2013; Steele, Feng et al., 2010), such as extensive dilation and inflammation of the large intestine with pseudomembrane formation and colonic mucosal haemorrhages, were not observed. Likewise, gross large intestinal lesions were uncommon, only discernable in a single test piglet, and small intestinal lesions were not found. These lesions were associated with piglets receiving a challenge inoculum of 10^8–10^9 spores, which was higher than the challenge dose in this study. Regardless, microscopic luminal ‘volcano’ lesions resulting from inflammatory influx of red and white blood cells, fibrin and bacteria (Rupnik, Wilcox et al., 2009) and the hallmark of severe CDI in piglets were identified in five animals, positive for AI 35 and RT 078 strains, in agreement with other challenge studies (Lizer, Madson et al., 2013) and naturally occurring infection (Songer, Post et al., 2000; Yaeger, Funk et al., 2002; Yaeger, Kinyon et al., 2007).

Disease severity differed between *C. difficile* positive piglets, which is consistent with CDI in piglets (Steele, Feng et al., 2010) and humans (Gebhard, Gerding et al., 1985).

Reasons for this spectrum of disease presentation in piglets have been proposed in Chapter 3, and should be subject to future research.

5.5.3.1.3 Histopathology

Classic microscopic lesions of porcine CDI (caecal and colonic lesions and mesentritis) were observed more often in piglets positive for a toxigenic strain of *C. difficile* than *C. difficile* negative piglets. CDI lesions were also significantly more severe in culture-positive animals. The likely reason *C. difficile* negative piglets had histopathology that resembled CDI was that *Salmonella* spp. was also isolated from their gut contents.

Lesions of porcine CDI are typically localised in the caecum and colon (Keel and Songer, 2006), which concurs with the findings of this study. One-third (8/24) of culture positive animals presented with neutrophilic infiltration of the small intestinal lamina propria. Four of these animals had a *Salmonella* spp. co-infection, which is associated with small intestinal neutrophil influx (Milo, Correa-Matos et al., 2004). The remainder had *C. difficile* gut toxin and/or CDI lesions of the caecum/large intestine, without isolation of any other enteric pathogen.

Although RT 078 produced a numerically greater mean microscopic CDI lesion score in *C. difficile* positive piglets, it was not significantly different between toxigenic strains with more than one score/strain. (RT 078: 8, AI 35: 3, VP27: 5, $p = 0.344$). This suggests that strain-dependent virulence was similar, but as RT 078 was acquired from the environment and differing numbers of spores of the other strains were administered, experimental confirmation of this is required. AI 35 produced more weight loss than CDI in a mouse model of infection, suggesting enhanced virulence, but these results indicate that AI 35 has a similar virulence potential to RT 078 in a piglet model.

5.5.3.1.4 Toxins

Toxin was identified in the gut of 67% (16/24) culture positive animals at 72 h and no culture-negative animals, but no statistical association could be found between the presence of toxin and other signs of CDI. As in previous findings regarding the use of toxin EIA for diagnosing *C. difficile* (this thesis), EIA for tcdA/B performed poorly (sensitivity, specificity, PPV and NPV were 70.8, 100, 46.1 and 100% respectively) when compared to DC on CA combined with detection of toxin genes by PCR.

5.5.3.1.5 Implications for CDI diagnosis in Australian piglets

There was a relationship between exposure of piglets, isolation of *C. difficile* and disease development, and a significant development of colonisation over time. All piglets presented with ill thrift, dehydration and weight loss at 72 h post-inoculation. If the assumption that the single non-colonised piglet was a sampling anomaly was correct, all piglets were positive for *C. difficile* at 72 h, with a significant increase in numbers of culture positive animals throughout the experiment period. When all culture positive animals were considered, 88% (21/24) had toxin in their gut contents, or microscopic lesions of CDI in the colon and caecum, or mesocolonic oedema, or a combination of these.

Other piglet challenge experiments determined the incubation period for CDI was 48 h in the gnotobiotic model (Steele, Feng et al., 2010) and 32–48 in the field (Hopman, Keessen et al., 2011). In these studies, 100% of animals were colonised with *C. difficile* or showed clinical and histopathological symptoms of CDI at 48 h. In contrast, only 88% (21/24) of animals in this study had features consistent with CDI at 72 h. Spore inoculum concentration is likely not a consideration as Steel et al. (2010) demonstrated CDI in all piglets at 48 h regardless of dose (test animals received either 1×10^8 or 1×10^5 spores/2 mL). Bolus administration of spores and overwhelming tcdA/B/CDT intoxication may have influenced disease outcome, however.

The lack of a dose–response colonisation relationship is borne out by conventional farrowing pen experiments where 100% of piglets were colonised with *C. difficile* after 48 h in a farrowing pen model, with asymptomatic sows also shedding *C. difficile* (Hopman, Keessen et al., 2011) (vs. 70% in this study). Spore density in an Australian 2 x 0.6 m pen ranged from 0–2.088×10^6/pen (this thesis), suggesting that rooting piglets ingest differing numbers of spores over a longer period rather than a single large dose. Although spore density was not calculated in the Keessen et al. (2011) investigation, their work demonstrated transmission and colonisation in a farrowing pen model.

The most plausible explanation for differences in temporal disease development is the colonisation and virulence proficiency of strains used in challenge studies. All piglet challenge studies to date have used the epidemic RT 078 (Arruda, Madson et al., 2013; Lizer, Madson et al., 2013) or RT 027 strains (Steele, Feng et al., 2010), or investigated

colonisation with a naturally-occurring RT 078 strain (Hopman, Keessen et al., 2011). A range of strains was utilised in our work, including the RT 078 strain that contaminated our study. RT 078 was isolated from more piglets at 48 h than other strains, and was the predominant strain at 72 h.

There may also be a dose-response disease severity relationship. Arruda et al. proposed this relationship although their results were not compelling due to statistical insignificance (Arruda, Madson et al., 2013). They did describe a dose-response trend, however, which may impact on the time frame selected for diagnosis. Although there was no statistically significant trend in disease severity between strains in this study, piglets positive for RT 078 had numerically greater histopathology and clinical signs scores. This concurs with other investigations where RT 078 and AI 35 were compared in a mouse model of infection (this thesis) and human disease where RT 078 strains cause CDI with similar severity to RT 027 strains (Goorhuis, Bakker et al., 2008).

These factors have important diagnostic implications in the Australian context. This study showed that disease consistent with CDI could be diagnosed in piglets within 72 hours after fewer spores of more genotypically diverse strains were administered than other challenge experiments. Strain-dependent differences in virulence and colonisation proficiency may impact on disease development timeline and severity. This is important in Australia where genotypically diverse strains circulate in piggeries, rather than just RT 078; hence, piglets selected for necropsy and bacteriology for CDI should be acutely affected for 48–72 hours to ensure development of symptoms and maximise diagnostic accuracy. A number of piglets from an affected herd should be selected for necropsy and the large intestine/caecum examined and tissue sections taken for histopathology where CDI is suspected.

5.5.3.2 Piglet model considerations

The snatch-farrowed, artificially reared (SNAR) model of piglet infection used in this study has several advantages over gnotobiotic or Caesarean-derived colostrum-deprived (CDCD) models. Lack of immunological development and normal flora exposure in Caesarean-derived piglets are postulated to present a higher risk of mortality and different immunologic responses than snatch-farrowed piglets delivered by spontaneous parturition at term via the vaginal canal (Butler, Weber et al., 2002; Fowden, Li et al.,

1998; Sangild, Holtug et al., 1997). SNAR piglets in this study additionally received a bolus of pooled colostrum from the disinfected teats of the farrowing sows to commence immunological development and enhance survival.

Logistically, the SNAR method is easier to manage and less expensive than CDCD models. There is no requirement for surgical facilities for sow Caesareans or a specialised facility with sterile isolators to house piglets, and sows are not culled after farrowing.

There are limitations to the SNAR model. Whilst it better represents on-farm conditions regarding *C. difficile* environmental contamination and transmission, mortality rates are higher due to infection from vaginal flora acquired during birth (Oliveira, Galina et al., 2003). Although housing animals in sterile compartments in a specialised facility is expensive and requires technical expertise, the positive pressure ventilation used in these facilities reduces cross-contamination during infection experiments.

5.5.3.2.1 Improvements for SNAR

With adaptations to overcome environmental contamination and non-specific scouring, the SNAR model used in this study is suitable for further study of disease pathogenesis in piglets. The model is especially suited to piglet CDI vaccine development, given its relevance to the on-farm situation, its ease of use, and the availability of pig-specific immune reagents to study host responses to CDI. The obvious advantage of using a piglet model to develop piglet vaccine is that there is no requirement to extrapolate disease pathology and immunological response from another species such as mice.

The source of the RT 078 infecting strain was not definitively identified but originated either in the piggery or experimental facility. Improved biosecurity measures to avoid cross-contamination with endemic environmental strains or spores shed from other piglets could include disinfecting piglets with 0.1% hypochlorite, removing them from the farrowing pen area immediately after birth, and transporting them in separate, disinfected crates to the facility. Environmental contamination of the experiment facility and housing should also be ruled out prior to commencing the study. Piglets receiving different strains, and negative controls, should be housed in different rooms.

Human infant milk replacer (Similac) has been successfully used in a gnotobiotic piglet model of CDI (Steele, Feng et al., 2010) where scouring occurred in all animals but correlated with culture and histopathology results. In this study piglets were fed three times daily. The same product was used in a twice-daily feeding regimen in a piglet EHEC infection model (Tzipori, Gunzer et al., 1995) and scouring was again consistent with colonisation sequelae. This feeding regimen was used in a gnotobiotic piglet model of the human adult and infant gastrointestinal tract (GIT) where human faecal microbiota was transplanted into the piglet GIT (Zhang, Widmer et al., 2013). Diarrhoea was not reported in this study. Use of human infant milk replacer should be considered in future piglet model investigations of CDI.

5.5.3.3 Conclusion

A snatch farrowed piglet infection model was successfully used to reproduce clinical CDI consistent with international reports using Australian strains of *C. difficile*. Despite infection with a diverse range of genotypes and a lower spore inoculum than other infection model experiments, piglets developed features of porcine CDI by 72 h post inoculation. An RT 078 endemic strain infected some piglets, with similar disease severity to that caused by Australian strains of differing genotypes and toxin production profiles.

Based on these findings, a definitive diagnosis of CDI should include the presence of symptoms in 1–7-day-old piglets (typically scouring in the farrowing herd with concomitant ill-thrift, dehydration, weight loss, anorexia), a faecal culture result positive for toxigenic *C. difficile,* and necropsy findings demonstrating classic CDI lesions. Several piglets should be chosen for examination to account for differences in CDI severity between affected piglets in the herd. Piglets selected for rectal swab and/or necropsy should have been acutely affected for at least 48–72 hours to maximise disease expression and diagnostic accuracy. The use of CA and toxin gene PCR is recommended for faecal culture and toxin gene detection. EIA for tcdA/B is not recommended due to its poor performance in detecting *C. difficile* in porcine faeces.

Chapter 6 Conclusions and Recommendations

Outside Australia there is a clear association between *Clostridium difficile* and enteric disease in neonatal piglets. Whilst this association has been well studied elsewhere, relatively little is known about porcine CDI in Australia despite reports of idiopathic scour. We hypothesised that *C. difficile* would be present in Australian pig herds but that the epidemiology would be different due to our geographic isolation, rigorous import restrictions on live animals, and low pig stocking density, limiting the applicability of available data to the local setting.

No systematic studies of *C. difficile* in the Australian pork industry have been undertaken until now. The dearth of local data is compounded by lack of diagnostic capacity for CDI in Australian piglets, including acceptance of this pathogen by industry stakeholders.

The Biosecurity CRC for Emerging Infectious Diseases funded the research reported in this thesis via a PhD scholarship (Project: P3.132EI), with contribution of funds from Australian pork industry revenue. APL provided funding for additional projects under the Herd Health Management program, which aims to enhance animal health whilst reducing routine antibiotic use in commercial pork production. This peak industry body for Australian pork, representing the interests of Australian pork producers, recognised that the impact of CDI in Australian piglets is impossible to assess without accurate epidemiological and clinical data, and so provided an opportunity to investigate *C. difficile* at the local level.

This research was designed to evaluate the following in Australian neonatal piglets:

- the epidemiology of *C. difficile* in farrowing units including risk factors and prevalence
- the characteristics of *C. difficile* strains isolated
- association between *C. difficile* and enteric disease,
- appropriate methods for diagnosis of CDI.

6.1 Aim 1: epidemiology of *C. difficile* in Australian farrowing units, including prevalence and risk factors: evidence summary

6.1.1 *C. difficile* was prevalent in scouring and non-scouring neonatal piglets in piggeries across Australia

This study provided the first data demonstrating *C. difficile* colonisation of neonatal piglets in Australia. Retrospective examination of diagnostic samples from neonates with symptoms that fit the case definition of porcine CDI revealed 60% prevalence of *C. difficile*. This was reinforced by an Australia-wide period prevalence study that showed a 67% prevalence rate in neonatal herds. These rates are higher than reported prevalence in both diagnostic and period prevalence studies from major pork-producing countries.

C. difficile colonisation is age-dependent, with neonates most commonly affected. Prevalence data was consistent with this: piglets ≤7 days old were more likely to be colonised with *C. difficile* than older pigs.

Like infection with other porcine enteropathogens such ETEC, STEC and *Salmonella* spp., asymptomatic colonisation of piglets within a scouring herd is common in CDI. This is the most commonly cited basis for lack of acceptance of *C. difficile* as a pathogen in Australian pig herds. There are several plausible explanations for asymptomatic carriage, all of which require experimental confirmation.

The caveat is that only 5% of Australia's total pork operations were surveyed in these studies, although facilities sampled included all methods of pig production and operations in all pork-producing states. The fact that 423 diagnostic samples were submitted in a two-year period to determine a cause for idiopathic scour cannot be ignored.

6.1.2 Antimicrobials were not statistically associated with scouring but there was reliance on agents of critical and high importance to human health to treat idiopathic scour in Australian piggeries

Dysbiosis of gut flora through antimicrobial use remains the most important risk factor for CDI in humans and older or hospitalised animals. There was no evidence in the literature that antimicrobials predispose naive piglets to CDI. Similarly, there was no

evidence that antimicrobial administration was a risk factor for *C. difficile* colonisation in scouring herds of neonates.

The Australian government and public are increasingly concerned about AMR in production animals. Although the impact of antibiotic use in food-producing animals on AMR infections in humans is unclear, there is general agreement that responsible use of antibiotics in agriculture is desirable. Analysis of clinical data submitted with samples in these prevalence studies revealed that the Australian pork industry relied on antimicrobials of critical and high importance to human health. These are important data as there is only a single published study describing antimicrobial use in Australian pigs (Jordan, Chin et al., 2009).

Ceftiofur use in the farrowing unit was reported in 53% and 28% of herds across the two prevalence studies. The use of this antimicrobial, to which *C. difficile* is intrinsically resistant, is likely a high-risk practice in animals older than neonates. Administration of antimicrobials such as ceftiofur to older animals (especially sows) may be the inciting event that predisposes the neonatal herd to a CDI outbreak. However, ceftiofur use may contribute to prolonged excretion of *C. difficile* in piglets and thus to the overall burden of environmental contamination.

6.1.3 *C. difficile* spore contamination of the farrowing unit environment was high and developed quickly but could not be explained by scouring piglets alone

Environmental spore contamination is considered the mode of transmission in porcine CDI (Hopman, Keessen et al., 2011). This study examined prospective data from a newly commissioned farrowing shed and demonstrated that spore contamination equating to 12,000 spores/pen developed in 61% of pens sampled in a farrowing shed where numbers had been below the detectable limit one month prior. Over the next three months, spore density increased to 400,000 spores/pen in 82% of pens sampled. This was the first quantitative examination of *C. difficile* spore contamination in the piggery environment.

Throughout the nine-month sampling period there were minimal numbers of scouring piglets in the shed. There was no correlation between spore load in pens containing scouring piglets and the near environment ($\leq$ 3 adjacent pens) compared with all other pens in the farrowing shed. Shed cleaning and disinfection prior to restocking continued

as normal. This led to the assumption that an extraneous source of spores was contributing to contamination.

6.1.4 *C. difficile* spores survived in effluent from farrowing sheds treated in a two-stage pond

This study demonstrated that *C. difficile* resisted pond-based effluent treatment and was likely disseminated into the environment via common re-use practices such as hosing and flushing of farrowing pens. Due to the clonal nature of *C. difficile* molecular types within piggeries, transmission dynamics could not be determined. This was a pilot study on a single farm, but it was the first quantitative examination of *C. difficile* spores in treated piggery effluent.

Only small numbers of spores survived in treated effluent. This was likely an under-representation of true *C. difficile* concentration as sampling logistics necessitated that settled spores from the most anaerobic fraction of stored effluent were not sampled. Vegetative cells were also not included. Constant incursion of small numbers of organisms into the sheds via hosing and flushing, in the absence of sporicidal cleaning, likely increases spore burden in the pens, compounded by amplification reservoirs such as scouring piglets.

A study with sample collection techniques designed to more accurately estimate spore density in treated effluent and effluent-applied soil has been funded by APL and is currently being conducted.

6.1.5 *C. difficile* spore eradication is largely ignored by the Australian pork industry

C. difficile is different to most other piggery pathogens because it produces highly infectious spores that are shed into the environment. Proper environmental cleaning and disinfection can reduce CDI in a veterinary hospital environment (Weese and Armstrong, 2003). No controlled studies have been undertaken to determine the impact of sporicidal cleaning on CDI rates in piggeries.

The Australian pork industry has adopted a best practice approach for the management of manure and effluent from intensive piggery operations; the risk posed by *C. difficile* spores in effluent management has been largely ignored, however. Detailed protocols

for sporicidal cleaning and disinfection of farrowing pens are absent from leading pork industry guidelines. Unsurprisingly, a literature review of disinfectants in common use in farrowing units (Microtech 7000, Farm Fluid S, Virkon S) revealed they are not effective against *C. difficile* spores.

C. difficile spores survive for months whilst maintaining their transmissible nature (Fekety, Kim et al., 1981). *C. difficile* could feasibly be disseminated to human populations by land application of contaminated effluent, contamination of waterways by run-off, particularly during flooding events, or water bodies that receive abattoir treatment plant effluents. There is no evidence regarding the fate of *C. difficile* spores in compost derived from pond sludge, the longitudinal effects of land application of either raw or treated liquid effluent or biosolids, the or the survival of *C. difficile* in effluent treated in storage pits, or in composted piglet carcasses. Airborne dissemination of *C. difficile* is also a possibility. Farm workers in direct contact with contaminated waste may pose a particular risk, especially where high-pressure hosing is employed.

6.2 Aim 2: characteristics of *C. difficile* isolated from Australian neonatal piglets: evidence summary

6.2.1 *C. difficile* ribotypes circulating in Australian piggeries in the sample cohort were unique and genotypically diverse

RT 078 is an A+B+CDT+ strain with similar virulence attributes to the outbreak strain RT027, and the almost universally isolated RT from swine in major pork-producing countries (Debast, van Leengoed et al., 2009a; Keel, Brazier et al., 2007; Norman, Harvey et al., 2009). RT 078 was not isolated from Australian piglets in these prevalence studies; instead, there was a heterogeneous mix of RTs, the majority of which (71% and 61% respectively) had not been previously described in animals or humans either locally or outside Australia.

A single clonal strain predominated at individual piggeries, with few exceptions. These were likely related to physical transfer of spores between farms by staff or veterinarians.

6.2.2 The majority of *C. difficile* strains circulating in Australian piggeries in the sample cohort were toxigenic

The symptoms of CDI result from elaboration of toxin A (tcdA) or toxin B (tcdB) from the vegetative form of *C. difficile* in the gut of animals. Animal strains typically produce an additional binary toxin (CDT) with a possible role in colonisation.

The majority (87%) of strains in the prevalence studies were toxigenic, producing tcdA and/or tcdB. Toxin profiles were more diverse than in overseas studies where RT 078 predominates. Accordingly, the isolation rate of toxigenic strains was less than the >99% reported in 078-dominant piglet studies (Avbersek, Janezic et al., 2009; Hopman, Keessen et al., 2011). When compared with piglet studies with greater RT diversity (Thakur, Putnam et al., 2010), toxigenic strain prevalence was higher in Australia (87% vs. 58%).

The distinct molecular epidemiology of porcine *C. difficile* in Australia was supported by other toxin gene findings. CDT+ strains were less common here than overseas, presumably due to the RT 078 effect. A-B+ strains are only rarely isolated in piglets elsewhere, but were a common feature of our study.

Toxigenic isolates were associated with scouring piglets in the diagnostic study, likely due to a sample bias towards sick piglets. There was no association between *C. difficile* colonisation and scouring in the period prevalence study, consistent with all other piglet studies worldwide. Non-toxigenic strains (A-B-CDT-) were significantly associated with non-scouring farms, suggesting that competitive exclusion of toxigenic strains occurred. This represents a possible prevention modality but is limited by the ability of non-toxigenic strains to acquire the PaLoc from toxigenic strains in vitro.

6.2.3 *C. difficile* strain AI 35, a representative Australian RT 237 strain from neonatal piglets, is genotypically unique, produced a variant toxin, and was more virulent than RT 078 strain in mice

Strain AI 35 was the first published *C. difficile* strain isolated from piglets in Australia. It was isolated from scouring herds and displayed a similar prevalence (77%) and asymptomatic carriage rate (100%) as other local and international studies.

As the first published Australian strain, AI 35 has been well studied phenotypically and genotypically, in this thesis and other investigations (Elliott, Dingle et al., 2014; Elliott, Squire et al., 2011). MLST analysis revealed that AI 35 belonged to clade 5 and sequence type 11, like RT 078 strains from the USA and Europe (Stabler, Dawson et al., 2012).

Genome sequencing of AI 35 showed a novel PaLoc structure, with *tcdA* and *tcdC* deleted, and a novel *tcdE*. Although *tcdB* was intact, AI 35 produced a variant CPE in cell culture, consistent with other *tcdB*-variant *C. difficile* strains 8864 and 1470 that produce *C. sordellii* lethal toxin-like CPE. The AI 35 CdTLoc was complete and contained an intact copy of the CDT expression regulator *cdtR.* This suggested that AI 35 may be a more proficient binary toxin producer, but this was not proven experimentally. The AI 35 strain retained a fragment of the *cdd1* gene whose acquisition has been phylogenetically dated to about 1,300 years ago, making it older than RT 078 clones (Elliott, Dingle et al., 2014).

AI 35 expressed toxin B at low levels; approximately 25-fold less than RT 027 and RT 078 strains, but similar levels to strain 630, a low toxin-producing strain. This did not correlate perfectly with clinical virulence in a mouse model; AI 35 produced more weight loss than an RT 078 strain, suggesting that toxin quantity is not associated with clinical outcome. In vitro, *tcdB*-variant strains had eight-fold greater cytotoxic potency than the high toxin-producing *C. difficile* strain VPI 10463 (A+B+CDT-). Other A-B+CDT+ strains demonstrated increased virulence when compared to A+B+CDT+ strains in animals (Kuehne, Collery et al., 2014) and humans (Kyne, Warny et al., 2001). Clearly, more controlled studies are required to determine the contribution of variant-TcdB and CDT to virulence in this strain.

How this strain emerged in an isolated area of Australia to become a potential pathogen of piglets is not clear. The answer may lie in AI 35's unique genome and the selection pressures that acted on it. This is a subject for further study.

6.3 Aim 3: diagnosis of *C. difficile* in porcine faecal samples: evidence summary

6.3.1 Confirmation that *C. difficile* prevalence in Australian piglets is high and genotypically diverse

Overall prevalence of 39.5% (by EC) in this study was lower due to greater numbers of non-toxigenic strains in the sample cohort than in previous prevalence studies (41.9% vs. 13%); this underscores the importance of broad sampling in prevalence studies. Regardless, prevalence is still similar to other major pork-producing countries.

Ribotype and toxin gene profiles were heterogeneous, consistent with previous prevalence study findings, although the RT distribution was different. The most prevalent RT was QX 006 (16.1%), followed by QX 207 (12.9%), UK 014 (11.3%), QX 057 (11.3%), and UK 020 (8.1%).

6.3.2 Culture on a C. *difficile* chromogenic medium (DC on CA) outperformed molecular- and toxin-based methods for detecting *C. difficile* in piglet faeces

The diverse strain population, broad geographic distribution of sampling sites, and sample transport logistics involved in this study provided a unique scenario for assessing the local performance of assays for detecting CDI in piglets.

Other studies have evaluated different human GDH- and toxin-based detection assays in animals, including piglets (Alvarez-Perez, Alba et al., 2009; Anderson and Songer, 2008; Chouicha and Marks, 2006; Keessen, Hopman et al., 2011; Medina-Torres, Weese et al., 2010; Post, Jost et al., 2002) with generally unsatisfactory results. Molecular-based assays have been assessed in animal faeces (pigs, horses, dogs) but performed poorly compared to detection in human faecal samples (Anderson and Songer, 2008; Chouicha and Marks, 2006; Keessen, Hopman et al., 2011). RT-PCR was 91.6% sensitive in detecting *C. difficile* in piglet faeces, but the study sample consisted of a single RT, RT 078 (Keessen, Hopman et al., 2011).

This study showed that toxin- and molecular-based assays (EIA-tcdA/tcdB, real-time PCR, and LMIA-PCR) performed poorly in detecting toxigenic and non-toxigenic *C. difficile* in porcine faeces. This was true for all RT in the genotypically diverse sample cohort.

Conversely, DC on a specific chromogenic medium (ChromID® *C. difficile* agar (CA), bioMerieux, Marcy l'Etoile, France) performed the best of all the comparator assays across all RT. The overall recovery of *C. difficile* by DC on CA was high (36.3%) and comparable to that by EC (39.5%) (96.8% concordance). It also had a high sensitivity (91.9%) and specificity (100.0%). This method has a number of additional benefits, including simplicity of use, low-cost, rapid turnaround and ability to isolate strains for toxin gene profiling and genotyping.

This study presented the first data worldwide on the performance of a chromogenic medium for recovery of *C. difficile* from animal faecal samples and represents a viable method for the veterinary laboratory.

6.4 Aim 4: association between *C. difficile* and enteric disease: evidence summary

6.4.1 *C. difficile* strain AI 35 isolated from scouring piglets caused more weight loss in mice than RT 078

We conducted experiments in mice to determine if strain AI 35 caused disease in a mouse model and compared its virulence with the known human and animal outbreak strains RT 027 and RT 078. All mice inoculated with RT 078 and AI 35 survived until the end-point but all RT 027 animals died, suggesting enhanced virulence. When AI 35 and RT 078 were compared in the same model, AI 35 caused significantly greater weight loss, suggesting greater disease severity. Although extrapolating the clinical relevance of a mouse model to piglet disease pathology requires caution, this was the first indication that AI 35 (A-B+CDT+) was as virulent as RT 078 (A+B+CDT+), despite its different toxin production profile. This is corroborated by human clinical studies suggesting that A-B+ strains cause more severe disease than A+B+ strains (Kyne, Warny et al., 2001).

6.4.2 Porcine CDI was reproduced in a piglet model of infection by 72 h post-inoculation with spores of genotypically diverse Australian strains isolated from scouring piglets

Spores of toxigenic strains of porcine *C. difficile* were inoculated into newborn piglets in a snatch farrowed model of infection. Piglets were monitored for 72 h for signs of

CDI then humanely euthanised, necropsied and examined for bacteriological, histopathological and grossly visible signs of porcine CDI.

All piglets presented with ill thrift, dehydration and weight loss at 72 h post-inoculation. Scouring was variably present.

Seventeen of 18 test piglets were positive for *C. difficile* by DC on CA at 72 h versus 64% (7/11) controls. The single culture-negative piglet at 72 h was culture positive at 48 h. When all animals positive for *C. difficile* at 72 h were combined, 67% had toxin in their gut contents and 30% had mesocolonic oedema. Toxin and mesocolonic oedema were not identified in any culture negative animal. Disease severity differed between *C. difficile* positive piglets, which is consistent with CDI in piglets (Steele, Feng et al., 2010) and humans (Gebhard, Gerding et al., 1985). Classic microscopic lesions of porcine CDI (caecal and colonic lesions and mesentritis) were observed more often in piglets positive for a toxigenic strain of *C. difficile* than in *C. difficile* negative piglets. CDI lesions were also significantly more severe in culture-positive animals. Microscopic luminal 'volcano' lesions resulting from inflammatory influx of red and white blood cells, fibrin and bacteria (Rupnik, Wilcox et al., 2009) and the hallmark of severe CDI in piglets were identified in five animals, positive for AI 35 and RT 078 strains, in agreement with other challenge studies (Lizer, Madson et al., 2013) and naturally occurring infection (Songer, Post et al., 2000; Yaeger, Funk et al., 2002; Yaeger, Kinyon et al., 2007). Toxin was identified in the gut of 67% (16/24) faecal culture positive animals and no culture-negative animals.

An RT 078 endemic strain infected some piglets. Although RT 078 produced a numerically greater mean CDI lesion score, the mean microscopic lesion score in *C. difficile* positive piglets was not significantly different between toxigenic strains with more than one score/strain (RT 078: 8, AI 35: 3, VP27: 5, $p = 0.344$). This suggested that strain-dependent virulence is similar, but further proof is required because of differences in spore inoculum concentration.

Scouring was not a good indicator of disease; it did not correlate with culture-positive animals. Although this has been previously reported on-farm in natural infection (Yaeger, Kinyon et al., 2007) and in previous infection experiments (Lizer, Madson et al., 2013), it may be a consequence of the feeding regime chosen for this experiment (Oliveira, Galina et al., 2003).

6.5 Recommendations

As a result of the outcomes in this study several recommendations are made.

6.5.1 Surveillance

The key to recognising pathogens and proactive intervention is surveillance, especially in determining the magnitude of the problem and evaluating interventions. There is an increasing body of literature regarding CDI in piglets, but the epidemiology of *C. difficile* in Australian piglets is clearly different to that described elsewhere. Decision-makers in the Australian pork industry have, for the first time, baseline data to assess risk and inform evidence-based action priorities. Our data highlight the need for information regarding strain types circulating in pig populations at the national level and local risk factors.

The pork industry peak bodies have played an historic role in early investigative efforts into this emerging pathogen, and should continue this role by coordinating ongoing surveillance and typing. The epidemiology of human *C. difficile* has changed dramatically over the last decade, primarily because of new, more virulent outbreak strains resulting from mutations and gene transfer. This is also a very real possibility in the pork production environment. Surveillance data are necessary for understanding risk factors contributing to *C. difficile* establishment and expansion within piggeries, and also for determining public health risks, if any.

Recommended surveillance activities:

- continued typing and epidemiological studies Australia-wide to provide impact data
- a central typing repository to monitor, actively analyse and disseminate epidemiological data. Molecular typing of strains can confirm a shift in the epidemiology of CDI, including outbreak strains with increased virulence
- reporting should include antimicrobial or other treatments.

6.5.2 Diagnosis

Accurate diagnosis is crucial to the acceptance of *C. difficile* as an enteric pathogen in Australian piglets and overall management of CDI. This study demonstrated that

Australian strains cause CDI in neonatal piglets, with bacteriological, clinical and histopathological signs evident by 48–72 hours. The poor performance of current commercially available non-culture based assays for detection of *C. difficile* in porcine faeces was also highlighted.

A reliable, cost-effective, and specific *C. difficile* detection method for use in the veterinary laboratory (CA) was identified in this study. CA, with its high sensitivity and NPV, could be used by veterinarians as a primary test to identify those piglets or herds with presumptive CDI, followed by PCR characterisation of the toxin genes.

Recommended diagnostic activities:

- CDI should be considered in any herd with scour in neonates ≤7 days of age.
- *C. difficile* testing should be added to the routine enteropathogen testing panel for scouring herds/piglets using the CA method described in this study.
- Culture of *C. difficile* is not sufficient evidence to support diagnosis of CDI. A positive diagnosis for CDI includes a history of herd scouring, clinical presentation, positive faecal culture, detection of toxin genes, and characteristic lesions on necropsy.
- Piglets selected for faecal culture or necropsy should have been acutely affected for at least 48–72 hours.
- Education of veterinarians, animal health groups and producers about CDI in the Australian context should include detection, diagnosis, prevention and dissemination of prevalence summary data.

6.5.3 Prevention and control

Complete eradication of *C. difficile* in the farrowing unit environment is not feasible given the persistent nature of the organism and the rapidity with which spore contamination develops in the farrowing unit. A more realistic aim would be to reduce intestinal colonisation of *C. difficile*, and subsequent faecal shedding. Measures should be taken to limit the persistence of *C. difficile* in the farm environment.

Recommended prevention and control activities:

- development of a *C. difficile* vaccine to reduce intestinal colonisation and faecal dissemination
- infection-control training for stock hands, especially correct hand washing and barrier precautions (such as disposable coveralls) when in contact with scouring animals with diagnosed CDI. Alcohol-based hand hygiene products do not kill *C difficile* spores
- funded PhD scholarships to investigate implications of environmental contamination including carcass management, sludge disposal and re-use, pond management in relation to waterways and run-off, airborne spores, abattoir effluent management, vector contribution to disease transmission, and quantitative research into environmental spore control in the farrowing unit. This would include whole genome single-nucleotide polymorphism (SNP) analysis to study the relatedness of isolated from humans and pigs. This will facilitate action priorities that are evidence-based and appropriate to the level of risk posed by a dissemination of environmental spores
- good management practices such as sporicidal cleaning of farrowing sheds, reconsidering the use of treated effluent for hose-downs, especially via high pressure apparatus, and identifying and removing environmental sources of *C. difficile* if outbreaks continue
- continued participation in national antimicrobial usage and antimicrobial stewardship initiatives
- Identification of on-farm practices at risk from the unique characteristics of spore-forming organisms such as *C. difficile*, specifically incorporating *C. difficile* risk management into pig husbandry and management policies and guidelines such as APIQ. This should include performance measures to ensure guidelines are translated into practice and measurement of the effectiveness of any changes.

6.5.4 Additional future research priorities

Many factors relating to CDI in piglets remain unknown, and there is scope for future research. CDI establishment and persistence in a herd is likely complex and multifactorial, involving bacterial genetics, hygiene, and husbandry practices.

Recommended future research:

- Determine transmission dynamics within piggeries using new modalities such as WGS, including laboratory transmission studies to determine infectious dose in piglets with focus on selection pressures that promote persistence of individual strains within piggeries.
- Determine why disease does not occur in every herd, and not in all piglets within the herd, especially factors related to asymptomatic carriage with focus on husbandry practices that contribute to CDI development, allowing management to assess and eliminate high-risk practice.
- Undertake research into non-antibiotic treatment modalities such as bacteriophage therapy and probiotics.
- Confirmation of porcine strain-specific virulence factors using WGS, phenotypic and infection studies.

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Appendix I: Farm questionnaire

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UWA (APL 00462) - The prevalence of *Clostridium difficile* in Australian piggeries

QUESTIONNAIRE
This document is to be completed by the attending veterinarian on day of collection and sent back to UWA with the collected samples.

Date of collection:

Name of collector:

Farm name and address:

Farm contact person/manager:

Farm type:

Number of sows:

Previous history of unexplained scouring in piglets <7 days of age? (Please circle)	Yes/no
Current mortality rate:	%
Onsite effluent treatment? (Please circle)	Yes/no
Treated effluent re-used within piggery? (Please circle)	Yes/no
Other animals (e.g. equine, bovine etc) kept in close proximity to the farrowing sheds? (Please give details below)	Yes/no

Antimicrobial use (general classes only – particularly cephalosporins, please give details below)