# Molecular epidemiology of *Clostridium difficile* isolated from piglets in Thailand and Malaysia

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## INTRODUCTION

*Clostridium difficile* is an important enteric pathogen of neonatal pigs[1]. Infection with *C. difficile* often occurs following a disruption in the gut flora, particularly after exposure to antimicrobial agents. In Asia, antimicrobial use in livestock remains a common practice[2]. Notwithstanding an increase in the number of publications relating to the epidemiology of *C. difficile* in animals and the environment in the recent years, such information is still largely unavailable for South-East Asian countries[2]. Given the high prevalence of indiscriminate and inappropriate use of antimicrobials in animals, it is highly probable that *C. difficile* is relatively common.

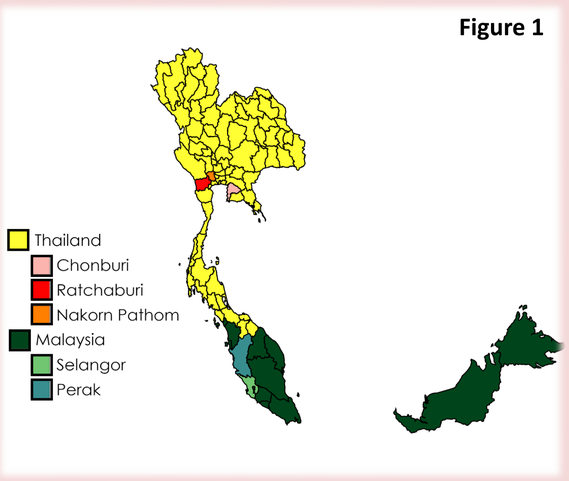
## STUDY OBJECTIVE

This study aimed

- to investigate the prevalence of *C. difficile* 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 in the region.

## MATERIALS & METHODS

**Collection of rectal swabs and epidemiological data**




A total of 165 and 59 piglet rectal swabs were obtained from piggeries located in Thailand (Chonburi, Ratchaburi and Nakhon Pathom provinces) and Malaysia (Perak and Selangor states), respectively, between September 2015 and June 2016 (**Figure 1**). Specimens were obtained from a minimum of six different litters per farm via random selection. To investigate environmental contamination with *C. difficile* in piggeries, soil (ca. 20 g) and effluent water samples (ca. 20 ml) from each farm were collected from sites surrounding farrowing sheds. Age of the piglets sampled was also recorded.

**Toxigenic culture**

Both direct (on ChromID agar) and enrichment cultures (in supplemented Robertson's cooked meat broth) were performed on the rectal swabs, and enrichment culture (in supplemented brain heart infusion broth) was performed on the environmental specimens as previously described[3]. PCR assays were performed on all isolates to determine the presence of *tcdA, tcdB, cdtA* and *cdtB*[3]. PCR ribotyping was performed on all isolates from Thailand and a subset of isolates (*n*=30) from Malaysia[4].

**Whole genome sequencing (WGS)**

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[5]. 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)].

## CONCLUSIONS

- This is the first report of the sole presence of non-toxigenic *C. difficile* strains in animals. Non-toxigenic strains occupy the same niche in the intestinal tract as that occupied by toxigenic strains[11]. Their dominance could protect the host against *C. difficile* infection via a competitive exclusion mechanism.
- WGS data did not indicate clonal transmission between humans and piglets, however, it implies that both piglet strains shared a common ancestor with the 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*.

## KEY RESULTS & DISCUSSION

**Prevalence of *C. difficile* in piglets and the environment**

- Overall, *C. difficile* was recovered from 35% (58/165) of piglets in Thailand.
- When stratified by age, 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). This correlates with the establishment of a healthy gut flora as the animal ages[6].
- *C. difficile* was recovered from 92% (54/59) of piglets in Malaysia, all of which were aged 7 days. The high prevalence of *C. difficile* among 7-day-old piglets is consistent with other studies[6].
- *C. difficile* was isolated from 8 of 9 environmental specimens (89%) from Thailand and all of the environmental specimens (*n*=14) from Malaysia.

**Molecular characteristics of *C. difficile* strains**

- All isolates in this study were non-toxigenic.
- The most common ribotype (RT) was 038 (ST48) accounting for 88% (51/58) and 78% (7/9), respectively, of piglet and environmental isolates from Thailand, and all (23 piglet and 7 environmental isolates) from Malaysia.
- Examination of the literature showed that non-toxigenic strains were found at relative higher prevalences among piglets in Asia (8-39%)[7-8] compared to piglets in North America, Europe and Australia (2-16%)[9-11].
- Our results contrast the high prevalence of toxigenic strains among piglets in other Asian countries (61% in Japan[8], 92% in Taiwan[7]).

**Comparative genomic analysis**

- *C. difficile* RT038 was also found at low prevalence in humans in Thailand (2%; 2/105)[12] and Indonesia (3%; 2/74)[13].
- We analysed 4 strains of *C. difficile* RT038 by WGS and found that piglet strains from Thailand and Malaysia were only 18 cgSNVs apart and both were, on average, 30 cgSNVs apart from RT038 strains isolated from humans in Thailand and Indonesia (**Table 1**).
- Based on approximations of the *C. difficile* molecular clock (1-2 cgSNVs per genome per year)[5], the WGS data did not indicate clonal transmission. However, 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.

**Table 1 Pairwise core genome single nucleotide variant distances between *C. difficile* RT038 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 | - | - | - |

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