Veterinary Microbiology 237 (2019) 108408

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Molecular epidemiology of *Clostridium difficile* isolated from piglets

Papanin Putsathit[a], Vasantha K. Neela[b], Narcisse M.S. Joseph[b], Peck Toung Ooi[c], Bhunika Ngamwongsatit[d], Daniel R. Knight[e], Thomas V. Riley[a,e,f,*]

[a] Edith Cowan University, School of Medical and Health Sciences, Joondalup, Western Australia, Australia
[b] Universiti Putra Malaysia, Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Serdang, Malaysia
[c] Universiti Putra Malaysia, Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Serdang, Malaysia
[d] Mahidol University, Faculty of Veterinary Science, Nakhon Pathom, Thailand
[e] Murdoch University, Medical, Molecular and Forensic Sciences, Murdoch, Western Australia, Australia
[f] PathWest Laboratory Medicine, Nedlands, Western Australia, Australia

ARTICLE INFO

Keywords:
Clostridium difficile
Thailand
Malaysia
Piglet
Environment
Epidemiology

ABSTRACT

Information on the epidemiology of *C. difficile* infection (CDI) in South-East Asian countries is limited, as is data on possible animal reservoirs of *C. difficile* in the region. We investigated the prevalence and molecular epidemiology of *C. difficile* in piglets and the piggery environment in Thailand and Malaysia. Piglet rectal swabs (n = 224) and piggery environmental specimens (n = 23) were collected between 2015 and 2016 from 11 farms located in Thailand and Malaysia. All specimens were tested for the presence of *C. difficile* with toxigenic culture. PCR assays were performed on isolates to determine the ribotype (RT), and the presence of toxin genes. Whole genome sequencing was used on a subset of isolates to determine the evolutionary relatedness of RT038 (the most prevalent RT identified) common to pigs and humans from Thailand and Indonesia. *C. difficile* was recovered from 35% (58/165) and 92% (54/59) of the piglets, and 89% (8/9) and 93% (13/14) of the environmental specimens from Thailand and Malaysia, respectively. All strains from Thailand, and 30 strains from Malaysia (23 piglet and 7 environmental isolates) were non-toxigenic. To our knowledge, this is the first and only report with a complete lack of toxigenic *C. difficile* among piglets, a feature which could have a protective effect on the host. The most common strain belonged to RT038 (ST48), accounting for 88% (51/58) of piglet and 78% (7/9) of environmental isolates from Thailand, and all 30 isolates tested from Malaysia. Piglet RT038 isolates from Thailand and Malaysia differed by only 18 core-genome single nucleotide variants (cgSNVs) and both were, on average, 30 cgSNVs different from the human strains from Thailand and Indonesia, indicating a common ancestor in the last two decades.

1. Introduction

Previously regarded as healthcare-associated, in recent years *Clostridium difficile* infection (CDI) has been increasingly diagnosed in the community and among individuals lacking traditional risk factors (Khanna et al., 2012). A large body of research is now dedicated to elucidating possible reservoirs of infection outside the hospital system (Hensgens et al., 2012; Rodriguez et al., 2016). So far, there are some convincing evidence suggesting CDI is a zoonotic disease. *C. difficile* PCR ribotype (RT) 078, which was highly prevalent among neonatal pigs (83% in North America (Keel et al., 2007) and 12–33% in Asia (Tsai et al., 2016; Usui et al., 2014; Wu et al., 2016)), was the third most common RT (8%; 31/389) among human patients in Europe (Bauer et al., 2011). Furthermore, some human and pig strains of *C. difficile* RT 078 are genetically identical by whole genome sequencing (WGS) (Knetsch et al., 2014). The widespread presence of *C. difficile* in domestic, zoo, wild and food animals could play a significant role in transmission events in the community and there have been concerns about food contamination with *C. difficile* (Weese, 2010). Indeed, *C. difficile* is widely present in retail food (Lim et al., 2018; Rodriguez et al., 2016). *C. difficile* is also abundant in community and natural environments (Rodriguez et al., 2016; Songer et al., 2009; Varshney et al., 2014). The recovery of toxigenic *C. difficile* RTs known to cause human disease from these sources led many to hypothesise that the increasing incidence of community-associated CDI (CA-CDI) was driven by exposures to contaminated food and the environment. To fully understand the epidemiology of CDI, surveillance of *C. difficile* in animals, food and the environment is essential. To date, the vast majority of such

* Corresponding author at: 2nd Floor J Block PathWest Laboratory Medicine, Queen Elizabeth II Medical Centre, Nedlands, 6009, Western Australia, Australia.
E-mail address: thomas.riley@uwa.edu.au (T.V. Riley).

https://doi.org/10.1016/j.vetmic.2019.108408
Received 17 June 2019; Received in revised form 2 September 2019; Accepted 3 September 2019
0378-1135/

Table 1
C. difficile culture results for rectal swabs collected from piglets in Thailand, by age group and farm (T1-T5).

Culture	Age group	N specimens					Total
		T1	T2	T3	T4	T5	
POS	1 to 7 days	0	7	10	0	2	19
	8 to 14 days	7	6	18	0	7	38
	15 to 23 days	0	0	0	0	0	0
	Unknown	0	0	0	1	0	1
	Total	7 (23.3)	13 (48.1)	28 (82.4)	1 (3.3)	9 (20.5)	58 (35.2)
NEG	1 to 7 days	0	7	6	0	10	23
	8 to 14 days	23	7	0	3	25	58
	15 to 23 days	0	0	0	24	0	24
	Unknown	0	0	0	2	0	2
	Total	23 (76.7)	14 (51.9)	6 (17.6)	29 (96.7)	35 (79.5)	107 (64.8)
Grand total		30 (18.2)	27 (16.4)	34 (20.6)	30 (18.2)	44 (26.7)	165 (100.0)

investigations have been conducted in North America, Europe and Australia, and very little is known about CDI in Asia in general, and specifically in animals. This study aimed to investigate the prevalence of CDI among piglets and the piggery environment in Thailand and Malaysia, to describe the molecular epidemiology of *C. difficile* strains isolated and to determine the evolutionary relatedness between *C. difficile* strains from humans, animals and the environment.

2. Materials and methods

2.1. Collection of rectal swabs and epidemiological data

Rectal swabs were obtained from piglets between September 2015 and June 2016. Specimens were obtained from a minimum of six different litters per farm *via* random selection. To investigate the environmental contamination of *C. difficile* in piggery, soil (ca. 20 g) and effluent water sample (ca. 20 ml) from each farm were also collected from sites surrounding farrowing sheds. Rectal swabs and environmental specimens were maintained at 4 °C during storage and at ambient temperature during transportation. Age and scouring status of the piglets sampled was also recorded.

2.2. Isolation and identification of C. difficile

Stool specimens were cultured both directly on *C. difficile* ChromID agar (bioMérieux, Marcy l'Etoile, France) and in a cooked meat enrichment broth containing gentamicin (5 mg/L), cefoxitin (10 mg/L), cycloserine (200 mg/L) and taurocholate (0.1%) for 48 h as previously described (Putsathit et al., 2015). Environmental specimens (10 g or 10 mL) were inoculated in pre-reduced brain heart infusion enrichment broths supplemented with taurocholate (1 g/L), cycloserine (250 mg/L) and cefoxitin (8 mg/L) and incubated anaerobically at 35 °C for 7 days. To select for spores, 1 ml of each enrichment broth was added to equal volumes of 96% ethanol, left at room temperature for at least 60 min and then plated onto *C. difficile* ChromID agar (bioMerieux). All plates were incubated in an anaerobic chamber (A35, 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 were identified as described previously (Putsathit et al., 2015).

2.3. Molecular characterisation of C. difficile isolates

All *C. difficile* from Thailand and a subset of strains from Malaysia were screened by in-house PCRs for the presence of toxins A and B genes (*tcdA* and *tcdB*, respectively), and binary toxin genes (*cdtA* and *cdtB*) (Putsathit et al., 2015), and PCR ribotyping was performed as previously described (Knight et al., 2013). Isolates that could not be identified with the reference library were designated with an internal nomenclature, prefixed with QX.

2.4. Whole genome sequencing

A subset of strains underwent WGS and were investigated by *in silico* multi-locus sequence typing (MLST) and core genome single nucleotide variant (cgSNV) analysis, as previously described (Knight et al., 2016). For cgSNV analysis, *C. difficile* strain 630 (sequence type [ST] 54, clade 1, accession AM180355) was used as a reference. WGS data have been submitted to the European Nucleotide Archive under study PRJEB32765 [sample accessions ERS3466610 (isolate I0020), ERS3466611 (MP001), ERS3466612 (TAP005), and ERS3466613 (THP196)].

3. Results

3.1. Prevalence and molecular epidemiology of C. difficile in piglets and piggery in Thailand

A total of 165 piglet rectal swabs were collected from five piggeries located in central and eastern provinces of Thailand, Chonburi (Farm T1), Ratchaburi (Farms T2-T3) and, Nakhon Pathom (Farms T4-T5) (Table 1). In total, 53 (32.1%) specimens were positive for *C. difficile* by direct culture and an additional five (3.0%) specimens grew *C. difficile* only after enrichment. Among farms, the highest prevalence was observed for Farm T3 (82.4%; 28/34), followed by Farms T2 (48.1%; 13/27), T1 (23.3%; 7/30), T5 (20.5%; 9/44) and T4 (3.3%; 1/30). All 58 *C. difficile* isolates were non-toxigenic (Table 2) and the majority belonged to RT 038 (87.9%; 51/58). The remainder belonged to RTs 010 (8.6%; 5/58), QX553 (1.7%; 1/58) and QX554 (1.7%; 1/58), all of which were isolated only from specimens collected from Farm T3 (Table 2). Age and scouring data were available for 162 animals. Piglets included aged between 1 and 23 days. When stratified by age, 25.9% (42/162), 59.3% (96/162) and 14.8% (24/162) of the piglets belonged to age groups

Table 2
C. difficile PCR ribotypes isolated from piglets in Thailand, by farm.

PCR ribotype[a]	N (%) specimens					
	Farm T1	Farm T2	Farm T3	Farm T4	Farm T5	Total
038	7 (100.0)	13 (100.0)	21 (75.0)	1 (100.0)	9 (100.0)	51 (87.9)
010	0 (0.0)	0 (0.0)	5 (17.9)	0 (0.0)	0 (0.0)	5 (8.6)
QX553	0 (0.0)	0 (0.0)	1 (3.6)	0 (0.0)	0 (0.0)	1 (1.7)
QX554	0 (0.0)	0 (0.0)	1 (3.6)	0 (0.0)	0 (0.0)	1 (1.7)
Total	7 (12.1)	13 (22.4)	28 (48.3)	1 (1.7)	9 (15.5)	58

[a] All isolates were non-toxigenic.

Table 3
PCR ribotypes of *C. difficile* isolated from the Thai piggery environment, by farm.

Farm	Sample type	Culture	PCR ribotype[a]
T1	soil	Positive	038 and QX612[b]
	water	Positive	038
T2	soil	Positive	038
	water	Positive	038
T3	soil	Positive	038
	water	Negative	–
T4	soil	Positive	QX107
T5	soil	Positive	038
	water	Positive	038

[a] All isolates were non-toxigenic.
[b] Soil sample contained two isolates.

1–7, 8–14 and 15–23 days, respectively (Table 1). The prevalence of *C. difficile* in these age groups was 45.2% (19/42), 39.6% (38/96) and 0.0% (0/24), respectively (Table 1). The decline in *C. difficile* colonisation with increasing age was significant (OR 0.88, $p = 0.001$) and the highest prevalence was seen in the 1-7-day age group. Scouring was observed in 72 (44.4%) of the piglets. *C. difficile* was more common among non-diarrhoeic than diarrhoeic piglets although this difference was not statistically significant (64.9%; 37/57 vs. 35.1%; 20/57, $p = 0.077$).

One soil sample and one effluent water sample were collected per farm, except for Farm T4 (only one soil sample). All environmental specimens (88.9%; 8/9) grew *C. difficile*, except for a water sample from Farm T3 (Table 3). A soil specimen from Farm T1 contained two isolates, RTs 038 and QX612. RT 038 (77.8%; 7/9) was the most prevalent RT and all environmental isolates were non-toxigenic (Table 3).

3.2. Prevalence and molecular epidemiology of *C. difficile* in piglets and piggery in Malaysia

A total of 59 7-day-old piglet rectal swabs were collected from Selangor (Farms M1-M4) and Perak states of Malaysia (Farms M5-M6) (Table 4). None of the piglets had diarrhoea. In total, 48 (81.4%) specimens were positive for *C. difficile* by direct culture and an additional six (10.2%) specimens grew *C. difficile* only after enrichment. All specimens from Farms M4, M5 and M6 were positive for *C. difficile*. The prevalence was 90.0% (9/10) for both Farms M2 and M3, and was 66.7% (6/9) for Farm M1 (Table 4). One soil sample and one effluent water sample were collected per farm, except for Farm M5 (only one effluent water sample and three farrowing shed swabs). All environmental specimens grew *C. difficile*, except for a water specimen from Farm M1 (prevalence of 92.9%; 13/14). Thirty isolates were selected for PCR ribotyping and toxin gene profiling, including 23 rectal swab isolates (5, 1, 4, 2, 7 and 4 from Farms M1 to 6, respectively) and 7 environment isolates (1 effluent water isolate each from Farms M2, M3, M5 and M6, 1 soil isolate from Farm M3, and 2 farrowing shed isolates from Farm M5). All of the isolates were the non-toxigenic RT 038. The RT banding patterns of *C. difficile* isolated from piglets and piggery environment are shown in Fig. 1.

3.3. Comparative genomic analysis

A total of 4 strains were investigated by WGS, including one strain each of RT 038 (the most common RT) from piglets in Thailand and Malaysia, and one strain each of RT 038 from humans in Thailand and Indonesia collected in previous studies (Collins et al., 2017; Putsathit, Maneerattanaporn, Piewngam, Kiratisin, et al., 2017a.2017b). *In silico* MLST analysis did not further differentiate the isolates with all RT 038 belonging to clade 1 ST 48. Core genome analysis showed that piglet RT 038 isolates from Thailand and Malaysia differed by only 18 cgSNVs. Moreover, these strains were, on average, only 30 cgSNVs different from the human strains from Thailand and Indonesia (Table 5).

4. Discussion

The emergence of CA-CDI (cases of CDI detected ≤48 h post admission) and the mounting evidence supporting CDI as a zoonotic disease has stimulated significant interest in finding reservoirs of *C. difficile* in the community (Bloomfield and Riley, 2016). Given the paucity of *C. difficile* epidemiological data in animals and the environment in Asia, this study aimed to describe the prevalence and molecular characteristics of *C. difficile* isolated from piglets in Thailand and Malaysia, and their surroundings.

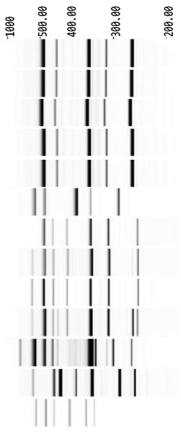
C. difficile was isolated from 35.2% and 91.5% of piglets in Thailand and Malaysia, respectively. The most commonly reported risk factors for CDI/colonisation in piglets included animal age, environmental contamination and antimicrobial exposure (Kiss and Bilkei, 2005; Moono, Foster, et al., 2016a). Neonatal piglets are highly susceptible to *C. difficile* colonisation due to the absence of an established gut flora, and *C. difficile* is most frequently observed among 1-7-day-old piglets (Songer and Anderson, 2006; Songer and Uzal, 2005). As described previously (Moono, Putsathit, et al., 2016b; Norman et al., 2009; Susick et al., 2012; Weese et al., 2010), the prevalence of *C. difficile* in piglets in Thailand significantly declined as their age increased (45.2%, 39.6% and 0.0% in piglets aged 1–7, 8–14 and 15–23 days, respectively; OR 0.88, $p = 0.001$). The high prevalence of *C. difficile* among 7-day-old piglets in Malaysia is also consistent with other studies (Moono, Putsathit, et al., 2016b; Norman et al., 2009; Susick et al., 2012; Weese et al., 2010).

Although direct comparison of prevalence data between studies cannot be made due to variations in risk factors, reports from other Asian countries suggest a similar decline in prevalence among older pigs. In Japan, 57.5% (69/120) of the < 12-day-old piglets from 12 Japanese farms tested positive for *C. difficile* (Usui et al., 2014). In Taiwan, 85.1% (114/134) of pigs from 16 piggeries were positive for *C. difficile*. The majority (64.2%; 86/134) of the pigs in the study were < 7 days old (Tsai et al., 2016). Another Taiwanese study reported 49.0% (100/204) overall prevalence of *C. difficile* among piglets from 13 commercial farms (Wu et al., 2016). When stratified, the prevalence was 52.6% (50/95), 25.0% (14/56) and 67.9% (36/53) among suckling pigs, nursery pigs and sows, respectively. The decline in prevalence with an increase in piglet age is consistent with our results.

The most striking difference between this study and studies conducted elsewhere, in Asia and other continents, was the absence of toxigenic *C. difficile*. All isolates from four out of five farms in Thailand and all Malaysian isolates were clonal (RT 038, an A-B-CDT- ST 48

Table 4
C. difficile culture results for rectal swabs collected from piglets in Malaysia, by farm.

Culture	N (%) specimens						
	Farm M1	Farm M2	Farm M3	Farm M4	Farm M5	Farm M6	Total
Positive	6 (66.7)	9 (90.0)	9 (90.0)	10 (100.0)	10 (100.0)	10 (100.0)	54 (91.5)
Negative	3 (33.3)	1 (10.0)	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (8.5)
Total	9 (15.3)	10 (16.9)	10 (16.9)	10 (16.9)	10 (16.9)	10 (16.9)	59 (100.0)



PCR ribotype	Source	Country	N (%)	Toxin profile
038	Piglet	Thailand	51/58 (87.9)[b]	A–B–CDT–
038	Piggery	Thailand	7/9 (77.8)[b]	A–B–CDT–
038	Piglet	Malaysia	23/23 (100.0)[b,c]	A–B–CDT–
038	Piggery	Malaysia	7/7 (100.0)[b,c]	A–B–CDT–
038	Human[a]	Thailand, Indonesia	2/105 (1.9), 2/74 (2.7)[d]	A–B–CDT–
QX107	Piggery	Thailand	1/9 (11.1)[b]	A–B–CDT–
QX553	Piglet	Thailand	1/58 (1.7)[b]	A–B–CDT–
010	Piglet	Thailand	5/58 (8.6)[b]	A–B–CDT–
010	Human[a]	Thailand	12/105 (11.4)[d]	A–B–CDT–
QX554	Piglet	Thailand	1/58 (1.7)[b]	A–B–CDT–
QX612	Piggery	Thailand	1/9 (11.1)[b]	A–B–CDT–
027	Human[ref]	-	-	A+B+CDT+
078	Human[ref]	-	-	A+B+CDT+

Fig. 1. PCR ribotyping patterns for *C. difficile* isolated from humans, piglets and the piggery environment in Thailand and Malaysia. Strain origin, prevalence and the corresponding toxin gene profile are also provided for each RT. [a] For comparative purposes, epidemic reference RTs 027 and 078 ([ref]), and human RTs 010 and 038 isolates are also added. [b] Data from the current study. [c] Not all Malaysian strains were typed. [d] Prevalence reported in previous human studies for Thailand (Putsathit, Maneerattanaporn, Piewngam, Kiratisin, et al., 2017a.b) and Indonesia (Collins et al., 2017). Top scale represents intergenic spacer region fragments sizes in kb.

Table 5
Pairwise core genome single nucleotide variant (cgSNV) distances between *C. difficile* RT 038 isolated from piglets and humans, and the associated epidemiological data.

I0020	MP001	TAP005	THP196	CD630	Lab ID	Origin	Year	Location
0	31	37	38	7977	I0020	Human inpatient	2014	Central Java, Indonesia
	0	18	23	7976	MP001	Piglet	2015	Selangor, Malaysia
		0	29	7982	TAP005	Piglet	2015	Ratchaburi, Thailand
			0	7983	THP196	Human inpatient	2015	Bangkok, Thailand
				0	CD630	–	–	–

lineage belonging to clade 1). Only Farm T3 exhibited greater strain diversity and an additional three RTs were recovered. These included internationally recognised non-toxigenic RT 010 and two novel RTs, both of which were non-toxigenic.

Non-toxigenic *C. difficile* was isolated from 10.0% (10/100) of the pigs in Taiwan (8.0%; 4/50 in suckling pigs, 35.7%; 5/14 in nursery pigs and 2.8%; 1/36 in sows) (Wu et al., 2016) and from 39.0% (39/100) of the piglets in Japan (RT 038 being the second most common strain, 20%; 20/100) (Usui et al., 2014). In the majority of pig studies conducted outside Asia, non-toxigenic *C. difficile* occurred at a relatively lower prevalence: 1.6% (4/245) in USA (North Carolina) (Susick et al., 2012), 5.0% (7/140) in Spain (Alvarez-Perez et al., 2009), 5.6% (1/18) in Belgium (Rodriguez et al., 2012), 13.3% (81/609) (Fry et al., 2012) − 15.3% (33/215) (Thakur et al., 2010) in USA (North Carolina and Ohio) and 16.2% (24/154) in Australia (Knight et al., 2015). The present study appears to be the first and only report with a complete lack of toxigenic *C. difficile* among piglet isolates, suggesting a possible unique *C. difficile* epidemiology among pigs in Thailand and Malaysia.

The high prevalence of non-toxigenic strain in this study is congruent with the relatively high prevalence of non-toxigenic strains observed among inpatients in Asia (15.6% or 66/422 in Thailand (Putsathit, Maneerattanaporn, Piewngam, Kiratisin, et al., 2017a,b) and 10.6%; 36/340 in Indonesia (Collins et al., 2017), including RT 038 (1.9% or 2/105 in Thailand (Putsathit, Maneerattanaporn, Piewngam, Kiratisin, et al., 2017a,b) and 2.7% or 2/74 in Indonesia (Collins et al., 2017). However, it is noteworthy that the prevalence and characteristics of non-toxigenic strains of *C. difficile* are not well understood in most of the world. CDI is a toxin-mediated disease and therefore non-toxigenic strains of *C. difficile* do not cause disease and studies usually do not report on non-toxigenic strains (Rodriguez et al., 2016). Furthermore, to detect non-toxigenic strains, culture has to be performed, a practice that is uncommon throughout the world (particularly in Asia) and confined to a handful of reference laboratories with anaerobic culture facilities. However, a high proportion of non-toxigenic strains of *C. difficile* isolated from humans in South-East Asia is resistant to multiple antimicrobials (Putsathit, Maneerattanaporn, Piewngam, Knight, et al., 2017a,b) and this knowledge may be important in an area of the world where antimicrobial misuse is a problem.

In addition, non-toxigenic strains of *C. difficile* are protective against colonisation with toxigenic strains. Oral administration of non-toxigenic *C. difficile* before challenging hamsters with a toxigenic strain reduced toxigenic *C. difficile* colonisation and increased survival (Wilson and Sheagren, 1983). This observation lead researchers to postulate that carriage of non-toxigenic *C. difficile* provides protection against subsequent CDI. Although the mechanism remains unknown, it was thought that both non-toxigenic and toxigenic *C. difficile* share the same niche and hence the presence of one can competitively exclude colonisation by another (Villano et al., 2012). Hospitalised patients colonised with *C. difficile* (either toxigenic or non-toxigenic strains) have a significantly lower risk of CDI compared to non-colonised patients in the same ward (Shim et al., 1998). Those carrying toxigenic strains were believed to be protected by the serum antibody against *C. difficile* toxin, which was higher in asymptomatic carriers compared to symptomatic patients (Kyne et al., 2000). Villano et al. (2012) orally administrated spores of non-toxigenic *C. difficile* to healthy adults. Persistent colonisation was observed on days 21–28 in 44% of the subjects, none of whom experienced serious adverse events. Oral administration of non-toxigenic *C. difficile* appeared to be a viable preventative measure against CDI in humans.

The lack of toxigenic strains suggested a lack of CDI among piglets in Thailand and Malaysia. Notwithstanding the unclear clinical impact, as previously described, colonisation with non-toxigenic strains could have a protective effect on the host. Songer et al. (2007) observed a marked increase in the number of weaned piglets per litter and higher average weaning weight among piglets that were administered spores of non-toxigenic *C. difficile* (10^6 spores orally within 24 h of birth). Oliveira Júnior et al. (2019) orally administered 1-day-old piglets in a commercial pig farm with 10^6 spores of non-toxigenic *C. difficile*, and reported a significantly lower incidence of CDI (25.0% and 7.8%, $p = 0.003$) and severity of diarrhoea among CDI cases ($p < 0.001$) in the treated compared to the control groups. Indeed, in the present study, *C. difficile* was more common among non-diarrhoeic than diarrhoeic piglets. The situation observed among piglets appears to be analogous to that seen in humans, and the presence of non-toxigenic strains in piglets and the environment could have a beneficial impact on the herd.

Contrasting our results was the high prevalence of toxigenic *C. difficile* seen in Japan and Taiwan. In Japan, 61.0% (61/100) of piglet strains were toxigenic (*tcdA* and *tcdB* positive), of which 42.6% (26/61) were binary toxin positive also (Usui et al., 2014). The third most common RT was 078 (12.0%; 12/100). Of great concern was their multiple-locus variable number tandem repeat analysis (MLVA) that revealed genetic identity between Japanese RT 078 piglet isolates and RT 078 isolates obtained from European patients, although this may indicate relatively quick clonal expansion of RT 078 around the world as described recently (Knetsch et al., 2018; Knight et al., 2019). Similar to Thailand, breeding pigs in Japan were imported from Europe and this suggests introduction of the epidemic strain *via* importation (Usui et al., 2014). This appeared to not be the case for Thailand.

WGS data revealed that RT 038 strains isolated from piglets in Thailand and Malaysia were only 18 cgSNVs apart and both were, on average, 30 cgSNVs apart from RT 038 strains isolated from humans in Thailand and Indonesia. Based on approximations of the *C. difficile* molecular clock (1–2 cgSNVs per genome per year), several studies have proposed a cut-off of ≤ 2 cgSNVs to signal a plausible clonal *C. difficile* transmission event (Knight et al., 2016). In our study, although the WGS did not indicate clonal transmission, the data implies that piglet strains from Malaysia and Thailand shared a common ancestor in the last two decades, and both strains shared a common ancestor with human strains in the past three decades. The presence of closely related strains in humans and animals further supports the growing view of the zoonotic potential of *C. difficile*.

This study provided the first data on *C. difficile* colonisation of piglets and *C. difficile* contamination of the farming environment in Thailand and Malaysia. It also provided internationally recognised *C. difficile* typing data on the isolates and demonstrated important differences between *C. difficile* epidemiology among piglets in Thailand and Malaysia compared to that seen in other countries.

Due to time and resource limitations, the study only included five farms located in three provinces of Thailand and six farms located in two states of Malaysia, and only a small number of environmental specimens was collected. It is uncertain whether the *C. difficile* colonisation dynamic would be different in farms in other parts of each country and the level of environmental contamination remains largely unexplored. Moreover, the investigated farms belonged to relatively smaller swine operations. Given the dominance of large swine enterprises, investigations involving these facilities may be more relevant from a public health standpoint. Access to these facilities, however, is limited by commercial concerns. Despite these limitations, the preliminary data obtained will assist veterinarians in animal care and researchers in designing future studies.

C. difficile was found at high prevalence in piglets and piggeries in Thailand and Malaysia. The complete lack of toxigenic strains correlated with the high prevalence of non-toxigenic *C. difficile* in humans in this region. As this is a relatively small study, the generalisability of the results remained to be confirmed. Although not found in the present study, toxigenic *C. difficile* may be present in livestock in Thailand and Malaysia. Only with further epidemiological studies will we fully understand the magnitude of the impact that *C. difficile* has on the health and well-being of humans and animals in this region.

Declaration of competing interest

TVR has received grants from Cepheid, Merck, Sanofi and Otsuka unrelated to the conduct of the study. Other authors declare no conflicts of interest relevant to this article.

Acknowledgement

This research was partly funded by Universiti Putra Malaysia through Geran Putra-Inisiatif Putra Siswazah: GPIPS/2016/9507100.

References

Alvarez-Perez, S., Blanco, J.L., Bouza, E., Alba, P., Gibert, X., Maldonado, J., Garcia, M.E., 2009. Prevalence of *Clostridium difficile* in diarrhoeic and non-diarrhoeic piglets. Vet. Microbiol. 137, 302–305.

Bauer, M.P., Notermans, D.W., van Benthem, B.H., Brazier, J.S., Wilcox, M.H., Rupnik, M., Monnet, D.L., van Dissel, J.T., Kuijper, E.J., Group, E.S., 2011. *Clostridium difficile* infection in Europe: a hospital-based survey. Lancet 377, 63–73.

Bloomfield, L.E., Riley, T.V., 2016. Epidemiology and risk factors for community-associated *Clostridium difficile* infection: a narrative review. Infect. Dis. Ther. 5, 231–251.

Collins, D.A., Gasem, M.H., Habibie, T.H., Arinton, I.G., Hendriyanto, P., Hartana, A.P., Riley, T.V., 2017. Prevalence and molecular epidemiology of *Clostridium difficile* infection in Indonesia. New Microbes New Infect. 18, 34–37.

Fry, P.R., Thakur, S., Abley, M., Gebreyes, W.A., 2012. Antimicrobial resistance, toxinotype, and genotypic profiling of *Clostridium difficile* isolates of swine origin. J. Clin. Microbiol. 50, 2366–2372.

Hensgens, M.P., Keessen, E.C., Squire, M.M., Riley, T.V., Koene, M.G., de Boer, E., Lipman, L.J., Kuijper, E.J., 2013. European Society of Clinical Microbiology and Infectious Diseases (ESCMID) Study Group for *Clostridium difficile* (ESGCD), 2012. *Clostridium difficile* infection in the community: a zoonotic disease? Clin. Microbiol. Infect. 18, 635–645.

Keel, K., Brazier, J.S., Post, K.W., Weese, S., Songer, J.G., 2007. Prevalence of PCR ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. J. Clin. Microbiol. 45, 1963–1964.

Khanna, S., Pardi, D.S., Aronson, S.L., Kammer, P.P., Orenstein, R., St Sauver, J.L., Harmsen, W.S., Zinsmeister, A.R., 2012. The epidemiology of community-acquired *Clostridium difficile* infection: a population-based study. Am. J. Gastroenterol. 107, 89–95.

Kiss, D., Bilkei, G., 2005. A new periparturient disease in eastern Europe, *Clostridium difficile* causes postparturient sow losses. Theriogenology 63, 17–23.

Knetsch, C., Connor, T., Mutreja, A., van Dorp, S., Sanders, I., Browne, H., Harris, D., Lipman, L., Keessen, E., Corver, J., Kuijper, E., Lawley, T., 2014. Whole genome sequencing reveals potential spread of *Clostridium difficile* between humans and farm animals in the Netherlands, 2002 to 2011. Euro Surveill. 19, 20954.

Knetsch, C., Kumar, N., Forster, S.C., Connor, T.R., Browne, H.P., Harmanus, C., Sanders, I.M., Harris, S.R., Turner, L., Morris, T., Perry, M., Miyajima, F., Roberts, P., Pirmohamed, M., Songer, J.G., Weese, J.S., Indra, A., Corver, J., Rupnik, M., Wren, B.W., Riley, T.V., Kuijper, E.J., Lawley, T.D., 2018. Zoonotic transfer of *Clostridium difficile* harboring antimicrobial resistance between farm animals and humans. J. Clin. Microbiol. 56, e01384–01317.

Knight, D.R., Kullin, B., Androga, G.O., Barbut, F., Eckert, C., Johnson, S., Spigaglia, P., Tateda, K., Tsai, P.J., Riley, T.V., 2019. Evolutionary and genomic insights into *Clostridioides difficile* sequence type 11: a diverse zoonotic and antimicrobial-resistant lineage of global one health importance. MBio 10, e00446–00419.

Knight, D.R., Squire, M.M., Collins, D.A., Riley, T.V., 2016. Genome analysis of *Clostridium difficile* PCR Ribotype 014 lineage in Australian pigs and humans reveals a diverse genetic repertoire and signatures of long-range interspecies Transmission. Front. Microbiol. 7, 2138.

Knight, D.R., Squire, M.M., Riley, T.V., 2015. Nationwide surveillance study of *Clostridium difficile* in Australian neonatal pigs shows high prevalence and heterogeneity of PCR ribotypes. Appl. Environ. Microbiol. 81, 119–123.

Knight, D.R., Thean, S., Putsathit, P., Fenwick, S., Riley, T.V., 2013. Cross-sectional study reveals high prevalence of *Clostridium difficile* non-PCR ribotype 078 strains in australian veal calves at slaughter. Appl. Environ. Microbiol. 79, 2630–2635.

Kyne, L., Warny, M., Qamar, A., Kelly, C.P., 2000. Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. N. Engl. J. Med. 342, 390–397.

Lim, S.C., Foster, N.F., Elliott, B., Riley, T.V., 2018. High prevalence of *Clostridium difficile* on retail root vegetables, Western Australia. J. Appl. Microbiol. 124, 585–590.

Moono, P., Foster, N.F., Hampson, D.J., Knight, D.R., Bloomfield, L.E., Riley, T.V., 2016a. *Clostridium difficile* infection in production animals and avian species: a review. Foodborne Pathog. Dis. 13, 647–655.

Moono, P., Putsathit, P., Knight, D.R., Squire, M.M., Hampson, D.J., Foster, N.F., Riley, T.V., 2016b. Persistence of *Clostridium difficile* RT 237 infection in a Western Australian piggery. Anaerobe 37, 62–66.

Norman, K.N., Harvey, R.B., Scott, H.M., Hume, M.E., Andrews, K., Brawley, A.D., 2009. Varied prevalence of *Clostridium difficile* in an integrated swine operation. Anaerobe 15, 256–260.

Oliveira Júnior, C.A., Silva, R.O.S., Lage, A.P., Coura, F.M., Ramos, C.P., Alfieri, A.A., Guedes, R.M.C., Lobato, F.C.F., 2019. Non-toxigenic strain of *Clostridioides difficile* Z31 reduces the occurrence of *C. Difficile* infection (CDI) in one-day-old piglets on a commercial pig farm. Vet. Microbiol. 231, 1–6.

Putsathit, P., Maneerattanaporn, M., Piewngam, P., Kiratisin, P., Riley, T., 2017a. Prevalence and molecular epidemiology of *Clostridium difficile* infection in Thailand. New Microbes New Infect. 15, 27–32.

Putsathit, P., Maneerattanaporn, M., Piewngam, P., Knight, D.R., Kiratisin, P., Riley, T.V., 2017b. Antimicrobial susceptibility of *Clostridium difficile* isolated in Thailand. Antimicrob. Resist. Infect. Control 6, 58.

Putsathit, P., Morgan, J., Bradford, D., Engelhardt, N., Riley, T.V., 2015. Evaluation of the BD Max Cdiff assay for the detection of toxigenic *Clostridium difficile* in human stool specimens. Pathology 47, 165–168.

Rodriguez, C., Taminiau, B., Van Broeck, J., Avesani, V., Delmee, M., Daube, G., 2012. *Clostridium difficile* in young farm animals and slaughter animals in Belgium. Anaerobe 18, 621–625.

Rodriguez, C., Van Broeck, J., Taminiau, B., Delmee, M., Daube, G., 2016. *Clostridium*

difficile infection: early history, diagnosis and molecular strain typing methods. Microb. Pathog. 97, 59–78.
Shim, J.K., Johnson, S., Samore, M.H., Bliss, D.Z., Gerding, D.N., 1998. Primary symptomless colonisation by *Clostridium difficile* and decreased risk of subsequent diarrhoea. Lancet 351, 633–636.
Songer, J.G., Anderson, M.A., 2006. *Clostridium difficile*: an important pathogen of food animals. Anaerobe 12, 1–4.
Songer, J.G., Jones, R., Anderson, M.A., Barbara, A.J., Post, K.W., Trinh, H.T., 2007. Prevention of porcine *Clostridium difficile*-associated disease by competitive exclusion with nontoxigenic organisms. Vet. Microbiol. 124, 358–361.
Songer, J.G., Trinh, H.T., Killgore, G.E., Thompson, A.D., McDonald, L.C., Limbago, B.M., 2009. *Clostridium difficile* in retail meat products, USA, 2007. Emerg Infect Dis 15, 819–821.
Songer, J.G., Uzal, F.A., 2005. Clostridial enteric infections in pigs. J. Vet. Diagn. Invest. 17, 528–536.
Susick, E.K., Putnam, M., Bermudez, D.M., Thakur, S., 2012. Longitudinal study comparing the dynamics of *Clostridium difficile* in conventional and antimicrobial free pigs at farm and slaughter. Vet. Microbiol. 157, 172–178.
Thakur, S., Putnam, M., Fry, P.R., Abley, M., Gebreyes, W.A., 2010. Prevalence of antimicrobial resistance and association with toxin genes in *Clostridium difficile* in commercial swine. Am. J. Vet. Res. 71, 1189–1194.
Tsai, B.Y., Ko, W.C., Chen, T.H., Wu, Y.C., Lan, P.H., Chen, Y.H., Hung, Y.P., Tsai, P.J., 2016. Zoonotic potential of the *Clostridium difficile* RT078 family in Taiwan. Anaerobe 41, 125–130.
Usui, M., Nanbu, Y., Oka, K., Takahashi, M., Inamatsu, T., Asai, T., Kamiya, S., Tamura, Y., 2014. Genetic relatedness between Japanese and European isolates of *Clostridium difficile* originating from piglets and their risk associated with human health. Front. Microbiol. 5, 513.
Varshney, J.B., Very, K.J., Williams, J.L., Hegarty, J.P., Stewart, D.B., Lumadue, J., Venkitanarayanan, K., Jayarao, B.M., 2014. Characterization of *Clostridium difficile* isolates from human fecal samples and retail meat from Pennsylvania. Foodborne Pathog. Dis. 11, 822–829.
Villano, S.A., Seiberling, M., Tatarowicz, W., Monnot-Chase, E., Gerding, D.N., 2012. Evaluation of an oral suspension of VP20621, spores of nontoxigenic *Clostridium difficile* strain M3, in healthy subjects. Antimicrob. Agents Chemother. 56, 5224–5229.
Weese, J.S., 2010. *Clostridium difficile* in food-innocent bystander or serious threat? Clin. Microbiol. Infect. 16, 3–10.
Weese, J.S., Wakeford, T., Reid-Smith, R., Rousseau, J., Friendship, R., 2010. Longitudinal investigation of *Clostridium difficile* shedding in piglets. Anaerobe 16, 501–504.
Wilson, K.H., Sheagren, J.N., 1983. Antagonism of toxigenic *Clostridium difficile* by nontoxigenic *C. difficile*. J. Infect. Dis. 147, 733–736.
Wu, Y.C., Lee, J.J., Tsai, B.Y., Liu, Y.F., Chen, C.M., Tien, N., Tsai, P.J., Chen, T.H., 2016. Potentially hypervirulent *Clostridium difficile* PCR ribotype 078 lineage isolates in pigs and possible implications for humans in Taiwan. Int. J. Med. Microbiol. 306, 115–122.