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Nationwide Surveillance Study of *Clostridium difficile* in Australian Neonatal Pigs Shows High Prevalence and Heterogeneity of PCR Ribotypes

Daniel R. Knight,[a] Michele M. Squire,[a] Thomas V. Riley[a,b]

Microbiology & Immunology, School of Pathology and Laboratory Medicine, The University of Western Australia,[a] and Department of Microbiology, PathWest Laboratory Medicine,[b] Queen Elizabeth II Medical Centre, Nedlands, Western Australia, Australia

***Clostridium difficile* is an important enteric pathogen of humans and the cause of diarrhea and enteritis in neonatal pigs. Outside Australia, prevalence in piglets can be up to 73%, with a single PCR ribotype (RT), 078, predominating. We investigated the prevalence and genotype of *C. difficile* in Australian pig herds. Rectal swabs ($n = 229$) were collected from piglets aged <7 days from 21 farms across Australia. Selective culture for *C. difficile* was performed and isolates characterized by PCR for toxin genes and PCR ribotyping. *C. difficile* was isolated from 52% of samples by direct culture on chromogenic agar and 67% by enrichment culture ($P = 0.001$). No association between *C. difficile* recovery or genotype and diarrheic status of either farm or piglets was found. The majority (87%; 130/154) of isolates were toxigenic. Typing revealed 23 different RTs, several of which are known to cause disease in humans, including RT014, which was isolated most commonly (23%; 36/154). RT078 was not detected. This study shows that colonization of Australian neonatal piglets with *C. difficile* is widespread in the herds sampled.**

Clostridium difficile, a ubiquitous spore-forming anaerobe, is the etiological agent of antibiotic-associated colitis and the most common cause of hospital-acquired infectious diarrhea in the developed world (1). It is spread oro-fecally through the ingestion of spores and opportunistically colonizes the gut of individuals with perturbed intestinal flora, where it can produce the large clostridial toxins TcdA and TcdB and sometimes a binary toxin (ADP-ribosyltransferase, or CDT) (2).

Natural infection of swine by *C. difficile* was first reported in 1983 (3). *C. difficile* has emerged since in swine-producing areas as a major cause of enteric disease in neonatal piglets (4). Pathology is similar to that in humans and includes colonic and cecal enteritis, colonic and mesocolonic edema, diarrhea, and anorexia (5). In piglets, signs of disease generally commence soon after parturition (5). Severe weight loss or anorexia is common, and there can be significant mortality (up to 16%) (4). 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), RT078, predominating in the majority of cases (6–12).

The *C. difficile* transmission cycle in a piggery may be perpetuated by multiple factors, including (i) the contamination of the environment with spores and (ii) increased susceptibility to colonization through immature endogenous microflora and/or exposure to antimicrobials. Environmental contamination occurs when *C. difficile* spores are shed in the feces of piglets with and without diarrhea (13) and when treated piggery effluent is reused within the farrowing facilities (14). *C. difficile* spores are hardy and remain viable in the environment for long periods. Furthermore, disinfectants commonly used in the Australian piggery environment are not sporicidal.

Increasingly, *C. difficile* infection (CDI) in humans is being reported in the community in populations without the classical risk factors of advanced age or immune suppression, although the source of *C. difficile* in this setting has not been clearly defined (15–17). RT078 is being found increasingly in cases of community-associated CDI (CA-CDI) outside Australia (1), suggesting that livestock, via the environment and/or food, are a reservoir for disease-causing strains in humans (18, 19). There is also mounting evidence from molecular typing (20) and highly discriminatory whole-genome sequencing (21) that human CDI is a potential zoonosis.

In this study, we investigated the prevalence and nature of gastrointestinal carriage of *C. difficile* in Australian neonatal pigs by culture of rectal swabs and characterization of the isolates.

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

MATERIALS AND METHODS

Study design. A total of 21 piggeries (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 diarrhea status. Farms were carefully 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 diarrhea for at least 6 months (experimental farms; $n = 12$) and those with no history of

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Address correspondence to Thomas V. Riley, thomas.riley@uwa.edu.au.

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TABLE 1 Summary of *C. difficile* isolate recovery in five states by toxigenic culture and direct culture and by piglet diarrhea status

Group and analysis method	No. of isolates in[a]:					No./total no. (%) [95% CI][b]
	NSW	QLD	SA	VIC	WA	
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. *, $P = 0.001$.

idiopathic neonatal diarrhea for at least 6 months (control farms; $n = 9$) were selected. Idiopathic diarrhea was defined as diarrhea of unknown etiology that veterinarians could not attribute to *Escherichia coli*, *C. perfringens*, *Isospora suis*, or rotavirus infection. From the 21 farms enrolled, piglets ($n \geq 10$) from a minimum of four different litters were randomly sampled by the attending veterinarian.

Sample collection. Fresh fecal 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 with charcoal (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.

Isolation and identification of *C. difficile*. The isolation of *C. difficile* was based on previously described methods (22), with some modifications. Feces were cultured both directly on *C. difficile* ChromID agar (CA; bioMérieux, Marcy l'Etoile, France) and in an enrichment broth containing gentamicin, cycloserine, and cefoxitin (GCC). After 48 h of incubation, to enhance spore selection, 1 ml of each enrichment broth was added to equal volumes of 96% alcohol, left at room temperature for at least 60 min, and then plated onto selective agar plates (cycloserine cefoxitin fructose agar containing sodium taurocholate [TCCFA]). All plates were incubated in an anaerobic chamber (Don Whitley Scientific Ltd., Shipley, West Yorkshire, United Kingdom) at 37°C in an atmosphere containing 80% N_2, 10% CO_2, and 10% H_2. Putative *C. difficile* colonies on either CA (direct) or TCCFA (enrichment) were subcultured onto blood agar and identified on the basis of their characteristic chartreuse fluorescence under long-wave UV light (~360 nm), colony morphology (yellow, ground glass appearance), and odor (horse dung smell). The identity of uncertain isolates was confirmed by Gram stain and the presence of the L-proline aminopeptidase activity (Remel Inc., Lenexa, KS, USA).

Molecular characterization of *C. difficile* isolates. All isolates were screened by PCR for the presence of toxin A and B genes (*tcdA-tcdB*) and binary toxin genes (*cdtA* and *cdtB*) and for changes in the repetitive region of the toxin A gene as previously described (23). PCR ribotyping was performed as previously described (23). PCR ribotyping reaction products were concentrated using the Qiagen MinElute PCR purification kit (Qiagen Sciences, Germantown, MD, USA) and run on the QIAxcel capillary electrophoresis platform (Qiagen Sciences, Germantown, MD, USA). The analysis of PCR ribotyping products was performed using the BioNumerics software package, v.6.5 (Applied Maths, Saint-Martens-Latem, Belgium). Dendrograms were generated for all isolates using an unweighted-pair group method using average linkages (UPGMA) and Dice coefficient to assess the clostridial diversity in the populations. PCR ribotypes were identified by comparison with banding patterns in our reference library, consisting of a collection of 15 reference strains from the European Centre for Disease Prevention and Control (ECDC) and the most prevalent RTs currently circulating in Australia (T. V. Riley, unpublished data). Isolates that could not be identified with the reference library were designated with an internal nomenclature, prefixed with QX.

Statistical analysis. Fisher's exact test was used to compare the prevalence of *C. difficile* in the sampled piggeries and the effect of diarrhea and geographic distribution on the number and types of RTs identified. A P value of <0.05 was considered significant.

RESULTS

Prevalence of *C. difficile* carriage. A total of 229 piglet fecal samples were collected in this study. *C. difficile* was isolated from 52.4% ($n = 120$) of the 229 samples of piglet feces by direct culture (*C. difficile* ChromID agar) and 67.2% ($n = 154$) by enrichment culture (GCC broth/TCCFA) ($P = 0.001$) (Table 1). All direct culture-positive samples also were positive on enrichment. Compared to enrichment culture, 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 diarrhea ($P = 0.141$) (Table 1). Prevalence varied across the five states (range, 50.9 to 82.5%).

Toxin gene profiles. Five combinations of *C. difficile* toxin genes (toxin profiles) were identified (Table 1). The majority (87%; 130/154) of strains were toxigenic, and the most common

TABLE 2 PCR ribotype distribution for 154 isolates of *C. difficile* recovered from Australian piglets

PCR ribotype	Toxin profile			No. of isolates in[a]:					Total (*n* [%])
	tcdA	*tcdB*	*cdtA-cdtB*	VIC	SA	QLD	WA	NSW	
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.

profile was $A^+B^+CDT^-$ (43.5%; 67/154). Nontoxigenic 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 nontoxigenic strains ($A^-B^-CDT^-$) were more prevalent in the control (nondiarrheic) farms ($n = 18/52$; 34.6%) than in the experimental (diarrheic) farms (6/102; 5.9%) ($P = 0.001$).

PCR ribotyping. Twenty-three RTs were identified (Table 2), nine of which were internationally recognized RTs. No RT078 or RT027 strains were identified. The most common RT was RT014 ($A^+B^+CDT^-$), representing 23.4% (36/154) of isolates. RT014 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%) (Table 2). The next most prevalent RTs were RT033 (13.0%), QX009 (12.3%), UK237 (10.4%), and QX006 (6.5%). Novel RTs QX006 (40% NSW/60% QLD) and QX009 (58% VIC/42% NSW) were restricted to smaller geographic areas. RT033, the second most commonly identified strain (13%; 20/154), was found equally between control ($n = 10/20$; 50%) and experimental farms ($n = 10/20$; 50%). RT033 was found in 19/40 samples from SA and a single sample from Victoria. RT237 was found exclusively in WA (Table 2). RT237, QX006, and QX009 were found only in experimental farms and were not associated with piglets with diarrhea.

DISCUSSION

This study presents data on the prevalence and genotypes of *C. difficile* in Australian piggeries. *C. difficile* prevalence in piglets aged less than 7 days was 67.2%. This is higher than reported in the United States (29.6%) (6), Slovenia (50.9%) (7), and the Czech Republic (56.7%) (8) and similar to recent reports from Sweden (67.2%) (11) and Germany (73%) (24). Recovery of *C. difficile* by enrichment culture was significantly better than direct culture on ChromID (52.4% versus 67.2%; $P = 0.001$), in accordance with studies of human CDI (25). Despite reduced sensitivity compared to enrichment culture, *C. difficile* ChromID represents a viable and cost-effective option for detecting *C. difficile* in piglets, particularly in the Australian veterinary setting. It is relatively cheap, can give answers in 24 h, and, in our experience, performs significantly better than molecular-based methods for the detection of *C. difficile* in porcine feces (26).

As with human CDI, piglets can be colonized with *C. difficile* but remain free of disease even when profound diarrhea is present in the herd (27, 28). We found no association between the presence of *C. difficile* and the diarrhea status of either individual piglets or farms. Toxigenic *C. difficile* isolates were common (85%), but the frequency was lower than the >99% reported elsewhere (7, 13). There was no association between the presence of toxigenic strains and diarrhea status for either farms or individual piglets. Significantly more nontoxigenic strains ($A^-B^-CDT^-$) were isolated from control farms. Given the sample population comprised of 79% apparently healthy piglets (no evidence of diarrhea at the time of collection), the finding of high numbers of toxigenic isolates is important. Similar results have been reported elsewhere (28), suggesting that the pathogenesis of CDI is complex in piglets, involving immune status and infectious dose, and requires further investigation. Colonization in piglets also may be transient or represent subclinical disease. This carrier state has implications for environmental contamination and the infectious

cycle. Vegetative cells and spores are shed into the immediate environment in the feces of piglets with and without diarrhea (13).

The difference in the proportion of toxigenic isolates seen here and elsewhere may be a consequence of the unique heterogeneity of the strains isolated in this study (23 different RTs). In Europe and North America, RT078 ($A^+B^+CDT^+$) predominates in most livestock animals, including pigs, chickens, and cattle (7, 9, 13, 29). No RT078 was isolated in this study. This was expected, since RT078 has not been found in any Australian livestock (23, 30), and it is not endemic in human populations in Australia (31).

RT033 ($A^-B^-CDT^+$), the second most prevalent RT identified in this study (13%), is rarely reported in the literature; however, it has recently been found in calves in both Germany (10) and Australia (23) and has been isolated from humans in Australia in the last decade (T. V. Riley, unpublished data). RT033, along with RT237, the fourth most prevalent RT in this study, belong to the multilocus sequence type 11 (ST11) group within the divergent clade 5 lineage (32), as does RT078. In Europe, RT078 is associated with CA-CDI, and in The Netherlands, strains of RT078 infecting both humans and animals are identical by multilocus variable-number tandem-repeat (MLVA) analysis (20). Whole-genome sequencing has revealed that strains of RT078 found in pig farmers and their families and pigs in The Netherlands are genetically identical (zero single-nucleotide polymorphism differences), suggesting zoonotic transmission, although the direction of the transmission is not known (21). However, given the high prevalence of *C. difficile* in pigs, the presence of *C. difficile* in pig farmers likely is the result of continuous exposure. In the absence of RT078 in Australia, other clade 5 strains, such as RT033, RT126, RT127, and RT237, appear to occupy the same ecological niche as RT078 and could contribute to disease in livestock and possibly humans (23).

RT237 has an uncommon toxin profile ($A^-B^+CDT^+$) as a result of a large deletion in the pathogenicity locus (PaLoc) and causes more weight loss in a mouse model of *C. difficile* infection than an RT078 animal strain (33, 34). Other variant strains of *C. difficile* (A^-B^+) also have been associated with an increasing incidence of clinically significant disease in humans (33) and animals (34). No other toxin variant strains were identified in this study.

In addition to RT033 and RT237, five RTs were identified as being binary toxin positive (CDT^+): QX009 (12.3%), QX027 (1.9%), QX209 (1.3%), QX147 (1.3%), and QX058 (0.6%). CDT^+ strains of *C. difficile* are strongly associated with animals, yet CDT^+ isolates comprised only 41% (63/154) of piglet isolates in this study and 54.5% (55/101) of the top five RTs. The remaining 59% were either $A^+B^+CDT^-$ or $A^-B^-CDT^-$. The lower prevalence of binary toxin-positive RTs was surprising and in contrast to previously published reports, both in Europe and North America, where CDT^+ strains predominate in piglets (75 to 100%), albeit mainly RT078 (7, 9, 13, 35). This is even more unusual in the Australian context given the predominance of CDT^+ strains in Australian cattle (23).

RT014 ($A^+B^+CDT^-$) was the most prevalent RT, comprising 23.4% of isolates. RT014 is the most common RT infecting humans in Australia (31) and in many countries in Europe, where it is also a leading cause of disease in the community (15). RT014 previously has been found in very small numbers in older cattle in Belgium (36) and in horses, domestic pets, and livestock in the Netherlands (37) and in retail meat in North America (38). The prevalence of RT014 reported in our study was higher than those of these earlier studies, 23.4% versus 1 to 2%.

Interestingly, two isolates of RT046 ($A^+B^+CDT^-$) were found in this study, both from Victoria. A Swedish study recently has isolated RT046 from both neonatal pigs (67%) and outbreak cases of human CDI, indicating a possible zoonosis (11). RT046 has been recovered from the stools of patients with CDI in Australia, although the number of cases was low (31). Fourteen unknown RTs were cultured in this study, comprising 42.8% of isolates; of these, 5 RTs (19 isolates) were CDT^+ and likely to belong to ST11.

A unique distribution of RTs throughout the states was observed. RT014 was found in all states but Western Australia, with the majority from Victoria (52.8%). RT033 was found in only two states, with the vast majority (95%) being from South Australia. RT237 was found exclusively in two of the four Western Australian piggeries, in keeping with our earlier finding (35). QX009 was equally distributed between Victoria and New South Wales, while QX006 was equally distributed between Queensland and New South Wales. There are factors that may account for the distributions seen here. Australian pig production operations typically are vertically integrated, so there is no requirement for movement or trade of pigs between piggeries. Australia is a big country (>6 million km^2 for QLD, VIC, WA, VIC, and NSW combined), and some of the piggeries sampled in this study were up to 4,000 km apart, further minimizing opportunities for the spread of strains between piggeries.

In conclusion, this study showed that colonization of Australian neonatal piglets with *C. difficile* was widespread in the herds sampled. Genotyping of isolates revealed (i) a heterogeneous population of strains, (ii) the absence of RT078 strains, which predominate in the Northern Hemisphere, and (iii) a smaller proportion of binary toxin-producing strains. The isolation of multiple strains of *C. difficile* known to cause disease in humans suggests that neonatal pigs are a source/reservoir for *C. difficile* infection in humans, although this requires further study. Additionally, a large number of toxigenic strains were found in piglets with subclinical disease, underscoring the importance of the carrier state in the transmission cycle.

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