Emergence of predominantly community-associated *Clostridium difficile* PCR ribotype 012 in Western Australia: Risk factors and relatedness to strains from Asia

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Background and aims

Since 2013, Western Australia (WA) has experienced an emergence of predominantly community-associated *C. difficile* infection (CA-CDI) caused by PCR ribotype (RT) 012. *C. difficile* RT 012 was first identified in WA in September 2012. This previously uncommon RT quickly rose to the equal seventh most prevalent RT in 2013 and the equal second most prevalent RT by the end of 2014 (unpublished data). The increased numbers of *C. difficile* RT 012 cases in WA are shown in **Figure 1**.

Following the isolation of *C. difficile* RT 012 from WA-grown organic beetroot in April 2015 [1], foodborne transmission of RT 012 was suspected. In response, a retrospective case-control study was carried out to determine whether consumption of certain vegetables or exposure to certain environmental factors increased the risk of CDI caused by RT 012. While this case-control study was taking place, a prevalence study in the Asia-Pacific region performed by our research group identified RT 012 as one of the most common RTs causing human CDI in Asia (16.7% in Hong Kong, 13.6% in Singapore, 11.4% in China, 6.8% in Taiwan and 5.3% in Thailand) [2]. Subsequently, the relatedness of Australian food and clinical RT 012 isolates to Asian clinical RT 012 was investigated using whole-genome sequencing (WGS) and high-resolution core genome phylogenetic analysis.

Materials and methods

A retrospective case-control study on emerging *C. difficile* RT 012 infection was conducted in WA to determine whether consumption of certain vegetables or exposure to certain environmental factors increased the risk of CDI caused by RT 012. Cases were defined as hospital patients who previously had RT 012 cultured from their stool samples and confirmed by molecular typing as previously described [1], between January 2014 and March 2015. Each case was matched with two controls for age (± 2 y) and gender. Controls were hospital patients identified in the same period of time, who previously had CDI caused by a RT other than RT 012. Statistical analysis was performed using SPSS. Characteristics of cases and controls were compared using Fisher's exact test. Univariate odd ratios (ORs) were calculated and used to identify risk factors for *C. difficile* RT 012 infection.

WGS and core genome single-nucleotide variant (SNV) analysis were performed on 11 *C. difficile* RT 012 strains (**Table 2**). Genomic DNA was extracted using lysing matrix B (MP Biomedicals) and the QuickGene DNA tissue kit S (Kurabo). Multiplex paired-end (PE) genome libraries were constructed using standard Nextera XT protocols (Illumina Inc.) and sequencing was performed on a MiSeq platform (Illumina) that generated 250 reads. WGS data was assembled and annotated, and *in silico* multilocus sequence typing (MLST), phylogenetic analysis and comparison of SNVs were performed as described [3].

Results

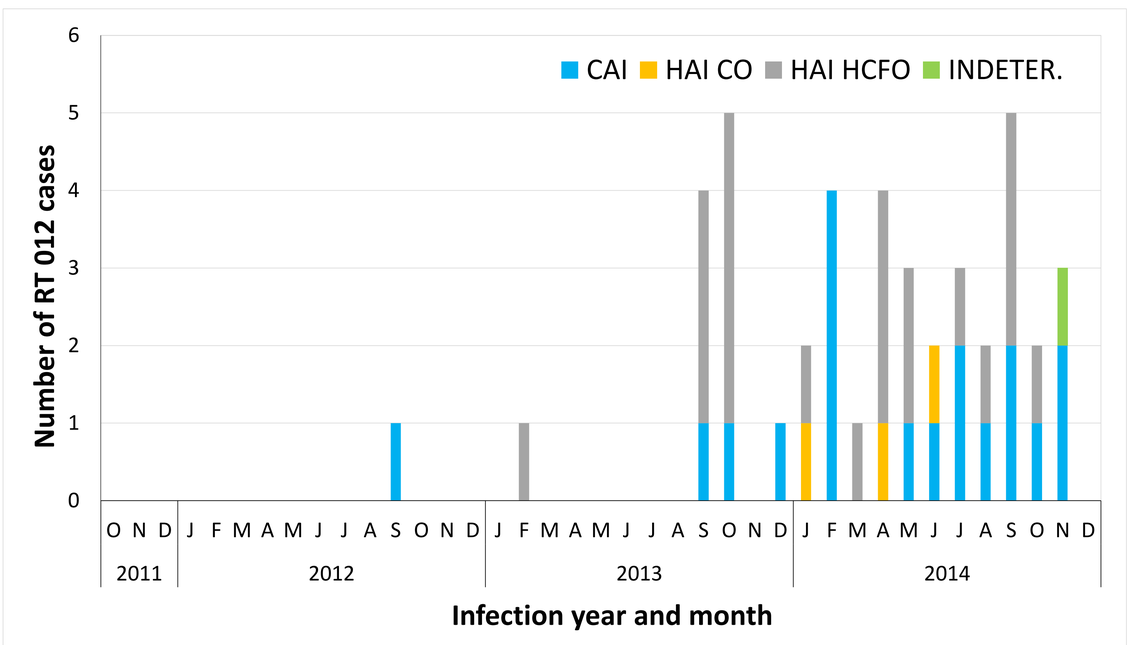

Figure 1 *C. difficile* RT 012 cases in WA in 2011 – 2014.

CAI, community-associated infection; HAI CO, hospital-associated infection community onset; HAI HCFO, hospital-associated infection healthcare facility onset; INDETER, indeterminate. Cases were defined according to McDonald *et al.* [4].

Table 1 Characteristics of CDI patients with RT 012 (cases) and with other RTs (controls).

Characteristic	Cases (*n* = 4)	Control (*n* = 19)	*p*-value[†]
Female	3 (75.0)	14 (73.7)	1.00
Median age (IQR)	71.0 (61.5 – 75.3)	69.0 (59.5 – 79.5)	0.84
Residence: Metro	3 (75.0)	14 (73.7)	1.00

CASE-CONTROL STUDY

- In total, 155 potential participants were contacted by questionnaire.
- Only 23 individuals responded to the survey and were enrolled in the study, a response rate of 14.8%.
- No significant differences in demographic characteristics were observed between those that were enrolled and those that were not enrolled.
- *C. difficile* RT 012 cases were more likely to be living in rural areas (65.4%, 34/52) compared to other CDI cases (26.2%, 27/103), although these differences did not reach statistical significance (Fisher exact $p = 0.08$).
- There were no significant differences in age, gender or area of residence between enrolled cases and controls (**Table 1**).
- By univariate analysis, eating raw non-organic home-grown vegetables (cases 75.0% vs. control 15.4%) and having contact with an individual that was living in a nursing home (cases 75.0% vs. control 15.4%) were associated with *C. difficile* RT 012 (OR 16.50, 95% CI 1.09 – 250.18 for both).
- Multivariate analysis was not performed due to the small number of enrolled cases and the broad confidence intervals for the only two significant variables.

DISCUSSION AND CONCLUSION

- Following the commencement of this case-control study, *C. difficile* RT 012 was isolated from compost and public lawn in WA [5]. However, with an overall prevalence of just 1.1% (3/274) among Australian foods [1] and environmental [5] isolates and a complete absence in Australian production animals [6-8], local establishment and wild-spread dissemination of *C. difficile* RT 012 in the community seems unlikely.
- While the prevalence of *C. difficile* RT 012 across Europe is relatively low (between 2.0% – 4.0%) [9, 10], studies since 2016 have shown that RT 012 is among the most prevalent RTs in Asia, accounting for nearly 20.0% of all CDI cases [2, 11, 12].
- It is possible that *C. difficile* RT 012 may be acquired from Asia where the prevalence is high, either ingested by returned travellers and/or via local foodborne transmission through seafood imported from Asia.
- SNV analysis (**Figure 2**) lends some support to the hypothesis that *C. difficile* RT 012 may have been acquired from Asia.

Table 2 *C. difficile* RT 012 collection for WGS.

Lab ID	Host	Sample type	Country	Source	CDI exposure	Sample date
FI 032	Food	Beetroot	Australia	WA	-	Apr-2015
WA2952	Human	Adult-CDI	Australia	WA	CAI	Feb-2014
WA3410	Human	Adult-CDI	Australia	WA	CAI	Jul-2014
WA3835	Human	Adult-CDI	Australia	WA	HAI-HCFO	Nov-2014
SQ477	Human	Adult-CDI	Australia	SA	UNK	May-2013
OT0066	Human	Adult-CDI	Hong Kong	Hong Kong	UNK	Jul-2014
OT0088	Human	Adult-CDI	Taiwan	Kaohsiung	UNK	Jul-2014
OT0104	Human	Adult-CDI	Singapore	Changi	UNK	Aug-2014
OT0171	Human	Adult-CDI	Thailand	Bangkok	UNK	Sep-2014
OT0184	Human	Adult-CDI	South Korea	Daejeon	UNK	Sep-2014
OT0405	Human	Adult-CDI	China	Shanghai	UNK	Nov-2014

WA, Western Australia; SA, South Australia; CAI, community-associated infection; HAI-HCFO, hospital-associated-healthcare facility onset; UNK, unknown.

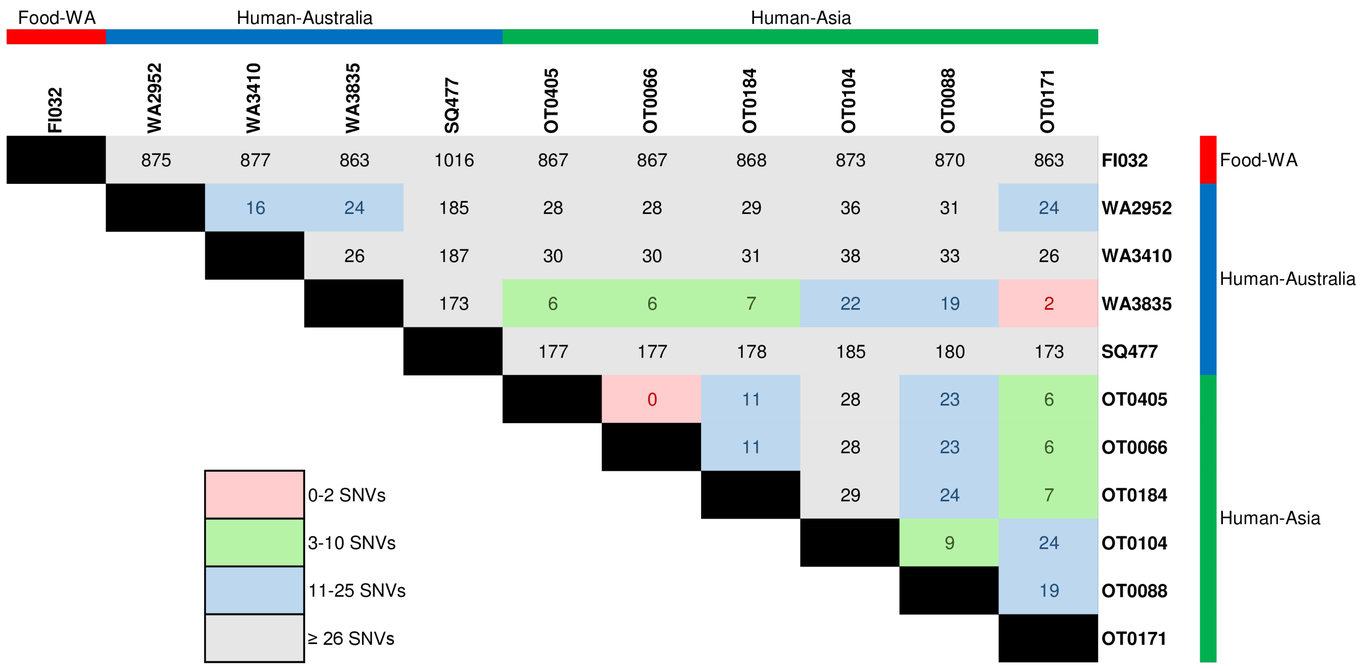

Figure 2 Core genome SNV distance between 11 *C. difficile* RT 012.

WGS AND COMPARATIVE GENOMICS

MLST

- All 11 *C. difficile* RT 012 belong to sequence type 54 within clade 1.

***In silico* antimicrobial resistance (AMR) profile**

- Of the 11 sequenced isolates, AMR genes were identified in 72.7% (*n* = 8) of the isolates.
- AMR genes encoding resistance to tetracycline (*tetM*), aminoglycosides (*Ant6-Ia, Aac6-Aph2, Sat4A* and *Aph3-III*), and macrolides-lincosamide-streptogramins (*ermB*) were detected in a food isolate from WA (FI032).
- Of the 4 human isolates from Australia, only WA3835 carried AMR genes (*ermB*, *tetM, Aac6-Aph2*).
- The human isolates from China, Hong Kong, Korea and Thailand (OT0405, OT0066, OT0184 and OT0171, respectively) carried genes encoding resistance to aminoglycosides (*Aac6-Aph2*) and macrolides-lincosamide-streptogramins (*ermB*).
- The Singapore (OT 0104) and Taiwan (OT0088) isolates were carrying *ermB*, *tetM* and *Aac6-Aph2* genes.

SNVs analysis

- A heatmap of pairwise SNV differences between the 11 genomes is shown in **Figure 2**.
- Two clonal groups were observed. First, a human isolate from Shanghai, China (OT0405) had zero SNV differences with a human isolate from Hong Kong (OT0066). These were isolated 5 months apart and separated by 1,226 km. Second, a human isolate from WA (WA3835) had a clonal relationship of ≤ 2 SNVs difference in their core genome with a human isolate from Thailand (OT0171).
- The food isolate from WA was genetically very distant from all other sequenced isolates, with an average SNV difference of 884.

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