

Evaluation of diagnostic tests to detect *Clostridium difficile* in piglets 2A-104

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By

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Executive Summary

Clostridium difficile is a Gram-positive spore forming bacterium and a well-known enteric pathogen of humans. Outside Australia *C. difficile* is reported as a major cause of high-morbidity enteritis and pre-weaning scour in neonatal piglets aged 1-7 days, with outbreak-associated mortality up to 50%. Piglets that recover from *C. difficile* infection (CDI) can be 10-15% underweight and take longer to wean. Investigations in our laboratory have confirmed that toxigenic *C. difficile* and idiopathic scour is present in many pig herds in Australia. *C. difficile* prevalence in Australian piggeries is as high as 70% and could potentially present a significant economic issue to the pork industry. To understand the role of *C. difficile* in pig disease it is essential to be able to detect the organism in a timely and cost-effective manner. Unfortunately veterinary laboratories in Australia have limited experience with this fastidious organism. Furthermore, few of the commercially available methods for detection of *C. difficile* in humans have been validated for animals, and none with the molecular types of *C. difficile* found in Australian piglets.

The project aim was to provide guidance to the pork industry, veterinarians and veterinary diagnostic laboratories about the suitability of currently available commercial assays to detect *C. difficile* in Australian piglets. To achieve this we evaluated the performance of four commercial assays to detect *C. difficile* in 157 specimens of piglet faeces obtained from neonatal piglets (49 scouring) aged <14 days during the period June 2012 to March 2013. The test population originated from 16 farms across five Australian states. Assays were performed according to manufacturers' instructions and compared against toxigenic culture (TC) as a "gold standard". Assays included two commercially available PCR methods for the detection of toxin A and B genes; illumigene® *C. difficile* amplification assay (IG, Meridian Bioscience) and BD GeneOhm™ Cdiff Assay (GO, BD Diagnostics), an enzyme immunoassay for toxins A and B (QC, TechLab *C. diff* Quik Chek™ (Alere) and culture on a chromogenic agar; *C. difficile* ChromID™ agar (CA, BioMérieux). Isolates were characterized by PCR ribotyping and PCR detection of toxin genes *tcdA* (toxin A), *tcdB* (toxin B) and *cdt* (binary toxin) which correlates with toxin production.

Overall, *C. difficile* was isolated by TC from 39.5% (n=62) of samples. PCR revealed 58.1% (n=36) of isolates were positive for at least one toxin gene (*tcdA/tcdB*). Five isolates (8.1%) had the uncommon genotype of *tcdA*⁻/*tcdB*⁻/*CDT*⁺ and the remainder (n=21, 33.9%) were negative for any toxin genes. Of the 62 isolates obtained from neonatal piglets, 19 different ribotypes (RTs) were detected, 8 of which were internationally recognised types. This heterogeneity in RT is consistent with previous studies in Australian pigs and is likely a result of the absence of RT078 which predominates in pig populations outside of Australia.

There was varied performance in detection of *C. difficile* by the assays tested. After correcting for the high number of non-toxigenic strains in the test population, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were, compared to TC, as follows: for CA; 100.0, 96.0, 88.9 and 100.0%; QC; 38.9, 92.6, 66.7 and 80.0%; GO; 42.9, 97.9, 88.2 and 82.3% and IG; 25.0, 95.8, 69.2 and 77.1%.

This study presents the first reported data worldwide on the performance of a chromogenic medium for recovery of *C. difficile* from animal faecal samples. CA performed the best of all the comparator assays with high sensitivity and specificity in recovery of *C. difficile* from piglet faeces irrespective of strain type. Culture on CA is highly selective, fast and does not require extensive training or expensive equipment. It is also suitable for culture of samples sent over long distances under suboptimal conditions. CA also allows for recovery of the organism for epidemiological typing. Thus, culture on CA presents a suitable detection and identification method for veterinary laboratories.

The performance of the molecular based assays (QC, GO and IG) in the detection of *C. difficile* in porcine faeces was unacceptably poor. Concordance with TC was low, due to a

large number of false negative results, which could be attributable to a number of host and/or microbial factors including strain type, faecal composition and sample deterioration. All of the above highlight the need for validation of existing assays and development of new assays for detection of *C. difficile* in piglets, particularly in light of the unique strain population present in Australian pigs. The results presented in this study underscore the importance of developing porcine-specific assays with high sensitivities, PPVs and NPVs for the rapid reliable detection of *C. difficile* and its toxins in porcine faeces.

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1. Introduction

Scope & benefit to industry

Clostridium difficile is reported outside Australia as a major cause of pre-weaning scour in neonatal piglets. Outbreak-associated mortality can reach 50% (1) but is generally much lower due to good stockmanship. Morbidity is high however. Piglets that recover from *C. difficile* infection (CDI) can be 10-15% underweight and take longer to wean (1). From an Australian standpoint this is significant as we compete in a global pig meat market and our existing slaughter weights are lower and weaning times longer when compared to international standards. Investigations in our laboratory have confirmed that toxigenic *C. difficile* is present in many pig herds in Australia. Unfortunately veterinary laboratories in Australia have limited experience with this organism. Furthermore, few of the commercially available methods for detection of *C. difficile* in humans have been validated for animals, and none with the molecular types of *C. difficile* found in Australian piglets. To understand the role of *C. difficile* in pig disease in Australia it is essential that veterinary diagnostic laboratories are able to detect the organism in a timely and cost-effective manner. This project will provide guidance to the pork industry, veterinarians and veterinary diagnostic laboratories about how to achieve this.

Human *C. difficile* disease and epidemiology

C. difficile is a ubiquitous, Gram positive spore-forming anaerobic bacillus that is widely recognised as the leading cause of infectious diarrhoea in human hospitalised patients in developed countries (2). It is spread oro-faecally through ingestion of spores which germinate in the gut, producing two major toxins, toxin A (an enterotoxin, TcdA) and toxin B (a cytotoxin, TcdB) encoded by the genes *tcdA* and *tcdB*, respectively, located within a 19.6kb pathogenicity locus (PaLoc) on the chromosome. In addition to TcdA and TcdB, some strains of *C. difficile* can produce a third unrelated toxin (an actin-specific ADP-ribosyltransferase known as binary toxin, CDT). Binary toxin producing strains of *C. difficile* are associated with increased severity of disease in humans (3) and are often found in animals (4).

C. difficile infection occurs opportunistically when the niche usually occupied by normal intestinal flora (microbiota) is disrupted, making it often associated with antibiotic exposure. Human disease is generally characterised by non-haemorrhagic watery diarrhoea, accompanied by fever and abdominal pain, commencing 48-72 h post infection, although a wide spectrum of disease presentation is possible from mild diarrhoea to life-threatening complications such as pseudomembranous colitis and toxic megacolon (5).

Due to its significant association with exposure to antimicrobial agents, CDI has long been considered a purely hospital-acquired (nosocomial) or healthcare-related infection. Although significantly lower in prevalence than healthcare-related CDI, community-associated CDI (CA-CDI) has been increasing globally, accounting for up to one-quarter of all diagnosed CDI cases (6). CA-CDI is significantly associated with younger otherwise healthy people often without prior exposure to antimicrobials (7, 8). Spillover of hospital strains does not fully explain CA-CDI as predominantly hospital strains such as epidemic PCR ribotype (RT) 027 have not been found in large numbers in the community setting. Community strains are also more heterogeneous, consisting of many previously unidentified RTs (9). This suggests that other reservoirs of infection contribute to CA-CDI. One possible explanation is exposure to animal sources of *C. difficile*.

C. difficile in animals

Animals may play a role in the epidemiology of human CA-CDI by acting as a reservoir for amplification of *C. difficile*. Transmission could occur by direct exposure to animals or their effluent as *C. difficile* spores survive in treated piggery effluent (10). Additionally, *C. difficile* spores have been recovered from a wide range of retail foodstuffs (11-13);

consequently contaminated meat products could be a potential source of CDI in humans. *C. difficile* causes intestinal disease in a variety of animals, including companion animals (cats, dogs, horses) and food animals (cattle, pigs). Disease pathophysiology and clinical presentation in animals are identical to humans, except that the site of intestinal lesions varies according to species and age, although it generally involves the distal gastrointestinal tract (5). Predisposing antimicrobial use is the most important risk factor for CDI development in adult and hospitalized animals, suggesting that non-judicious antibiotic use may be driving *C. difficile* colonisation in animals as it has in humans (14).

In the USA and Europe, RT078 is the predominant strain of *C. difficile* in animals, particularly cattle and pigs (15). RT078 is increasingly associated with CA-CDI (7, 16), is currently the 3rd most common strain of *C. difficile* isolated from humans in Europe (17) and is being increasingly isolated from humans in the USA (18). In the Netherlands, where infections with RT078 increased more than four-fold from 2005 to 2008, patients infected with this RT were younger and had CA-CDI more frequently, particularly if they lived in rural pig producing areas (19). RT078 strains from Dutch humans and pigs were subtyped by multi-locus variable number tandem repeat analysis (MLVA) and were indistinguishable (20). In the USA, RT078 prevalence increased from 0.02% to 1.3% from pre-2001 to 2006 and was increasingly associated with CA-CDI cases. Comparative analysis to animal RT078 strains by PFGE demonstrated that these strains were indistinguishable or very closely related (21). This suggestion of a potential zoonosis has recently been given further weight in another study from The Netherlands (22). In this study, *C. difficile* RT078 isolates from pigs and pig farmers were analysed by whole genome SNP (single nucleotide polymorphism) typing. There were zero SNP differences between isolates from pigs and their farmers, which means transmission between animals and humans did indeed occur, although the direction of transmission remains unclear.

***C. difficile* in pigs**

In the USA and Europe, *C. difficile* has been recognized as an enteric pathogen in porcine neonates for more than a decade (23) and it is now the most commonly diagnosed cause of enteritis in pigs in this age group in the USA (24). Outside Australia the predominant RT in pigs is RT078 that accounts for up to 100% of isolates (25). In Australia there are different ribotypes (RTs) circulating among livestock (sheep, cattle, pigs), including RT033, RT126, RT127 and RT237 (26, 27). Like human neonates, piglets are gnotobiotic at birth and do not develop a normal microflora until around 5 days of age. This lack of colonisation resistance in the early days of life means piglets are particularly susceptible to *C. difficile* colonisation, and the potential for colonisation will increase with exposure to antimicrobials during piglet processing. *C. difficile* colonisation frequency in piglets decreases with age from 74% at 2 days of age to 3.7% at 62 days of age (28). This suggests that all piglets in an affected farrowing facility may be colonised soon after birth, presumably via ingestion of environmental spores, and colonisation spreads throughout the herd (29). Although colonisation rates are high not all piglets will manifest clinical infection (30). The mechanism by which toxin-positive piglets remain asymptomatic, despite having toxin A receptors, is not yet understood but is likely to relate to maternal immune response. It is possible that subclinical disease might occur (31). The infectious cycle is maintained by contamination of the environment with spores which are shed in the faeces of both symptomatic and asymptomatic piglets, or continually reintroduced by cleaning with treated effluent. *C. difficile* spores are resistant to most disinfectants commonly used in Australian piggeries.

Porcine CDI is generally characterized by profuse non-haemorrhagic yellow pasty-to-watery scour. Extra-intestinal symptoms such as anorexia, dehydration, ascites/hydrothorax, scrotal oedema and dyspnoea have also been described and may be attributable to systemic sepsis or circulating toxin (32, 33). Symptoms are limited to neonatal piglets and generally commence soon after birth (23, 34). Lesions of porcine CDI are well-described and include inflammation and oedema of the colon (colitis) and caecum (typhlitis) and

mesocolonic oedema. Infiltration of neutrophils into colonic and caecal lamina propria, and mucosal ulcerations, are found microscopically (33, 35, 36).

Porcine CDI presents a significant economic issue to the pork industry, which operates with very tight margins. Disease-associated mortality in neonatal piglets can reach 50%, and those that survive remain, on average, 10%-15% underweight and take additional time to wean (1). In addition, oral rehydration and antimicrobial treatment is administered by oral drenching to each piglet, making it time-consuming and expensive. Microbiological diagnosis is problematic; culture of the organism alone from affected animals is not diagnostic since it may be carried asymptotically in high numbers. A final diagnosis of CDI requires a positive bacteriologic culture and toxin detection from intestinal contents as well as necropsy with gross morphological and histological examination of the caecum and colon to identify the characteristic lesions of CDI.

The development of effective vaccines and vaccination strategies to control *E. coli* scour in neonatal pigs has provided enormous benefits to the pig industry. Nevertheless mortality from pre-weaning scour is still a problem in many Australian piggeries. These cases are not associated with the usual serotypes of enterotoxigenic *E. coli*, and do not improve following vaccination. Clinical presentation differs from that of other enteric pathogens in this age group such as *C. perfringens* and rotavirus and investigations for these are usually negative. A diagnosis of CDI cannot be confirmed in these cases due to the lack of diagnostic capacity in Australia. Our observation, based on considerable discussions with specialist pig veterinarians, animal health groups and producers, is that many are not aware of *C. difficile* as a pathogen in this age group, nor is there expertise to culture this fastidious anaerobe or detect its toxins.

***C. difficile* detection in the laboratory**

In humans, detection of toxigenic *C. difficile* in faeces or intestinal contents is the gold standard for diagnosis of CDI in the laboratory. Culture provides a high level of sensitivity and offers the added advantage of recovering isolates for further characterisation and epidemiological analysis. However, there is a long turnaround time which is not ideal in outbreak situations. Enzyme immunoassay (EIA) kits targeting *C. difficile* toxins or “common” glutamate dehydrogenase antigen (GDH) have been available for many years and are used widely in both human and veterinary settings (38). These assays are relatively easy to use and provide rapid results; however they are not without significant limitations (38); the clinical sensitivity of these assays may be suboptimal, GDH can be found in both toxigenic and non-toxigenic strains and there may be cross-reactivity with *C. sordellii*. Consequently, a two-step algorithm is recommended comprising an initial GDH based EIA (as a screening test for CDI), with a confirmatory test such as a toxin EIA or a PCR based test for detection of toxin genes performed either concurrently or subsequently on all positive assays (38-41). In the last decade, the use of commercially available PCR assays, targeting the toxin-encoding genes *tcdA* and/or *tcdB* have, with high sensitivity and a rapid turnaround time, dramatically improved detection of CDI (38). There are however still limitations with this approach as some strain-types (A B⁺) may not be identified by assays targeting only *tcdA* and *in vitro* detection of toxin genes may not always reflect clinical manifestation of disease (41).

Currently, no guidelines are available for diagnosing CDI or detecting *C. difficile* in animals, and literature on this topic is scarce. Although commercially available detection methods for *C. difficile* have been comprehensively evaluated for use in human infections, their performance with animal samples has not been validated. A number of studies, both in Europe and North America, have reported varied performance for assays in detecting *C. difficile* in animal faeces, including RT078 strains which are not found in Australian animals (Table 1).

Table 1 - Summary of studies which evaluated the performance of commercial assays for detection of *C. difficile* in animal faeces

Species	N	Type	Assay	Sensitivity	Specificity	PPV	NPV	Ref
Canine	143	EIA	Techlab toxA/B	33.0	65.0	-	-	(42)
	143	EIA	Premier toxA/B	27.0	95.0	-	-	(42)
	143	EIA	Premier toxA	29.0	90.0	-	-	(42)
	143	EIA	BD toxA	7.0	100.0	-	-	(42)
	143	EIA	Triage ToxA	60.0	98.0	-	-	(42)
Porcine	50	EIA	Unknown	91.0	86.0	84.0	86.0	(43)
	172	EIA	Premier Toxins A&B	80.3	27.7	43.8	66.7	(44)
	172	EIA	ICTAB	80.0	46.2	52.8	75.4	(44)
	172	EIA	VIDAS <i>C. difficile</i> A&B	56.4	89.8	77.5	76.7	(44)
	115	EIA	Gastro-tect <i>C.diff</i> A&B	39.0	100.0	87.0	100.0	(45)
	115	EIA	<i>C. difficile</i> Tox A/B II	91.0	86.0	86.0	84.0	(45)
	172	PCR	GeneOhm (TcdB)	93.0	34.7	50.0	87.5	(44)
	32	PCR	In house (tpi, TcdA/B)	100.0	100.0	-	-	(46)
Equine	72	EIA	Techlab <i>C. diff</i> A/B II	84.0	96.0	-	-	(47)

2. Methodology

The objective of this study was to evaluate the performance of four commercially available tests to detect *C. difficile* in neonatal porcine faecal samples. The performance of the assays was compared with toxigenic culture (TC), a commonly used “gold standard” (48) for diagnosis of disease in humans.

The assays evaluated included two commercially available PCR assays for the detection of toxin A or B genes; illumigene® *C. difficile* amplification assay (IG, Meridian Bioscience) and GeneOhm™ Cdiff Assay (GO, BD Diagnostics), an enzyme immunoassay for toxins A and B (QC, TechLab *C. diff* Quik Chek™ (Alere) and culture on chromagar; *C. difficile* ChromID™ agar (CA, BioMérieux).

Assays were evaluated using recognised parameters for measuring the performance of a diagnostic assay. The sensitivity and specificity of an assay measures, respectively, the proportion of actual positives and negatives which are correctly identified as such. The positive predictive value (PPV) and negative predictive value (NPV) measure, respectively, the proportion of test results that are true positives and negatives.

Sample collection

A total of 157 faecal samples (swabs) were obtained from neonatal piglets (49 scouring) aged <14 days during the period of June 2012 to March 2013. The test population originated from 16 farms across five Australian states: New South Wales (NSW, n=2), Queensland (QLD, n=6), Victoria (VIC, n=4), South Australia (SA, n=1) and Western Australia (WA, n=3). All samples were transported under ambient conditions to The University of

Western Australia. The average transport time from farm to laboratory was 8 days. Upon receipt, all samples were stored at 4°C and prepared for analysis within 24 h.

Sample preparation

Upon receipt of the samples in the laboratory, sample slurries were prepared by suspension of the faecal swab in 800µL of phosphate buffered saline (PBS). The samples were vortexed briefly to create a homogenous suspension and split into 200µL aliquots. One part each was used for the two PCR based assays and stored at -20°C until use, after which point a single freeze thaw cycle was implemented as per the assay recommendations. One aliquot each was immediately used for toxigenic culture and direct culture by ChromID and finally one aliquot was stored at 2 to 8°C for use with the enzyme immunoassay and processed within 48 h.

Toxigenic culture

The method to isolate *C. difficile* was based on previously described methods (49). Faeces were cultured in an enrichment broth containing gentamicin, cycloserine and cefoxitin (GCC) (48, 50). After 48 h incubation, all enrichment broths were alcohol shocked, to enhance spore selection and sub-cultured onto selective agar plates [cycloserine cefoxitin fructose agar (CCFA)] containing sodium cholate. Plates were incubated in an anaerobic chamber (Don Whitley Scientific Ltd., Shipley, West Yorkshire, UK) at 37°C, in an atmosphere containing 80% nitrogen, 10% hydrogen and 10% carbon dioxide. Putative *C. difficile* colonies on CCFA were subcultured onto blood agar and identified on the basis of their characteristic colony morphology (yellow, ground glass appearance), odour (horse dung smell) and their characteristic chartreuse fluorescence under long-wave UV light (~360nm). The identity of uncertain isolates was confirmed by presence of the L-proline aminopeptidase activity (Remel Inc., Lenexa, KS, USA) and Gram stain.

C. difficile ChromID™ agar

C. difficile CA (BioMérieux, Marcy l'Etoile, France) is a relatively new chromogenic medium, containing taurocholate (a germinant) and a proprietary chromogen mix that allows rapid and reliable isolation and presumptive identification of *C. difficile* strains in 24 h (51). On CA, *C. difficile* appears as black colonies on a clear background. An example of *C. difficile* growth on CA is shown in Figure A1; appendix I. Direct culture was performed according to the manufacturers' recommendations. Plates were incubated in an anaerobic chamber at 37°C, in an atmosphere containing 80% nitrogen, 10% hydrogen and 10% carbon dioxide. Putative *C. difficile* colonies on ChromID were subcultured onto blood agar and processed as described above.

C. diff Quik Chek™

The *C. diff* Quik Chek™ Complete test (Alere North America, Inc. Orlando, FL) is a lateral flow membrane-type enzyme-linked immunosorbant assay (ELISA). The test uses antibodies specific for glutamate dehydrogenase (GDH), and toxins A and B of *C. difficile*. Whilst QC shows an improvement in performance over earlier toxin-only platforms (with GDH sensitivity similar to stand-alone GDH assays); the sensitivity of the toxin EIA component appears to suffer from the same sensitivity issues as conventional toxin EIAs (52). All assays were carried out according to the manufacturers' recommendations. Results were recorded as either positive or negative for GDH and/or toxins A/B.

illumigene® *C. difficile* assay

The illumigene® *C. difficile* amplification assay (IL) (Meridian Bioscience, Inc. Cincinnati, OH) is a nucleic acid amplification technique (NAAT) based upon the principle of loop-mediated isothermal amplification (LAMP). This assay detects toxigenic *C. difficile* by targeting a conserved 5' 204 bp sequence of *tcdA* (53). Assays were performed on the illumipro-10™ according to the manufacturers' instructions. The software of the illumipro-10™ recorded the results of the PCR assay as positive or negative.

GeneOhm™ Cdiff Assay

First FDA approved in 2009, the GeneOhm™ Cdiff Assay (Becton Dickinson, La Jolla, CA) (GO) is a NAAT using real time PCR (RT-PCR) technology to amplify conserved regions of *tcdB* and detects the amplified products by using fluorogenic target-specific hybridization probes (molecular beacons) (54). Assays were performed according to the manufacturers' instructions on a SmartCycler (Cepheid, United Kingdom; supplied by Becton Dickinson at the time of the study). The software of the SmartCycler recorded the results of the PCR assay as positive, negative, or unresolved.

Toxin profiling and PCR ribotyping of *C. difficile* isolates

All isolates were screened by PCR for the presence of toxin A and B genes, binary toxin genes (both *cdtA* and *cdtB*) and the repetitive region of the toxin A gene using previously described methods (55-57). Verification of true toxin A/B negative (A⁻B⁻) isolates was achieved by amplification of the Pathogenicity Locus integration region as previously described (58).

PCR ribotyping was performed as previously described (59). PCR ribotyping reaction products were concentrated using the Qiagen MinElute PCR Purification kit (Ambion Inc, Austin, Texas) and run on the QIAxcel capillary electrophoresis platform (Ambion Inc, Austin, Texas). Analysis of PCR ribotyping products was performed using the Dice coefficient within BioNumerics™ software package v.6.5 (Applied Maths, Saint-Martens-Latem, Belgium). RTs were identified by comparison of banding patterns with our reference library, consisting of a collection of the most prevalent RTs currently circulating in humans and animals in Australia (unpublished data) and a collection of 15 reference strains from the European Centre for Disease Prevention and Control (ECDC). Isolates that could not be identified with the reference library were designated with internal nomenclature.

Statistical analysis

The sensitivity and specificity were calculated for each kit against the gold standard assay (toxigenic culture). The sensitivity and specificity data were used to calculate the PPV and NPV. Fisher's exact test was used where appropriate to compare the recovery of *C. difficile* in the test systems with the recovery of *C. difficile* by toxigenic culture.

3. Outcomes

Detection of *C. difficile*

Toxigenic culture

C. difficile was isolated from 39.5% (n=62/157) of samples (range 26.0%-54.5%) (Figure 1). The recovery of *C. difficile* from piglets with (36.7%) and without (40.7%) diarrhoea was not significantly different (p=0.141).

ChromID™ agar

C. difficile was detected in 36.3% (n=57/157) of samples. Compared with toxigenic culture, the recovery of *C. difficile* was not significantly less (p= 0.56). Concordance with TC was 96.8%. There were no false positive results.

C. diff Quik Chek™ assay

C. difficile was detected in 21.0% (n=33/157) of samples. Compared with toxigenic culture, the detection of *C. difficile* was significantly less (p= <0.001). Concordance with TC was 73.9%. There were seven false positive results (21.2%).

GeneOhm™ Cdiff assay

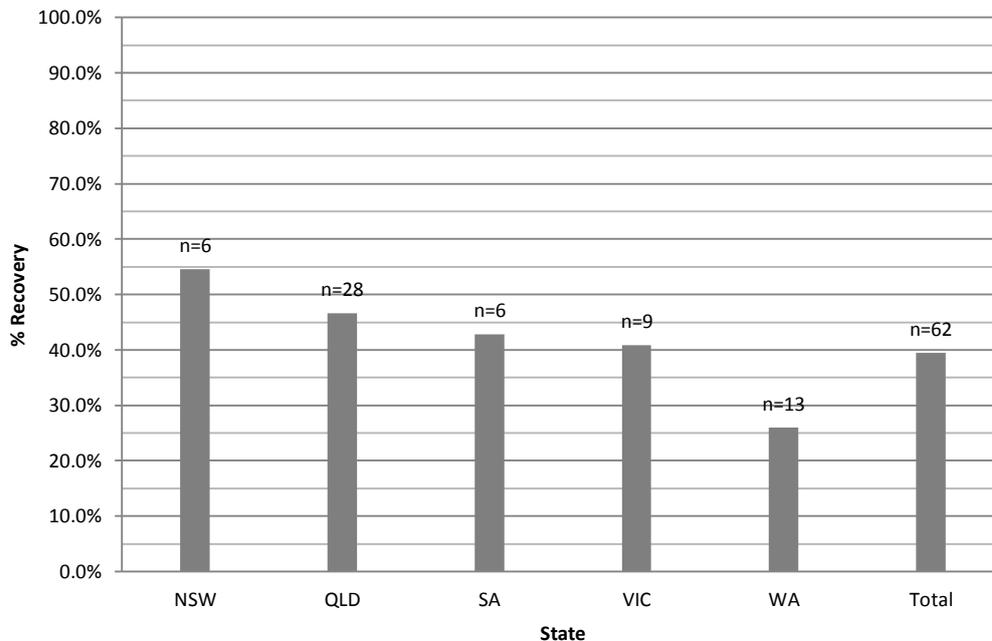
C. difficile was detected in 12.1% (n=19/156*) of samples (*there was a single unresolved result). Compared with toxigenic culture, the detection of *C. difficile* was significantly less

($p = <0.001$). Concordance with TC was 70.5%. There were two false positive results (10.5%).

illumigene® *C. difficile* assay

C. difficile was detected in 8.9% ($n=14/157$) of samples. Compared with toxigenic culture, the detection of *C. difficile* was significantly less ($P = <0.001$). Concordance with EC was 64.3%. There were four false positive results (28.6%).

Fig. 1 - Recovery of *C. difficile* by toxigenic culture, by State.



Toxin profiles

PCR revealed 58.1% ($n=36$) of isolates were positive for at least one toxin gene (*tcdA/tcdB*). Five isolates (8.1%) had the uncommon genotype of $A^-B^-CDT^+$ and the remainder ($n=21$, 33.9%) were negative for any toxin genes (Table 2).

Table 2 - Summary of *C. difficile* toxin profiles.

Toxin A gene (<i>tcdA</i>)	Toxin B gene (<i>tcdB</i>)	Binary toxin genes (<i>cdtA/B</i>)	Toxin Profile	N	%
+	+	-	$A^+B^+CDT^-$	33	53.2
-	-	-	$A^-B^-CDT^-$	21	33.9
-	-	+	$A^-B^-CDT^+$	5	8.1
+	+	+	$A^+B^+CDT^+$	2	3.2
-	+	-	$A^-B^+CDT^-$	1	1.6
Total				62	

Correction for a biased strain-type population

The prevalence of non-toxigenic (A^-B^-) strains of *C. difficile* in this study was high (42%). This observation raises the possibility of a population bias favoring strain-types that do not have the toxin gene targets which the non-culture methods (QC, GO and IL) are designed

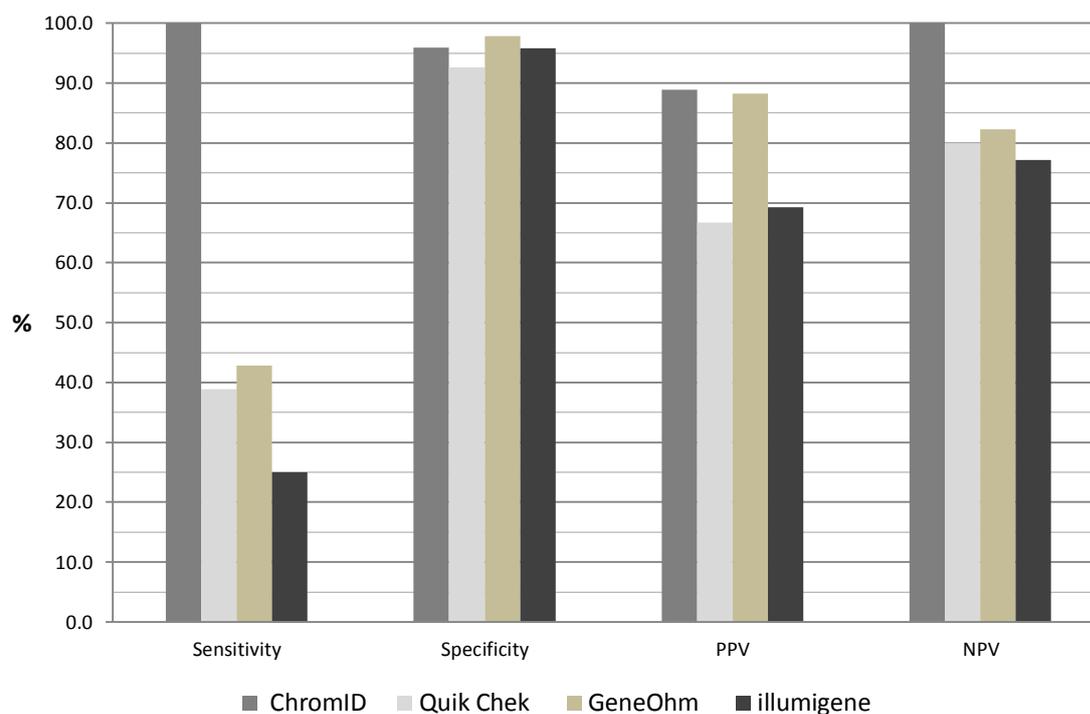
to detect. To fairly evaluate these three assays, the sensitivity, specificity, NPV and PPV for all assays was recalculated to exclude A⁻B⁻CDT⁻ (n=21) and A⁻B⁻CDT⁺ (n=5) strain-types (Figure 2). This would reduce the number of false negative results attributable to toxin-negative strains to zero. The original and corrected values for sensitivity, specificity, PPV and NPV for all assays are shown in Table 3 below. Table 4 shows the performance of each assay with different strain-types.

Table 3 - Performance of commercial assays for detection of *C. difficile* in animal faeces

Assay	% ^{a/B}			
	Sensitivity	Specificity	PPV	NPV
CA	100.0/100.0	95.0/96.0	91.9/88.9	100.0/100.0
QC	41.9/38.9	92.6/92.6	78.8/66.7	71.0/80.0
GO	27.9/42.9	97.9/97.9	89.5/88.2	67.9/82.3
IG	16.1/25.0	95.8/95.8	71.4/69.2	63.6/77.1

a = uncorrected, B = corrected

Fig. 2 - Sensitivity[‡], specificity[‡], PPV[‡] and NPV[‡] for four commercially available assays for the detection of *C. difficile* in porcine faeces, compared to toxigenic culture.



[‡] corrected values

Table 4 - Summary of *C. difficile* toxin profiles.

Toxin Profile	N Isolates (N RTs)	TP n(%)			
		ChromID	Quik Chek EIA	GeneOhm	illumigene
A-B-CDT ⁺	5 (3)	5 (100.0)	2 (100.0)	0 (0.00)	0 (0.00)
A-B-CDT ⁻	21 (4)	20 (95.2)	9 (42.9)	2 (9.5)	1 (4.8)
A-B+CDT ⁻	1 (1)	0 (0.00)	1 (100.0)	0 (0.00)	0 (0.00)
A+B+CDT ⁺	2 (1)	1 (50.0)	0 (0.00)	0 (0.00)	0 (0.00)

Toxin Profile	N Isolates (N RTs)	TP n(%)			
		ChromID	Quik Chek EIA	GeneOhm	illumigene
A+B+CDT-	33 (10)	31 (93.9)	13 (39.4)	15 (45.4)	9 (27.3)
Total	62 (19)	57	26	17	10

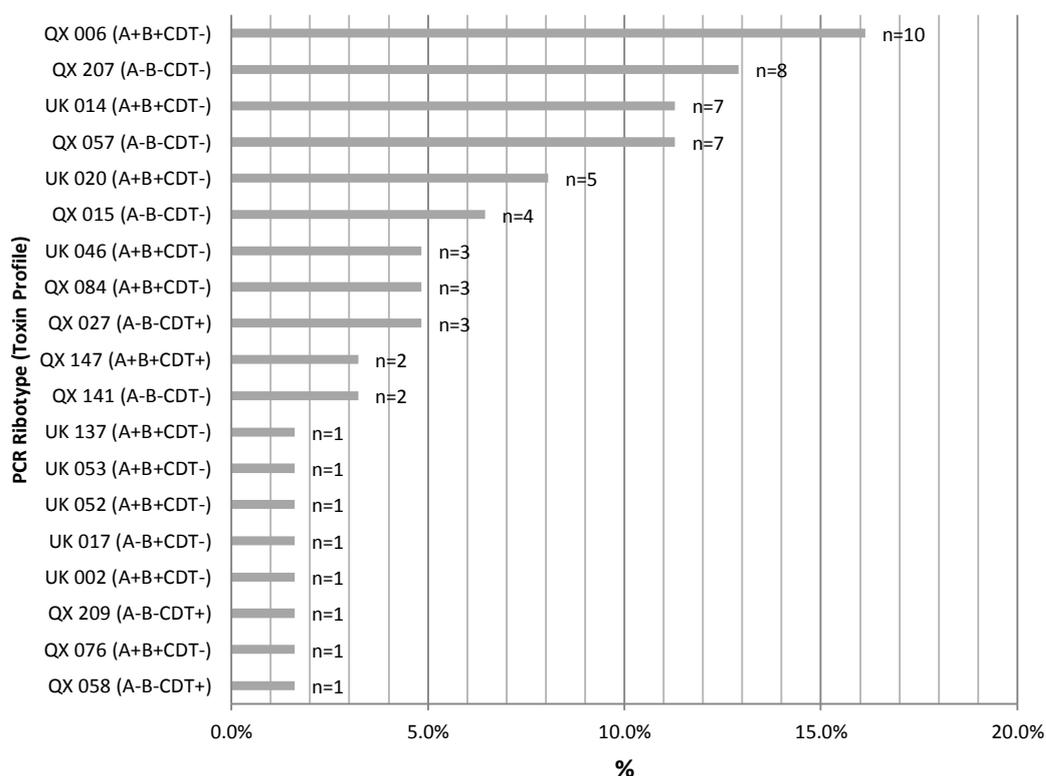
TP - number of true positives

PCR ribotyping

Multiple RTs were identified (Figure 3). Of the 62 isolates obtained from neonatal piglets, 32.3% (n=20) were assigned one of 8 internationally recognised RTs. No RT027 or RT078 was identified. The remaining isolates were assigned the prefix QX and given an internal number. QX006 (A⁺B⁺CDT⁻) was the most common RT found overall representing 16.1% (10/62) of isolates. After QX006, the next four most prevalent RTs were; QX207 (12.9%), QX057 (11.3%), UK014 (11.3%) and QX020 (8.1%).

There was varied performance in detection of *C. difficile* with different toxin profiles /PCR ribotypes (Table 3). ChromID performed well across the range of toxigenic and wildtype RTs. The PCR based assays performed poorly across all toxin profiles and RTs.

Fig. 3 - Summary of PCR ribotypes and toxin profiles from recovered *C. difficile* isolates.



4. Application of Research

This study presents the first evaluation of several commercially available diagnostic assays for detection of range of *C. difficile* genotypes from Australian neonatal piglets. Of the 157 samples collected in this study, 62 (39.5%) were positive for *C. difficile* by toxigenic

culture. These prevalence data are similar to reports from the USA (29.6%) (60) and from lesser pork-producing countries like Slovenia (50.9%) (61) and the Czech Republic (56.7%) (62). *C. difficile* in Australian neonatal piglets is widespread and prevalent. The prevalence of *C. difficile* detected in this study is, however; lower than reported in a recent study conducted in Australian piggeries (APL study #00462, 2013). In that study an overall prevalence of 67.2 % was seen. A reason for the difference in prevalence in these studies could be the composition of the test population. The earlier study involved a large scale surveillance of Australian piggeries. This current study included some samples derived from the same surveillance study but also many opportunistic samples sent to our laboratory by veterinarians. When a 'sick' piglet was identified and sampled by the veterinarian, in some cases many of the piglet's litter-mates were sampled and sent for testing. This may have resulted in the introduction of bias into the test population. Notwithstanding, the prevalence of *C. difficile* carriage reported in the current study was still high.

We evaluated the performance of two PCR based methods for the detection of toxin A and B genes (illumigene® *C. difficile* assay and BD GeneOhm™ Cdiff Assay), an enzyme immunoassay for toxins A and B (TechLab *C. diff* Quik Chek™) and direct culture; *C. difficile* ChromID™ agar). All of these assays were compared against toxigenic culture). To date few studies have evaluated this medium (51, 63, 64) and, of those, only one included samples (isolates) from animal origin (64). This current study presents the first reported data worldwide on the performance of a chromogenic medium for recovery of *C. difficile* from animal faecal samples. Of all the comparator assays, CA performed the best and recorded a high sensitivity (100.0%) and specificity (96.0%). Overall recovery of *C. difficile* by CA was high (36.3%) and similar to that obtained by EC (39.5%) (96.8% concordance). These findings are consistent with studies performed on human faeces (51) (63). Culture on CA presents a suitable identification method for veterinary laboratories. CA outperformed not only the molecular methods assessed in this study, but other media by negating the need for pre-reduction, alcohol shock or the additional 24h incubation time. CA plates are highly selective; by limiting the growth of endogenous flora *C. difficile* colonies are easy to identify and sub-culture prior to epidemiological typing. Other benefits of this medium are its relatively low cost and no requirement for expensive and accurately maintained anaerobic cabinets; rather a gas jar with gas packs can be used.

The performance of the molecular based assays (QC, GO and IG) in the detection of *C. difficile* in porcine faeces was poor, in particular the two PCR based assays (GO and IG). Concordance (detection of *C. difficile*) of these assays with EC was low (QC 73.9%, GO 70.5% and IG 64.3%). Sensitivity ranged from 25.0 - 42.9% which is also unacceptably low. Even though there was a high prevalence of *C. difficile* in the population, the PPVs and NPVs for the molecular based assays were unacceptably low (PPV range 66.7-88.2%, NPV range 77.1-82.3%). This finding is in accordance with other studies reporting the performance of molecular assays (PCR and ELISA based) in faecal samples of dogs, horses and pigs was less than in human faecal samples (42, 44, 45, 65). These data presented herein further provide evidence that current commercially available molecular based assays routinely used for detecting CDI in humans are not suitable for testing porcine faeces.

Overall concordance of all assays with TC was 60.5% (95/157). The poor performance of these assays is primarily due to the high number of discordant results, principally false negatives and could be attributable to a number of factors. Several host/environmental factors thought to influence the performance of human diagnostic assays have been suggested and may also apply in animal studies. First, Gumerlock *et al.*, 1993, proposed that the presence of faecal proteases degrade levels of toxins in the stool (66). It is possible that the length of time the samples spent in transit had a detrimental effect on toxin levels in the faeces to reducing them below the level of detection of ELISA based assays. This could also explain the difference between the poor results presented here for

the QC assay and those reported in a study by Keessen *et al.*, 2011 (ELISA sensitivity 80-90%) (44), in which faeces were collected in April and transported under refrigeration from a farms within the relatively small geographic area of The Netherlands. In our study samples were transported over large distances and in sub-optimal (ambient) storage conditions. This is an important observation and likely reflects the circumstances in which samples are routinely transported from the site of collection to the veterinary laboratory. The fact that CA worked so well under these conditions underscores its suitability as a diagnostic test for *C. difficile* in Australia. Second, Lyerly *et al.*, 1992 found that low specificity in an assay of this kind may be attributed to toxin degradation due to multiple freeze thaw cycles (67). This is unlikely to account for the discordant results seen in this study as samples were thawed only once, as per the manufacturer's recommendations. Third, non-specific binding of host faecal proteins to toxin in the gastrointestinal tract results in low levels of free unbound toxin in the sample (affecting ELISA and cytotoxic assays) (68). Finally, low specificity seen with commercial assays testing animal faeces is potentially caused by the presence of inhibitory substances or inactivating enzymes (45, 67). To date there are limited data in the literature to support this hypothesis, however it is conceivable that inherent differences in the composition of the faeces between animals and humans influence the binding of primers or in the case of ELISA assays, antigens.

Whether *C. difficile* diagnosis and typing will be an Australian Animal Health Laboratory (AAHL) reference function or conducted at the network laboratory level will impact choice of diagnostic assay. Capital equipment and consumable budgets and technical expertise at each location will be critical factors. Whilst the QC and CA assays are relatively fast and easy to perform, requiring little technical expertise, the PCR based assays (IG and GO) have several steps comprising the extraction and PCR reaction mixture set up stages which provide an opportunity for contamination and/or technician error. In particular, there are two critical steps that could lead to a higher-than-expected "invalid" rate if not performed correctly (69). Although it would need to be validated for animal use, the new BD MAX system provides an easy platform that automates sample preparation for use in the BD GeneOhm assay. The extraction, detection, and interpretation of the signals take place within a sealed cartridge to prevent contamination. Regardless of diagnostic choice, culture and typing by a recognized method (i.e. PCR ribotyping and toxin gene PCR) should be included for surveillance and epidemiological purposes.

The statistics used to measure the performance of an assay are ultimately influenced by the choice of reference standard comparison. Historically there have been two assays considered as gold standards. The cell culture cytotoxicity neutralization assay (CCCNA) is performed by preparation of a stool filtrate, which is then applied onto a monolayer of an appropriate cell line such as such as Vero cells or McCoy cells. Cells are examined microscopically at 24 and 48 h for evidence of toxin-induced cytopathic effect (CPE) (cell rounding). The neutralization step uses either *C. sordellii* or *C. difficile* antiserum and is performed to ensure that the CPE is attributable to *C. difficile* toxins rather than nonspecific toxicity. This assay has historically been considered the gold standard for *C. difficile* detection in human stool samples. Reported sensitivities for CCCNA are in the region of 65 to 90% (70). Many experts feel this is an inadequate level of sensitivity for a gold standard and, combined with the relatively prolonged turnaround time (24 to 48 h) and the requirement for expertise in maintenance of cell cultures and interpretation of results has meant CCCNA has fallen out of favour as a routine diagnostic test (70). In addition, no standard protocol currently exists, making comparison of results between laboratories around the world difficult (54, 70).

Toxigenic culture (TC) has not only high sensitivity and specificity for *C. difficile* but it has the benefit recovery of the isolate, which can be used for future epidemiological typing and antibiotic susceptibility testing (70). The basis of this is broth culture with selective and differential agents to inhibit overgrowth of other faecal flora while enhancing the recovery of *C. difficile*. Exposure to heat or alcohol enhances spore recovery. Cultures are

typically incubated for a minimum of 48 h and frequently held for up to 7 days before being reported as negative (42, 70). These two assays essentially detect different targets, one the presence of the organism in the stool (TC), the other the presence of *C. difficile* toxins (primarily toxin B, but also toxin A). It is therefore not surprising that studies comparing diagnostic assays are often not in perfect agreement.

It is important that diagnostic tests perform well, independent of the strain types present in the test population. This study identified numerous PCR ribotypes, some of which were internationally known strains. The most prevalent RT was QX006 (16.1%), followed by QX207 (12.9%), UK014 (11.3%), QX057 (11.3%) and UK020 (8.1%). These top five RTs comprised 60% of the isolates recovered by TC. RT014 and RT020 are often grouped together due to their very similar PCR ribotype fingerprint. RT014/020 is the most common RT in many countries including the Netherlands (17) and Australia (Riley et al. unpublished data). Not only is RT014 well established in nosocomial cases of CDI, but it is also the leading cause of disease in the community (17) and has been found in a small number of livestock (71, 72) and retail meats (73). Overall, 58% of isolates were positive for one or both *tcdA* and *tcdB*; the remainder, including about half of the isolates comprising the top five RTs, were non-toxigenic (A-B-CDT-) strains. These data indicate heterogeneity in the test population and are consistent with the epidemiological data in the recent APL study. Of the limited number of studies performed in animals to date, a single study by Keessen *et al.* (2011) provided typing data which indicated homogeneity of *C. difficile* strains in the test population (44). In that study, 99% (70/71) of isolates recovered from samples of porcine faeces were RT078, the predominant RT circulating in animals in Europe. The same study reported significantly higher sensitivity for the GO assay and for a range of ELISA platforms. It is possible their results were biased because of the single strain population; in particular there may be potential differences in the antigenic features of the strains and toxins that are expressed, and in the PaLoc primer binding sites. This idea has also been suggested by Tenover *et al.* who found significantly lower sensitivity with RT-PCR and EIA for RT078 than many other ribotypes including worldwide epidemic strain RT027 (74), although the Keessen *et al.* study looked at almost exclusively RT078 strains. No RT078 was found in this or any previous studies in Australian livestock. Furthermore, RT078 has not become endemic in human populations in Australia (unpublished data). In our study, CA performed consistently well across all the 19 RTs and this result is similar to the findings of Eckert *et al.* (51) who found no relationship between PCR ribotype and isolate recovery using chromogenic agar. Conversely, the non-culture methods evaluated in this study, IG, GO and QC, performed consistently poorly across all the 19 RTs. Furthermore, the finding of three novel porcine *tcdB*⁺ strains (QX076, QX084 and QX147) may represent variant strains that do not signal in GO like RT237 (26). The cost of a diagnostic test is an important consideration. CA plates are about AU\$3 each but of course there is a significant labour cost attached to processing samples this way and at least 48 h is required before presumptive results are available. Both molecular tests cost about AU\$25 each with a 1-2 h turnaround time, while the EIA is about AU\$15 per test with a 1 hour turnaround time.

The detection of *C. difficile* toxins by veterinarians is of utmost importance. The presence of A+B+ strains of *C. difficile* in this test population, combined with the absence of toxin detected in the QC assay (3/157) possibly reflects asymptomatic carriage rather than disease. In humans, toxigenic strains can be carried asymptotically by up to 50% of elderly patients who are residents of a long-term care or nursing home facility. *C. difficile* spores and vegetative cells are shed into the immediate environment in the faeces of both scouring and non-scouring pigs, underscoring the importance of high carriage rates in apparently healthy piglets (75). The carrier state is also emphasized in mouse studies that demonstrate a marked increase in spore shedding when antibiotics are administered to asymptomatic carrier mice. Subsequent spore-mediated transmission to immunosuppressed mice led to severe intestinal disease (76). Further studies are needed to ascertain the role of *C. difficile* in disease and in colonisation of pig populations.

In conclusion, this study highlights the high prevalence and unique strain types of *C. difficile* present in Australian neonatal piglet populations. The poor performance of commercially available non-culture based tests reported in this study should be of concern for veterinary laboratories. In human cases of CDI, discordant test results have major implications for patient care. False positives can lead to unnecessary treatment and isolation, and false negatives increase risk of delay in treatment and cross-infection (2). Diagnosis of *C. difficile* infection in a piglet, herd or on a farm may not be as time critical nor are there current isolation/ infection control methods hinging on test results as is the case in the human setting, however, the results presented in this study underscore the importance of developing novel porcine-specific assays with high sensitivities, PPVs and NPVs for the rapid reliable detection of *C. difficile* and its toxins in porcine faeces.

5. Recommendations

As a result of the outcomes in this study we recommend:

- This study demonstrates the poor performance of current commercially available non-culture based assays for detection of *C. difficile* in porcine faeces. These results underscore the need for developing novel porcine-specific assays for the detection of *C. difficile*.
- Whilst CA has a number of benefits which make it a promising method for use by veterinarians, the identification of *C. difficile* toxin and/or its genes is considered to be of primary importance for correct diagnosis of CDI. As with human CDI, a two-step process is needed. CA, with its high sensitivity and NPV, could be used by veterinarians as a primary test to identify those piglets or herds that require a second 'definitive' test for toxigenic *C. difficile* such as TC followed by PCR characterisation of the toxin genes.
- The epidemiology of *C. difficile* in Australian piglets is unique and clearly necessitates further investigations into disease phenotype, molecular epidemiology and development of new diagnostic assays. There is an urgent need for proactive surveillance at national and local levels of the strain types circulating in Australian pig populations as there will most likely be temporal changes both in the strains of *C. difficile* found in the piglets and the risk factors contributing to their establishment and spread in piggeries. This data is also necessary for analyzing public health risks, if any.
- Given the findings of this study and the increasing body of literature in this field, both global and domestic, every effort should be made by the pork industry to increase awareness among veterinarians, animal health groups and producers of *C. difficile* as a pathogen of piglets and the challenges of detection and diagnosis.

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8. Intellectual Property And Disclosures

None

9. Publications Arising

Preliminary results of this investigation have been submitted for presentation at the 8th International Conference on the Molecular Biology and Pathogenesis of the *Clostridia* (CLOSTPATH), Palm Cove, Queensland, October 2013.

A manuscript for submission to a peer-reviewed journal is currently being prepared.

10. Appendix 1

Fig. A1 - Growth of *C. difficile* on ChromID agar at 24 and 48h.

