Genomic investigation of a sequence type 67 *Clostridium difficile* causing community-acquired fulminant colitis in Hong Kong

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ABSTRACT

In 2017, we identified a *Clostridium difficile* strain HKCD4 that caused community-acquired fulminant colitis in a previously healthy child. Phylogenetically, it belonged to clade 2, sequence type 67 and was resistant to fluoroquinolone and tetracycline. The strain was pathogenicity locus and binary toxin positive. It has a mutation in the trehalose repressor *treR* leading to the L172I substitution that was previously reported in the epidemic ribotype 027 lineage. HKCD4 has a *tcdB* sequence that shared very high identities with 3 highly virulent reference strains. It has a CpG depleted genome that is characteristic of hypervirulent *C. difficile*. The emergence of ST67 lineage with molecular feature of hypervirulence in the community is concerning and emphasizes the need for full characterization of strains causing severe disease in patients without classical risk factors.

1. Introduction

Clostridium difficile (also now named *Clostridioides difficile*) infection (CDI) is the most common cause of antibiotic-associated diarrhea and colitis. Since the early 2000s, the incidence and severity of CDI have increased, both in community and hospital settings (McDonald et al., 2018). In the United States and in Europe, this is partly caused by the emergence and spread of two virulent ribotypes 027 and 078 (Collins et al., 2018; Couturier et al., 2018; Dingle et al., 2014). Data involving the epidemiology of CDI in Asia are limited. In Hong Kong, the *C. difficile* ribotype 027 was first identified in 2008 (Cheng et al., 2011). Two recent studies have shown that the ribotypes of *C. difficile* in our locality were diverse (Cheng et al., 2011; Chow et al., 2017). Ribotype 002 was the most common ribotype identified, comprising 10−13% of all isolates (Cheng et al., 2011; Chow et al., 2017). The other ribotypes that occurred at >5% frequencies included 012, 014, 017 and 020. Only two of the 629 isolates were of ribotype 027, and ribotype 078 remained unobserved (Cheng et al., 2011; Chow et al., 2017). No major outbreaks of CDI have occurred in our locality and the great majority of CDI were healthcare-associated, affecting patients with underlying conditions (Cheng et al., 2015). In an analysis of a public hospitals database, only 5% of the 15,753 CDI cases during 2006−2014 were classified as community-acquired (Ho et al., 2017). Severe CDI cases were rare, and restricted to patients with major comorbidities (Cheng et al., 2011; Wong et al., 2016).

CDI in children usually occurs in the healthcare settings and involving those with severe underlying diseases (Noor and Krilov, 2018). Recently, we encountered a case of community-acquired, fulminant *C. difficile* colitis in a previously healthy, 6-year-old girl. In PCR ribotyping, the isolate could not be assigned to any of the ribotypes commonly encountered in our locality (Cheng et al., 2011). In view of the unusual severity of the *C. difficile* disease; the isolate was investigated further by whole genome sequencing in this report.

2. Methods

2.1 Patient description

In 2017, a previously healthy, 6-year-old girl was admitted with a 4-day history of abdominal pain with repeated vomiting and diarrhea which was treated by a general practitioner with anti-motility agents including atropine, diphenoxylate, dimenhydrinate and methylscopolamine. Five days prior to the onset of diarrhea, she had an episode of upper respiratory tract infection with fever, cough and sputum, thus was given a course of amoxicilin-clavulanate by another general practitioner.

On arrival, the abdominal X-ray showed a markedly dilated colon. Blood tests showed leukocytosis (58 x 10^9/L) and a 2 fold increase in creatinine (93 µmol/L, normal: 19-59 µmol/L). In the subsequent 24 hours, she deteriorated rapidly with septic shock and toxic megacolon, and necessitated intensive care unit admission. CT scan of the abdomen showed ascites, ileus and colonic dilations (supplementary file, Figure S1). The disease continued to progress despite ileostomy for decompression and treatment with metronidazole, vancomycin and meropenem. Therefore, subtotal colectomy was performed on post-admission day 2. Multiple stool and ileostomy output samples were positive for *C. difficile* by culture, nucleic acid testing, with toxin production confirmed by cytotoxin assays. Histological examination of the resected colon demonstrated features consistent with pseudomembranous colitis. Eventually, the patient improved and was discharged from the ICU on day 19 and sent home on day 27.

2.2 Microbiological studies

Stool samples were cultured using chromID *C. difficile* chromogenic agar (CCFA, Oxoid, UK). A Bruker MALDI-TOF system was used for bacterial identification (Chen et al., 2017). Antimicrobial susceptibility against metronidazole and vancomycin were performed

using Etest strips (BioMerieux Inc., USA) (Cheng et al., 2011). Stool samples were tested by the VIDAS *C. difficile* Toxin kit (BioMerieux Inc., USA) and the cell culture cytotoxicity neutralization assay (Cheng et al., 2011). Besides stool samples, stationary-phase *C. difficile* culture supernatant was tested by the VIDAS *C. difficile* Toxin kit and the test value was used as proxy to estimate the level of production (Cheng et al., 2011). Two strains, including a locally prevalent *C. difficile* ribotype 002 (RT002) and the ribotype 027 ATCC 1870 (RT027) were included for comparison.

2.3 Whole genome sequencing and bioinformatics

An isolate from a stool sample of the patient was sequenced using an Illumina platform at the Genome Research Center of the University of Hong Kong at >200 fold coverage. A commercial software package (CLC Genomics Workbench 9.01) was used for *de novo* assembly and further improved using a Sanger pipeline (Page et al., 2016). The genome of strain CD630 (ST54/ribotype 012, GenBank AM180355.1) and R20291 (ST1/ribotype 027, GenBank FN545816) were used as references in the analysis. Accessory genomic elements were identified and annotated by a previously described method (Kociolek et al., 2018). Online databases, including the ResFinder 3.0 and the CARD (comprehensive antibiotic resistance database) v3.0.0 database were used to identify and annotate acquired resistance genes and chromosomal mutations associated with resistance determinants, respectively (Cheng et al., 2019).

To place our *C. difficile* strain into the context of the population clades (Janezic et al., 2018), 109 completed and draft genomes deposited in the GenBank were downloaded and the sequence type (ST) of each assigned using the *C. difficile* PubMLST database (https://pubmlst.org/). The genomes were further analyzed for variant sites using ParSNP v1.1.2. Afterwards, a maximum likelihood phylogenetic tree was constructed and edited using iTOL (http://itol.embl.de). Metadata of all genomes were retrieved from GenBank

using in-house python scripts (Cheng et al., 2019). The CpG content for all genome sequences and coding DNA sequences was calculated and indicated as a ratio as previously described (Kamuju et al., 2018). Single nucleotide polymorphisms (SNP) across the PaLoc were called by alignment to the clade 1, ST2 reference strain 217B as previously described (Lewis et al., 2017). The variant pattern of the PaLoc in HKCD4 (designated from the acronyms of the locality, Hong Kong and the species name *C. difficile* with the serial number of isolates) was compared to 12 published strains of different levels of disease severity in human and mouse models (supple file, Table S1) (Lewis et al., 2017).

The genome sequence of HKCD4 has been deposited in the GenBank under Bioproject PRJNA526488.

3. Results

The levels of toxin A/B production by ELISA in strain HKCD4, RT002 and RT027 were 1.1, 5.7 and 10.0 units, respectively. HKCD4 was susceptible to both vancomycin (MIC 0.5 μg/ml) and metronidazole (MIC 0.094 μg/ml).

The genome of strain HKCD4 was assembled. The circular chromosome has a size of 4,100,689 bp, GC content of 28.6% and it shared 99.0% identities with the reference CD630. SNP calling and phylogenetic analysis revealed that our strain belongs to clade 2 and ST67 (Figure 1). It is most closely related to three strains of ST67 (02493) or its single locus variant ST41 (00224 and 02439) originating from Oxfordshire in United Kingdom in 2008−2009 (Figure 1).

In HKCD4, the pathogenicity locus (PaLoc) has a size of ~18.5 kb and included an array of genes in the same order as in strain CD630 and sequence identity of 95.0%. These included the genes encoding toxin A and B (*tcdA* and *tcdB*) and the three putative regulatory elements (*tcdR*, *tcdE* and *tcdC*). The putative negative regulator *tcdC* was intact without any deletion. Figure 2 shows an alignment of the PaLoc sequences from HKCD4 and 12 strains

with different levels of disease severity in human and mouse models (supplementary file, Table S1). HKCD4 has a PaLoc nucleotide sequence that shared high identities with three high virulence strains of ST41 (WUp8 (98.9%) and ST1 (WUp14, 97.4% and WUp4, 97.2%). The *tcdB* gene in HKCD4 is almost identical to WUp8 over the catalytic and protease domains (nucleotide position 1-2406, 99.8% identity). The sequence identities over the translocation and receptor binding domains (nucleotide position 2407-7098) with WUp8, WUp14 and WUp4 were 97.9%, 98.5% and 98.5%, respectively (Figure 2).

The intact binary toxin locus (CdtLoc) was present with a length of ~6.2kb and a nucleotide sequence identity of 99.9% with the ribotype 027 strain R20291. In the trehalose operon, the critical substitution L172I (leucine to isoleucine) was found in transcription repressor *treR* as in strain R20291. The same L172I substitution was shared by the aforementioned three ST67/ST41 strains (02493, 00224 and 02439) from the United Kingdom. The CpG ratio of HKCD4 for complete genome sequence and coding sequence were 0.29 and 0.28, respectively.

Beside the Paloc and CdtLoc, additional accessory genomic elements included three prophages including a ϕCDHM19-like (~27.3kb) , a ϕMMP01- like (~44.4kb) and a CD27-like (~69.2kb) prophages and a Tn*916*-like transposon (~28kb) carrying a tetracycline resistance determinant. In GyrA, the fluoroquinolone resistance conferring substitution Thr82Ile was found. No mutations were found in the other chromosomal genes included in the CARD database: *gyrB*, *rpoB* and *cedA*.

4. Discussion

The isolate which caused community-acquired fulminant colitis in a healthy 6 year-old girl with preceding antibiotic exposure as the only risk factor of CDI was identified as ST67 (clade 2) with PaLoc and binary toxin. By comparison, the 002, 027 and 078 ribotypes correlate with ST8 (clade 1), ST1 (clade 2) and ST11 (clade 5), respectively (Janezic et al.,

2018). Due to limited availability of reference ribotypes, we are not able to determine the ribotype of the isolate. ST67 is rare and only a few isolates have been described in the published literature. In a study of 1290 *C. difficile* isolates from the United Kingdom during 2006−2009, only one ST67 isolate of ribotype 019 was identified (Dingle et al., 2011). In another study of 58 toxigenic *C. difficile* isolates from Thailand during 2006-2008, one isolates of ST67 (ribotype QX 319) was identified in a patient with cancer (Ngamskulrungroj et al., 2015). In both reports, no information is available on the severity of CDI caused by ST67 (Dingle et al., 2011; Ngamskulrungroj et al., 2015). In the MLST database, only one of the 1,623 *C. difficile* isolates was of ST67 (https://pubmlst.org/, last accessed on 11 March 2019).

HKCD4 contains a single point mutation in the trehalose repressor *treR* that is shared by the epidemic ribotype 027 strains (Collins et al., 2018). In the presence of trehalose, this mutation confers a competitive advantage over other lineages in growth experiments as well as increasing the severity of CDI disease in animal models (Collins et al., 2018). Sequence alignment of the trehalose operon (*treR*-*treA*) across 1010 sequenced *C. difficile* strains revealed the same mutation of L172I in all ribotype 027 strains, as well as ribotype 244 that has caused community-acquired epidemic outbreak in Australia, and ribotype 176 which has caused epidemic outbreaks in the Czech Republic and Poland, such mutation was not found in the other non-epidemic strains (Collins et al., 2018; Eyre et al., 2015; Polivkova et al., 2016). Of note, the same mutation in *treR* was identified in all ST67/ST41 strains compared in this study.

HKCD4 produced a lower level of TcdA/B toxins than the ribotype 002 and 027 strains. In the past, toxin production has been the main focus of study when addressing the virulence of *C. difficile*. However, hyperproduction of toxin is only a feature of some ribotype 027 strains and toxin production was not correlated with in vivo colonic pathology and survival (Lewis et al., 2017). Instead, certain sequence patterns of *tcdB* have been proposed

to contribute to *C. difficile* virulence. HKCD4 has a *tcdB* sequence pattern that shared very high identities with 3 highly virulent strains of ST1 (WUp4 and WUp14) and ST41 (WUp8). ST1 is a lineage that contains the epidemic ribotype 027 while ST41 is a single locus variant of ST67. Further studies should investigate whether ST67/ST41 represents another lineage comprising strains of high CDI disease potential. HKCD4 has a low CpG ratio that is also characterized the hypervirulent *C. difficile* strains (Kamuju et al., 2018). It has been proposed that CpG depletion from hypervirulent *C. difficile* ribotypes may facilitate escape from innate immune responses such as ZAP that targets CpG-containing mRNA (Ashkar and Rosenthal, 2002; Kamuju et al., 2018).

In conclusion, this study described the genomic features of a ST67 *C. difficile* strain causing community-acquired fulminant colitis. Further epidemiological studies are required to define the geographical distribution of this lineage and the spectrum of CDI that it caused.

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Conflicts of interest

None

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Figure 1. Maximum likelihood phylogenetic tree of 109 *C. difficile* isolates based on total core genome SNP without showing short branch lengths to clearly exhibit topology. The strain in this study is represented by red font. Each strain labeled with the name and sequence type (ST). Solid circles on the nodes represent credible statistical supports (>80%).

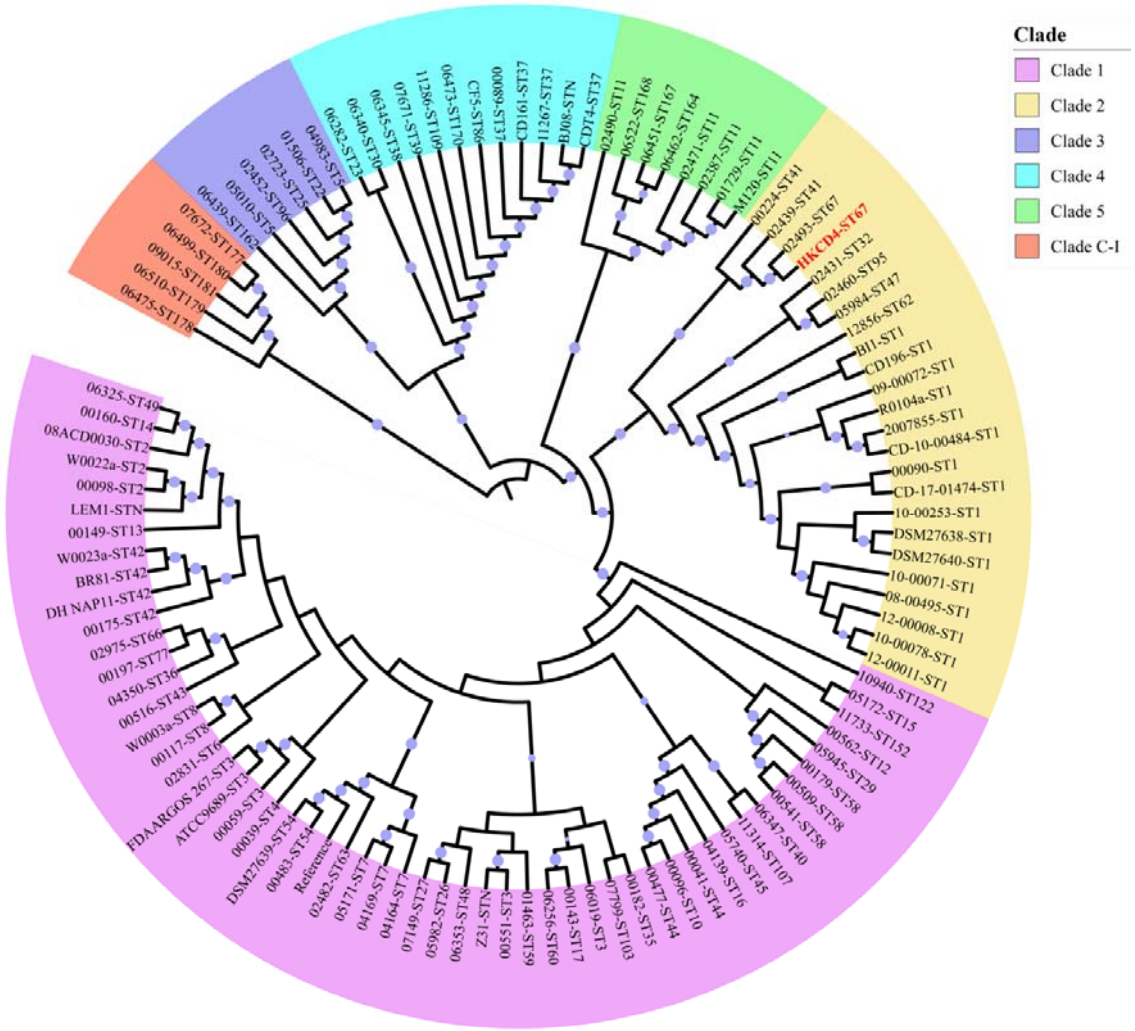

Figure 2. Analysis of *Clostridium difficile* PaLoc sequences. Single nucleotide variant differences in the PaLoc sequences, in relation to the reference strain 217B, are compared and illustrated. Each variant nucleotide is indicated by a small vertical line. The strain name, following by the sequence type (ST) was indicated on the left and colors based on the isolate's clade (red, clade 2; purple, clade 1 and green, clade 5). Sequence from our patient is on the top and the other sequences are arranged in descending order of the acute disease scores (Lewis et al., 2017), which are labeled on the right.

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