Reservoirs of *Clostridium difficile* in the community

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Background

Clostridium difficile is a spore-forming anaerobe bacterium that is a well-known cause of infectious diarrhoea in hospitalised patients. Since 2003, there has been a change in the global epidemiology of *C. difficile* infection (CDI)[1]. Strains of hypervirulent *C. difficile* have emerged and spread throughout North America and Europe, causing significant morbidity and mortality[1]. Currently, the global incidence of previously rare community-associated CDI (CA-CDI) has also risen to as high as 41%[2].

Reports of *C. difficile* being isolated from production animals, retail meats, vegetables and the environment in Europe and North America have raised concerns regarding foodborne and environmental transmission of *C. difficile*[3].

In previous studies, we found *C. difficile* in 30% of root vegetables[4], 27% of compost (unpublished data) and 59% of lawn[5] samples. Many of the isolated ribotypes (RTs) were also common in production animals and humans in Australia, such as RT 014 and RT 056 (**Figure 1**). The isolation of indistinguishable RTs suggests possible foodborne and environmental transmission, however, in Australia the assessment of genetic overlap has been limited to low resolution genotyping tools such as analysis of the 16S – 23S rRNA intergenic spacer region. In this study, to detect evidence of potential transmission routes, whole-genome sequencing (WGS) and high resolution core genome phylogenetics were performed on a collection of *C. difficile* RT 056 from WA of human, food and environmental origins.

Materials and methods

WGS and single-nucleotide variants (SNVs) analysis were performed on 29 *C. difficile* RT 056 of human (*n* = 21), food (*n* = 4) and environmental (*n* = 4) origins. 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. Sequencing yielded a median PE read count of 99% ≥ Q30, resulting in a theoretical coverage of 99× across all isolates. WGS data was assembled and annotated as previously described[6]. *In silico* multilocus sequence typing (MLST), phylogenetic analysis, comparison of SNVs and agar dilution were performed as described[6].

Results and discussion

Table 1 Antimicrobial susceptibility of 29 *C. difficile* RT 056.

Agent	Breakpoints† S	I	R	MIC range (mg/L)	MIC50 (mg/L)	MIC90 (mg/L)	GM (mg/L)	Resistance % (*n*)	Non-resistance % (*n*)
Fidaxomicin	-	-	≥ 1	0.002 - 0.12	0.03	0.06	0.03	0 (0)	100 (29)
Vancomycin	≤ 2	-	> 2	1 - 2	2	2	1.86	0 (0)	100 (29)
Metronidazole	≤ 2	-	> 2	0.12 - 0.5	0.25	0.25	0.26	0 (0)	100 (29)
Rifaximin	-	-	≥ 32	0.002 - 0.008	0.004	0.004	0.004	0 (0)	100 (29)
Clindamycin	≤ 2	4	≥ 8	0.12 - > 32	4	8	4.41	51.7 (15)	48.3 (14)
Erythromycin	-	-	> 8	0.5 - > 256	1	2	1.45	10.3 (3)	89.7 (26)
Amoxicillin/clavulanate	≤ 4	8	≥ 16	0.25 - 0.5	0.5	0.5	0.45	0 (0)	100 (29)
Moxifloxacin	≤ 2	4	≥ 8	1 - 2	2	2	1.86	0 (0)	100 (29)
Meropenem	≤ 4	8	≥ 16	2 - 4	4	4	3.23	0 (0)	100 (29)
Tetracycline	≤ 4	8	≥ 16	0.06 - 0.5	0.25	0.5	0.21	0 (0)	100 (29)

† Breakpoints are recommended by EUCAST[17] and CLSI[18].

MLST

- All 29 *C. difficile* RT 056 belong to sequence type 34 within clade 1.
- All sequence types display allelic conservation in the seven housekeeping genes (*adk*1, *atpA*5, *dxr*7, *glyA*1, *recA*1, *sodA*3 and *tpi*1).

In silico and *in vitro* antimicrobial resistance (AMR) profile

- Of the 29 sequenced isolates, only a single human isolate (WA0707) was carrying an antimicrobial resistance gene (*ermB*).
- Summary antimicrobial susceptibility data are presented in **Table 1**.
- All isolates were fully susceptible to the first-line treatment agents (fidaxomicin, vancomycin and metronidazole).
- *In vitro* antimicrobial susceptibility profiles were largely in agreement with the results of resistance gene profiling.
- While 15/29 (51.7%) isolates displayed phenotypic resistance against clindamycin, all but WA0707 had a MIC of only 8 mg/L.
- Two isolates (WA3223 and WA3461) showed phenotypic resistance to erythromycin (MIC = 128 mg/L) with no known AMR gene. This is consistent with increased reports of *ermB*-negative *C. difficile* resistance to macrolide-lincosamide-streptogramin B from Europe and North America.

SNVs analysis

- A heatmap of pairwise SNV differences is shown in **Figure 2**.
- Three clonal groups (CGs) were identified (**Figure 3**).
- CGs II and III include clusters of human and food/compost strains.
- 19% of human strains showed a clonal relationship with one or more food/compost strains.
- CG I comprised of two human isolates collected 2-year apart (Sep-12 – Sep-14). Both were HA-CDI cases but originated from different hospitals, 13.0 km apart.
- CG II comprised of two human and one food (organic potato) isolates collected over 2.5-year period (Feb-12 – Aug-15).
- CG III comprised of two human, two compost and one food (organic potato) isolates collected over a 3-year period (Jan-13 – Dec-15).
- Half of the human isolates within CGs II and III were classified as CA-CDI with no recent healthcare exposure but were very closely-related.
- The occurrence of closely-related (≤ 2 SNVs) human, food and environmental strains, collected 2 – 3 years apart, strongly suggest that over an extended period of time there has been frequent long-range transmission of *C. difficile* RT 056 from food and the environment to humans.
- The food and/or environmental isolates in CGs II and III were collected 2 – 3 years after the corresponding human isolates. This could infer directionality but it is important to note that food and environmental *C. difficile* are generally assumed to be in spore form, during which evolution is effectively suspended in time.

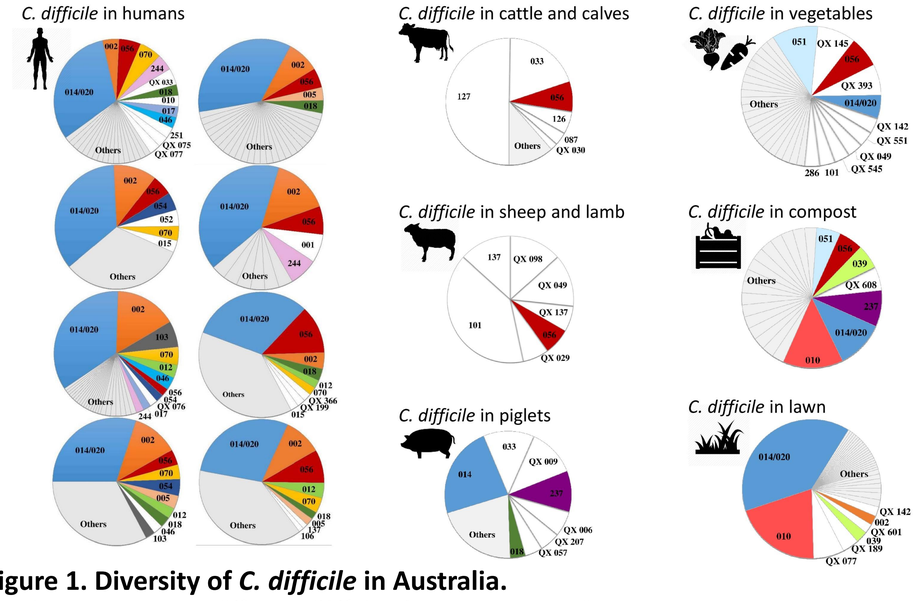

Figure 1. Diversity of *C. difficile* in Australia.
Data from previous human[7-13], animal[14-16], food[4] and environmental[5] studies.

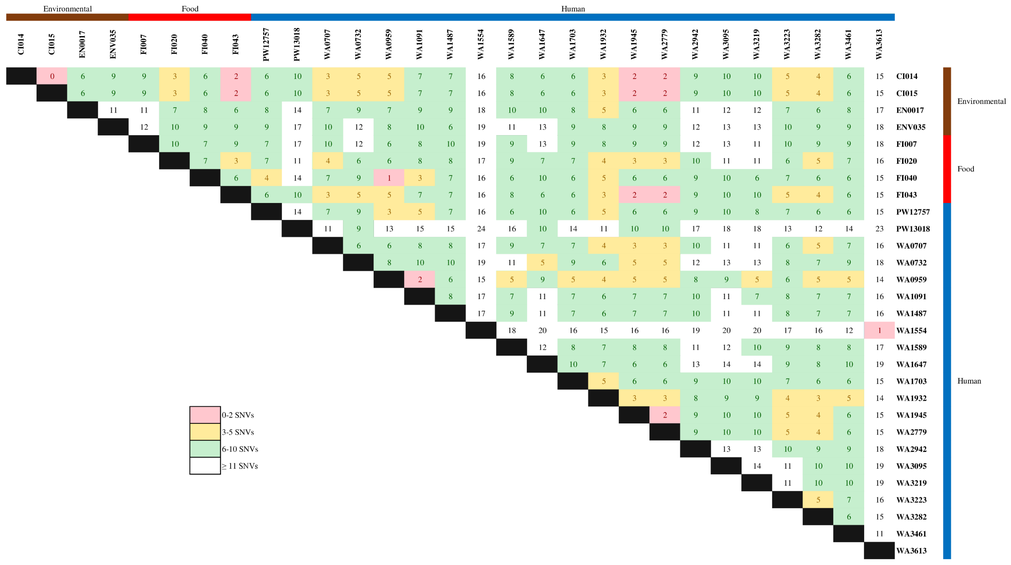

Figure 2 Core genome SNV distances between 29 *C. difficile* RT 056.

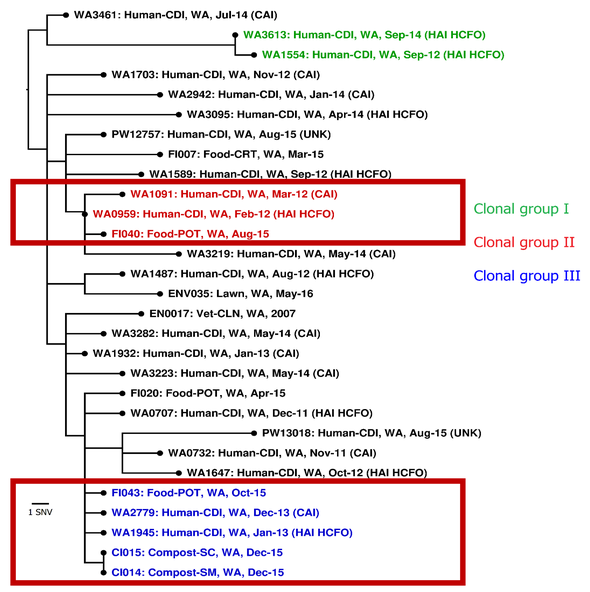

Figure 3 SNV analysis of 29 *C. difficile* RT 056.
Taxa labels include ID: origin, state, isolation date and acquisition status (if known). CRT, carrot; POT, potato; CLN, clinic; SC, soil conditioner; SM, sheep manure. Three clonal groups where isolates differ by ≤ 2 SNVs are colored green, red and blue.

Conclusion

- This study demonstrate multiple clonal populations of human and food/environmental *C. difficile* strains in WA, indicative of a recent shared common ancestry and supports the hypothesis of foodborne and environmental transmission of *C. difficile*.
- Performing WGS and high resolution core genome phylogenetics on a larger collection of human, animal, food and environmental isolates would paint a clearer picture of transmission.

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