Evaluation of Diagnostic Tests to Detect *Clostridium difficile* in Piglets

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BACKGROUND

Clostridium difficile is a Gram-positive spore forming bacterium and a well-known enteric pathogen of humans. Outside Australia *C. difficile infection* (CDI) is reported as a major cause of high-morbidity enteritis and pre-weaning scour in neonatal piglets aged 1-7 days [1,2]. We have confirmed that toxigenic *C. difficile* and idiopathic scour is present in pig herds in Australia and *C. difficile* prevalence in Australian piggeries is as high as 70% [3].

To understand the role of *C. difficile* in pig disease it is essential 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.

AIM

To evaluate the performance of four commercial assays to detect *C. difficile* in 157 specimens of piglet faeces.

METHODS

Sample population. 157 specimens were collected by rectal swab 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: 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).

Tests. Assays were performed according to manufacturers' instructions and compared against enrichment culture (**EC**) as a "gold standard" [4]. 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 direct culture; *C. difficile* ChromID™ agar (**CA**, BioMérieux).

Characterisation of Isolates. PCR for toxin genes *tcdA* (toxin A), *tcdB* (toxin B) and *cdtA/B* (binary toxin), which correlate with toxin production, and PCR ribotyping were performed as previously described [5].

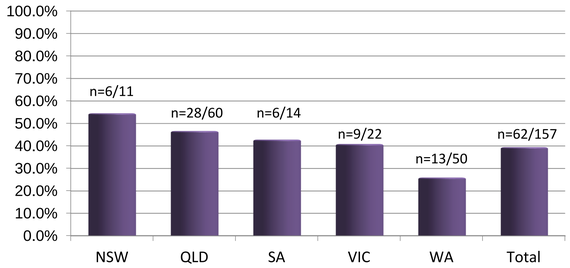
Sample processing. All samples were transported under ambient conditions to The University of Western Australia (mean transport time of 8 days). Upon receipt, sample slurries were prepared by suspension of the faecal swab in 800µL of phosphate buffered saline. The samples were vortexed briefly to create a homogenous suspension and split into 200µL aliquots. One part each was used for IG and GO and stored at -20°C until use, after which point a single freeze thaw cycle was implemented. One aliquot each was immediately used for toxigenic culture and direct culture by CA and finally one aliquot was stored at 2 to 8°C for use with QC and processed within 48 h.

Statistics. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for each assay against the gold standard assay (EC). 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.

RESULTS – *C. difficile* prevalence & characterisation

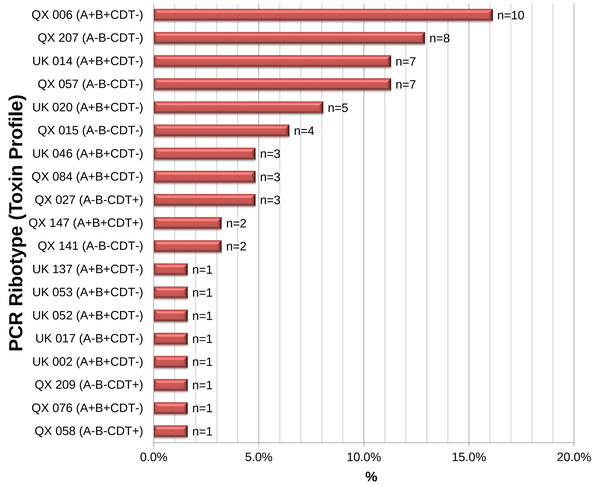
Prevalence. Overall, *C. difficile* was isolated by EC from 39.5% (n=62/157) of samples (Figure 1).

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



PCR ribotypes. Of the 62 isolates obtained from neonatal piglets, 19 different ribotypes (RTs) were detected, 8 of which were internationally recognised types (Figure 2). ***Toxin genes.*** PCR revealed 58.1% (n=36) of isolates were positive for at least one toxin gene (*tcdA*/*tcdB*) (Table 2, Figure 2). Five isolates (8.1%) had the uncommon genotype of *tcdA*$^-$/*tcdB*$^-$/cdt$^+$. The remainder (n=21, 33.9%) were negative for any toxin genes (Table 2).

Figure 2 - Summary of PCR ribotypes and toxin profiles from recovered *C. difficile* isolates.



RESULTS – assay performance

There was varied performance in detection of *C. difficile* by the assays tested (Tables 1 and 2).

Table 1 – Assay result concordance with enrichment culture.

Assay	N positive	% Concordance	P-value	N False Positives (%)
CA	57	96.8	p= 0.56	0 (0.00)
QC	33	73.9	P= <0.001	7 (21.2)
GO	19	70.5	P= <0.001	2 (10.5)
IG	14	64.3	P= <0.001	4 (28.6)

Table 2 - Summary of *C. difficile* toxin profiles and true positives.

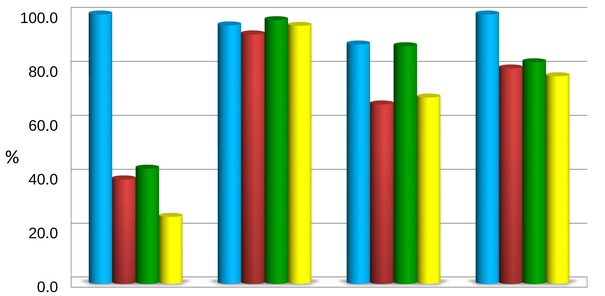
Toxin Profile	N Isolates (N RTs)	CA	QC	GO	IG
A-B-CDT+	5 (3)	5 (100.0)	2 (100.0)	0 (0.00)	0 (0.00)
A-B-CDT-	21 (4)	20 (95.2)	9 (42.9)	2 (9.5)	1 (4.8)
A-B+CDT-	1 (1)	0 (0.00)	1 (100.0)	0 (0.00)	0 (0.00)
A+B+CDT+	2 (1)	1 (50.0)	0 (0.00)	0 (0.00)	0 (0.00)
A+B+CDT-	33 (10)	31 (93.9)	13 (39.4)	15 (45.4)	9 (27.3)
Total	62 (19)	57	26	17	10

Population bias. The prevalence of non-toxigenic (A-B-) strains of *C. difficile* in this study was high (42%) and possibly introduces a population bias favouring 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, thus reducing the number of potential false negatives to zero (Table 2 & Figure 3).

Table 2 and Figure 3‡ - Sensitivity, specificity, PPV and NPV for four commercially available assays for the detection of *C. difficile* in porcine faeces, compared to enrichment culture (EC).

Assay	%α/β			
	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

α = uncorrected, β = corrected



‡ Final corrected values (n=36)

DISCUSSION & CONCLUSIONS

High prevalence and unique strain epidemiology

Prevalence of *C. difficile* was high and is similar to previous international & local studies (3, 6-8). No PCR ribotype 078 or 027 were found and thus this was a different sample population for validating the assays compared to previous studies (9-12). The observed heterogeneity in PCR ribotypes is consistent with previous studies in Australian pigs (3) and is likely a result of the absence of RT078 which predominates in pig populations outside of Australia (10).

Poor performance of molecular assays

The performance of the molecular based assays (QC, GO and IG) to detect *C. difficile* in porcine faeces was poor, in particular the two PCR based assays (GO and IG). Sensitivity ranged from 25.0 – 42.9% which is also low. Even though there was a high prevalence of *C. difficile* in the population, the PPVs and NPVs for the molecular based assays were low (PPV range 66.7-88.2%, NPV range 77.1-82.3%).

Discordant results

Concordance with EC 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 (presence of inhibitory substances/differences in specific antigens or primer binding sites) and sample deterioration. 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 in Australia are routinely transported from the site of collection to the veterinary laboratory.

Good performance of C. difficile ChromID™ agar

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. Furthermore the performance of CA appeared not to be compromised by long sample transport times.

Cost

The cost of a diagnostic test is an important consideration. CA plates are approximately AU$3 each but resource intensive in terms of labor and time (48 hrs for presumptive ID). 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 h turnaround time.

Need for development of porcine specific assays

Notwithstanding the good performance by CA, this study clearly highlights the need for validation of existing assays and development of new porcine-specific assays with high sensitivities, PPVs and NPVs for the rapid reliable detection of *C. difficile* and its toxins in porcine faeces, particularly given the unique strain population present in Australian pigs.

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REFERENCES

1. Squire M, Riley TV. *Clostridium difficile* Infection in Humans and Piglets: A 'One Health' Opportunity. Current Topics in Microbiology and Immunology. 2012 Epub June 2012.
2. Songer JG, Anderson MA. *Clostridium difficile*: an important pathogen of food animals. Anaerobe. 2006;12:1-4.
3. DR. Knight, M. Squire and TV. Riley. Prevalence and molecular characterisation of *Clostridium difficile* in neonatal piglets in Australia. Abstract submitted for presentation at Manipulating Pig Production XIV, APSA 2013.
4. Brazier JS. The diagnosis of *Clostridium difficile*-associated disease. J Antimicrob Chemother. 1998 May; 41 Suppl C:29-40.
5. Knight DR, Thean S, Putsathit P, Fenwick S, Riley TV. Cross-sectional study reveals high prevalence of *Clostridium difficile* non-PCR ribotype 078 strains in Australian veal calves at slaughter. Appl Environ Microbiol. 2013;79:2630-5.
6. Susick E, Putnam M, Bermudez D, Thakur S. Longitudinal study comparing the dynamics of *Clostridium difficile* in conventional and antimicrobial free pigs at farm and slaughter. Vet Microbiol. 2012;157:172-8.
7. Avbersek J, Janezic S, Pate M, Rupnik M, Zidaric V, Logar K, *et al*. Diversity of *Clostridium difficile* in pigs and other animals in Slovenia. Anaerobe. 2009;15:252-5.
8. Norén T, Johansson K, Unemo M. *Clostridium difficile* PCR ribotype 046 is common among neonatal pigs and humans in Sweden. Clin Microbiol Infect. 2013 Jun 14. doi: 10.1111/1469-0691.12296. [Epub ahead of print]
9. Post K, Jost H, Songer J. Evaluation of a test for *Clostridium difficile* toxins A and B for the diagnosis of neonatal swine enteritis. J Vet Diagn Invest. 2002;14:258-9.
10. Keessen EC, Hopman NEM, Leengoed LAMGv, Asten AJAMv, Hermanus C, Kuijper EJ, *et al*. Evaluation of four different diagnostic tests to detect *Clostridium difficile* in piglets. J Clin Microbiol. 2011;49:1816-21.
11. Anderson M, Songer J. Evaluation of two enzyme immunoassays for detection of *Clostridium difficile* toxins A and B in swine. Vet Microbiol. 2008;128:204-6.
12. Alvarez-Perez S, Alba P, Blanco J, Garcia M. Detection of toxigenic *Clostridium difficile* in pig feces by PCR. Vet Med. 2009;54:360-6.