



Comparative Genomic Diversity of *Clostridium difficile* During Recurrences of Infections

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Abstract | *Clostridium difficile* is an important emerging infectious agent and might colonize in digestive tract of humans. *C. difficile* exhibits a low level of gene conservation. Consequently, antibiotic therapy result has demonstrated in 15-30% cases. In the current review we summarized up to date information about the drug resistance genotypes of *C. difficile* for the first time. Our study revealed that there was a steady difference among various genotype. Which demonstrated that mutations in the DNA genes was very common in the antibiotic resistance phenotypes. Together our data revealed that antibiotic resistivity poses selective pressure on the genome of *C. difficile* that could lead to more adoptable drug resistance strains. This study may open a new avenue for the research in understanding the mechanism of *C. difficile* adaptation to antibiotics and the development of new antibacterial. However, their further research is needed to exploring *C. difficile* genomic diversity changed from molecular typing assays to total-genome sequence comparisons and comparative genome microarrays.

Keywords | *Clostridium difficile*, Ribotypes, Whole genomic analysis, BLAST, Genome

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INTRODUCTION

Clostridium difficile (*C. difficile*) is an anaerobic, Gram-positive and spore-forming bacteria. Which is the causative factor of pseudomembranous colitis. Clinically, it shows different severity from mild diarrhea to severe and hard colitis included with toxic megacolon (Borriello, 1998; Rupnik et al., 2009). Toxin A (TcdA) and toxin B (TcdB) are the essential virulence factors of *C. difficile* (Kuehne et al., 2010; Voth and Ballard, 2005). Therefore, some strains show a dual toxin (CDT), which is related to enhanced virulence (Inns et al., 2013; McDonald et al., 2005; Schwan et al., 2009). *C. difficile* infection (CDI) is transmitted by the realization of spore through the fecal-oral route. Continue germination in small intestine, the

vegetative cells generate illness, toxins and eventually sporulation in the large intestine before being unleashed into the environment, which may make the disease of new individuals (Koenigsknecht et al., 2015; Paredes-Sabja et al., 2014; Shen, 2015). The master regulator of sporulation is Spo0A (Pereira et al., 2013). Which also might be a regulator of other supposed virulence factors (Mackin et al., 2013).

Endospores of *C. difficile* are favorable resistant to environmental stress, such as oxygen, heat, and sanitizers. Furthermore, they can stay for long period, which predisposes for nosocomial transmission. It was thought that *C. difficile* agent is trans-locating predominantly. However, the endemic spreading of this agent has

barricaded recognition of accurate sources of infection and the evaluation of the efficiency of interventions. Many occurrences of *C. difficile* infection were believed to have resulted from new possession within a health care setting. Prevention scrambles have therefore concentrated on symptomatic patients, their immediate environment, and the judicious use of antimicrobial medicine (Cohen et al., 2010; Vonberg et al., 2008). Human to human transmission of *C. difficile* agents and encompassing contamination have been widely documented (Dubberke et al., 2007; McFarland et al., 1989; Samore et al., 1994; Vonberg et al., 2008). Therefore, there are several other potential origins, including sick with asymptomatic colonization (Clabots et al., 1992; Muto, 2007), and broad environment sources, such as farm or pets animals, food and water (Hensgens et al., 2012). The contribution of occurrences that were came from these sources to the overall burden of illness is unknown, especially with enhancing reports of society associated with *C. difficile* infection.

In the previous studies assembling data from hospital registration and genotyping have been showing that transmission through the hospital and clinic-based collision with *C. difficile* patient's number was less than 25% of recent cases (Norén et al., 2004; Walk et al., 2012). Although such studies have not conclusively explained the role of symptomatic patients in transmission, they did not count for potential extend across hospitals and clinics by the travel of patients, workers and instruments (Harbarth and Samore, 2012) or for possible spread from social contacts. Horizontal transmission from symptomatic patient were the important source of several cases of the disease, and it is the basis for recent prevention guidelines (Surawicz et al., 2013).

The evaluation of hospital broad transmission with the usage of multi-locus sequence typing or ribotyping has prevented by the massive number of sicker who share a genotype and hospital contact. Nevertheless, whole-genome sequencing illustrated that considerable genetic diversity taking place, even within segregates of the same genotype (Didelot et al., 2012) to quantify the feature of symptomatic patients in the transmission of *C. difficile* leading to disease and to recognize such transmission has different over time (Eyre et al., 2013).

In current years, the incidence and mortality rates of CDI have been enhancing (Redelings et al., 2007). Moreover, the frequency of community-acquired diseases and CDI of the adult and healthy have been increasing (Kuntz et al., 2011; Lessa et al., 2015). PCR ribotyping in Europe is the standard assay for genotyping of *C. difficile* isolates. Nevertheless, whole-genome sequencing will become the assay of option in near next time. Ribotypes 001,027, 014 and 078 are the most prevalent ones in Germany (Sim et

al., 2017). Some of them, like 027 (BI/NAP1) and 078 were binary toxin positive and have related to enhanced virulence (Goorhuis et al., 2008; McDonald et al., 2005; Warny et al., 2005). Ribotype 126, which is less prevalent is considered as potentially hypervirulent since it shares 99.7% of its genes with ribotype 078 (Kurka et al., 2014). Ribotype 027 has spread around the world since its first emergence. In addition, the prevalence of hypervirulent strains continues to increase (Freeman et al., 2010; Goorhuis et al., 2008). In these futuristic two year study in China, the incidence of CDI among 276 patients with mild diarrhea was 23.1%. The absence of diagnostic testing for CDI was associated with in-appropriate management in 26.4% of patients, risk of nosocomial transmission from the absence of segregation caution, mind risk of society transmission from discharging symptomatic toxigenic *C. difficile* carriers (Zhang et al., 2016).

The overall prevalence of CDI and feasible risk factors between hospitalized patients who had watery diarrhea in Wuhan, China was 28%. The discovery of this study expects the prevalence of CDI in hospitalized patients with diarrhea is higher than what has been previously reported in the present literature (Galaydick et al., 2015). However, the available data on an association ribotypes with severe infections are contradictory (Carlson Jr et al., 2013; Goorhuis et al., 2008; Walk et al., 2012). Ribotypes 078 and 126 represent a high genetic variation compare to many other recognize ribotypes (Kurka et al., 2014). Infections with ribotype 078 strains are most usual in animals (Goorhuis et al., 2008; Hensgens et al., 2012), have illustrate that the incidence of human-to-human transmission might be overestimated (Eyre et al., 2012). Instead, the transmission may often happen via the foodborne or zoonotic routes (Hensgens et al., 2012; Rodriguez-Palacios et al., 2013). CDI was highly related to antibiotic pretreatment affecting the intestinal microbiota (symbiosis) (Borriello, 1998; Buffie et al., 2015). Therefore, huge transmission range in hospitals, enhancing infection rates, and the socioeconomic burden of CDI to the health systems steadily increasing (DePestel and Aronoff, 2013; Dubberke and Olsen, 2012; Lessa et al., 2015).

Moreover, recurrent infections are difficult to treat as the microbiome. May be insistently affected due to re-emergence association of CDI, despite successful treatment. This often leads to an ongoing cycle of symptoms, treatment, relief of symptoms and recurrence.

Epidemiological correlation among genetically dependent cases were classified as "ward contact", while incidents happened in two patients who had been present in the same hospital ward at the same time and this period of time was stable with among of patient transmission.

For happened transmission, it was supposed that cases were infectious from one week earlier diagnosis through eight weeks after the determination of the disease (Jinno et al., 2012; Sethi et al., 2010; Walker et al., 2012), with 0 to 12 weeks incubation period (Cohen et al., 2010; Walker et al., 2012). Sampled patients, considered to be infectious for eight weeks after their last positive diagnostic test. If not existed ward contact, patients might be connected by time (as above) within the same hospital or be exposure to the same ward, but with a respite of until twenty-eight days isolating the discharge of the first patient and the admission of the second patient. The cases of those patients who have classified as social contact, obtained from the same area or lived in the same district (Eyre et al., 2013).

The first approach to analyze *C. difficile* genomic diversity was based on various molecular typing assays. They were targeting either the whole genome (limitation endonuclease analysis, primed PCR, REA, pulsed-field gel electrophoresis, APCC), multiple-locus variable-number tandem repeat analysis (MLVA), different loci (multilocus sequence typing (MLST), or a single region (surface layer protein A (slpA) typing, PCR ribotyping, toxin typing) (Janezic and Rupnik, 2010; Knetsch et al., 2013). The first explanation about *C. difficile* genome was published in 2006 (Sebahia et al., 2006). Eventually, microarray-based comparative genome hybridization (CGHs) were utilized in different researches, on genetic diversity belong to host specificity, and strain virulence (Janvilisri et al., 2009; Marsden et al., 2010; Scaria et al., 2010; Stabler et al., 2006). With the evolvement of next-generation sequencing (NGS) techniques, the comparative genomics also made advances in *C. difficile* study. The first publication illustrated the analysis of less than ten strains (Didelot et al., 2012; Marsden et al., 2010; Stabler et al., 2010), but the numbers of sequenced and analyzed partial genomes quickly enhanced (Didelot et al., 2012; Dingle et al., 2013; Elliott et al., 2014; Eyre et al., 2013a, b, c; He et al., 2010, 2013; Kurka et al., 2014). Despite this increase, and although more than 300 different *C. difficile* PCR ribotypes are right now itinerating within the human society. The absolute majority of comparative genomic studies to date have focused solely on some strains/types that have more repeatedly related to CDI outbreaks, with a central role assigned to the PCR of 027 ribotype (Eyre et al., 2013; He et al., 2013; Stabler et al., 2010).

Similar to previous MLST and microarray-based studies, whole-genome comparisons mainly focused on exploring the phylogeny, population structure of *C. difficile*, more recently epidemiology and in particular transmission (Didelot et al., 2012; Dingle et al., 2013; Elliott et al., 2014; Eyre et al., 2013a, b; He et al., 2010, 2013; Janvilisri et al., 2009; Kurka et al., 2014; Stabler et al., 2009).

Here we present an overview of *C. difficile* genomic diversity studies, with a concentrate on phylogenetic and epidemiological aspects and the diversity of virulence correlated regions (Janezic and Rupnik, 2015). This review aims to highlight the comparison among ten isolates acquired from 8 episodes of CDI in one patient to contribute the identification of CDI reoccurring phenomenon (Sachsenheimer et al., 2018).

C. DIFFICILE PAN AND CORE GENOME

On a species plane, *C. difficile* exhibits a low level of gene conservation. CGH studies have estimated that only 16-32% of genes were conserved in *C. difficile* (Forgetta et al., 2011; He et al., 2010; Janvilisri et al., 2009; Marsden et al., 2010; Scaria et al., 2010; Stabler et al., 2006).

In one study, the pan-genome (complete gene pool found in a species) of *C. difficile* was estimated at a level of 9640 genes (Scaria et al., 2010). Feeble gene conservation among *C. difficile* and other clostridial species has also reported. It matching with the proposal that those genes of *C. difficile* (together with its close dependent within-cluster XI, as determined by Collins) (Collins et al., 1994), which have conserved between *C. difficile* shown homologues with those genes which involved in housekeeping function (DNA degradation, replication, cell division, biosynthesis, transcription and metabolism) and were found outside the regions that have horizontally obtained DNA (Janvilisri et al., 2009; Sebahia et al., 2006). Divergent genes could found to be disseminated throughout the whole genome and functional domains, but predominated in elements of extrachromosomal origin (Janvilisri et al., 2009).

HIGH GENOME PLASTICITY OF C. DIFFICILE AND SCOPE OF RECOMBINATION

Based on whole-genome sequences, the *C. difficile* genome length is from 4.1-4.3 Mbp (Megabase Pair) (He et al., 2010; Sebahia et al., 2006; Stabler et al., 2009). Variations in genome length are mainly attributable to move genetic elements, basically putative conjugative transposons and bacteriophages shown 11% organization with *C. difficile* genome (Janvilisri et al., 2009; Mullany et al., 2015).

POPULATION STRUCTURE OF C. DIFFICILE SPECIES

Even though a high plane of genomic diversity, genome sequencing indicated and multi-locus sequence typing illustrated that the structure population of *C. difficile* is clonal. Initial, the multi-locus sequence typing scheme for *C. difficile* was demonstrated by Lemee et al (Griffiths et al., 2010). To appraise the genetic correlation and structure population of *C. difficile* isolates from different hosts (animals, humans), whit various toxigenic conditions and various geographic places (Griffiths et al., 2010).

C. DIFFICILE EVOLUTION THROUGH DIVERSE LINEAGES

Research studies utilized comparative phylogenomics, entire genome and MLST comparison relying on single nucleotide polymorphism (SNP) identity, the core genome illustrated that the assess of *C. difficile* happened through various lineage (Dingle et al., 2013; Griffiths et al., 2010; He et al., 2010; Knetsch et al., 2012; Stabler et al., 2006, 2012).

EXPANSION OF C. DIFFICILE PCR RIBOTYPE 027 ON A GLOBAL LEVEL

Worldwide phylogeny, which relies on the core genome of 151 isolates of polymerase chain reaction (PCR) ribotype 027/NAPI/BI. Illustrated the presence of two genetically different epidemic lineages namely; FQR1 and FQR2 that appeared newly from strains cluster near the root of the phylogenetic tree, so-called pre epidemic strains. The two epidemic progenitors had various templates of worldwide expansion and representing limited land clustering, insinuate prevalent massive range transmission between humans and also in a limited amount, spreading among humans, food and animals. Nevertheless, isolates in both progenitors were tremendous resistant to fluoroquinolones and accomplished the same mutation in DNA gyrase, which was obtained independently after the segregation almost twenty years ago. Both progenitors could also share a similar conjugative transposon (Tn6192). These were solely two genetic features segregation FQR1 and FQR2 progenitors from the pre epidemic 027 isolated and were most fundamental changes related to the quick egress of 027/NAP1/B1 (He et al., 2010, 2013).

MACRO MORPHOLOGY, DIAGNOSTIC AND CLINICAL DATA, ANTILOGRAMS, ANAMNESTIC, AND RIBOTYPING

Whole 10 *C. difficile* isolated cases were consecutively isolated from feces of 73-year-old humans during 58 weeks after appearing manifestations of nausea and diarrhea with the drastic underlying situation. Including heart failure, chronic kidney illness, and myeloproliferative neoplasms. The patient firstly cared in the clinic for staphylococcus septicemia using rifampicin and flucloxacillin for four weeks. The initial episode of CDI happened after clinic discharge. The patient individual has continuously improved watery diarrhea after cefuroxime treatment in the beginning.

Interestingly, oral treating of vancomycin play significant role in treatment. The duration and drastic among episodes of CDIs are highly different. The manifestation caused by isolate 10 were just mild intestinal inconvenience without diarrhea which latterly resolved automatically. Treatment of the CDIs containing fidaxomicin, vancomycin, and rifaximin. Some episode resolute automatically. Whole hospital isolates had identified by PCR ribotyping, multiplex

PCR for the existence of common toxin (tcdA and tcdB) and double toxin (cdtA and B) genes and by antibiotic susceptibility examination. Solely first and second isolates, which shown moxifloxacin and erythromycin resistance. All other isolates were completely susceptible to the checked antibiotics. The initial two dyadic toxins positive, which should be related to higher virulent ribotypes. The 10 isolated were not epidemic and associated with a still non-classified ribotype with rare toxin pattern (Indra et al., 2008; Sachsenheimer et al., 2018; von Müller et al., 2015).

GENETIC ASSOCIATIONS AMONG WHOLE 10 ISOLATES

To acquire an in profoundness scheme of the genetic association among the sick isolates, and to research potent microevolution during persistence infection, entire genome sequencing of all isolates have accomplished. Hybrid assemblies of the studies acquired with 4554 and Illumina technique resulted in 114-470 contains with at least 500 bp length. According to these shotgun genome sequences, analysis of SNPs and MLST analysis and gaps were carried out. MLST entirely confirmed the ribotyping outcomes but shown an additional close association among isolates 1 and 2 on one side and isolate 10 on the other side (Griffiths et al., 2010). Another tight association was revealed among isolates 7, 8, and 9.

Genome comparisons of entire isolates against each other were accomplished by mutually mapping the Illumina and 454 reads generated from each isolate onto the shotgun genomes assembled for the whole of an isolate. In that isolates relating to ST-10, ST-14 and ST-76 sharer among 54 and 60% of their genomes in ROIs. If isolates have associated with the equal ST, more than 99% of their genomes were recognized as ROIs without in the case of isolate 10, determining the feasibility that the second and third CDI episodes reasoned by these isolates were re-substituting infections. However, isolate 10, which was isolated after 54 or 58 weeks than first and second isolate, and which the time of ribotyping was recognized as variant RT, shown solely among approx. 87-92 % determining parts with the first two isolates. SNP numbers among isolates of the same MLST sequence type were tremendously low (0-2 SNPs per comparison). Threatened parts involving the active center of a glucose-particular phosphotransferase system, in which one of the conserved amino acids was inverted form G-W in isolate 2.

The other SNP among these two strains outcome in one amino acid invert in an ORF annotated as feasible permease in isolate 1 and as a transporter in isolate 2. Including ST 14, isolate 6 also presented an SNP within the conserved domain of a template efflux transporter, which happened in a no conserved residue. The SNP recognized between the ST 76 isolates threatened two-component sensor histidine kinase. Therefore, whole regions in which SNPs

among isolates of the equal ST coincided with ROIs among isolates of various STS were placed in isolate 10. By testing the alignment of the corresponding ORFs from whole isolates.

Therefore, ORFs with determinable amino acid translations can be recognized utilizing the BLAST tools tblastn. Whenever entire strains included most resembling sets of competence associated genes, transposon associated genes were frequently ST or particular RT.

An exception was transposases associated protein invent in ST 76 strains and strain 4 (ST14), it has happened in two various small copies. One of these copies has been determinable in the ST 76 ORFs on the amino acid and nucleotide levels. A BLAST analysis for other sequences more than 70% equal to the strain 4 sequences did not recognize and other homologs between the left draft genomes, determining this protein may have been newly integrated into strain 4 (Sachsenheimer et al., 2018).

BLAST ALIGNMENTS OF WELL-KNOWN VIRULENCE GENES

The scheme genome screened for a set of motility, toxin and cell division associated genes utilizing the BLST tools tblastn. Isolates 1, 2 and 10, have been related to ST 11, encode the binary toxin genes *cdtA* and *cdtB*, which are thought-out to be correlated with higher virulence. Entirely, the last isolate in this line of the patient, isolate 10 was not solely identified by a novel non-epidemic ribotype but also a peculiar toxin locus. This identified locus had no *tcdB* and individually regions of *tcdA* with matched parts beginning 820 amino acids after the protein in reference strain 360 and strain R20291, and absence a probable start codon (Bouvet and Popoff, 2008; Ransom et al., 2014; Sachsenheimer et al., 2018).

ANTIBIOTIC RESISTANCE GENES

In order to, after alignment of the eleven 23S rDNA genes to reference assembly *C. difficile* 630 (NC-009089.1) including the 23S rDNA segments recognized in assemblies, were invent the sequence fragment in asking (GTGCGGA or GTGTGGA) to be placed one bp forward 3' in more *C. difficile* 630 gene copies and three bp forward 3' in the alignment. The sequence was polymorphous among the eleven *C. difficile* 630 genes with seven genes copies having the sequence GTGCGGA and 4 having the sequence GTGTGGA. Therefore, between assemblies, this part was covered solely in erythromycin sensitive isolates, but both (GTGCGGA and GTGTGGA) were differently invented. Whenever, the GTGCGGA difference was recognized in third and fourth isolates, and GTGTGGA different was recognized in isolates 7, 8 and 9. Then, cannot prevent differentiation among the single 23S rDNA copies

of entire isolates (Marosevic et al., 2017; Sachsenheimer et al., 2018; Schmidt et al., 2007; Spigaglia, 2016).

CONCLUSION

C. difficile causes recurrent infections in 15-30% patients (Lübbert et al., 2016; Maroo and Lamont, 2006). Several patients were suffered from a mix of recurrence and reinfection in a huge amount of relapses infections. Entirely, isolates seven, eight, nine and ten were mostly obtained from the community. Antibiotic therapy has recognized as one of the basic risk factors for CDI (Rupnik et al., 2009). If CDIs re-happened, the feasibility of recurrent, reinfections and their mortality rate likely to increase with each infection. Have counted in a follow-up series of sick with relapses infection that opportunity for a first relapse is 18.2 percent (222/1223), 28.4 percent (63/222) of those patients population would have a second relapse (third CDI episode). Of these groups, 30.2 percent (19/63) would have a third relapse (fourth DCI episode). In routine diagnosis, these mixed infections have most likely underestimated. In most laboratories, diagnosis were based on toxin testing (for TcdA and TcdB), detection of the glutamate dehydrogenase or nucleic acid amplification test (NAATs) (Tenover et al., 2011). Ribotype 078, which approximately determinable with 126, has also related to an enhanced virulence (Goorhuis et al., 2008), and it has recognized in animal's infection. Such as calf and pigs (Hensgens et al., 2012). Proteins have a SPOR domain are often part of the septal ring that mediates cell division in several other bacteria (Ransom et al., 2014; Yahashiri et al., 2015). Our review revealed that the genome of whole patient isolates except isolate ten having the toxigenic *C. difficile* locus PaLoc. Since, the patient had severe symptoms during CDI episodes one, two and seven, but lightly affected during other remains episodes. The intensity of CDI has mostly related to antibiotic dependent combination of microbiome. Therefore, immunogenic status and comorbidities of the host. Complete genome sequencing of *C. difficile* strains have the fruitfulness of all PCR based ribotyping approaches to path patient to patient conduction events and to more accurately differentiation recurrent form reinfection (Kumar et al., 2015; Mac Aogáin et al., 2015; Sim et al., 2017; Stevenson et al., 2015). Genomic comparisons of the most associated isolates within one ST revealed little prove for microevolution. However, most of the identified high-confidence SNPs resulted in changes in the amino acid level. They had placed within conserved domains of the respective proteins suggesting that they might affect protein function. Screening of entire strains for genomic expansion that may have lately converted among the bacterial dynasty did not produce evidence for coming genome exchange. Previously methods to the recognition of genomic diversity of *C. difficile* have relied on molecular

typing approaches or sequence identification of selected genes. The first genome of *C. difficile* was augmented in 2006 and has resulted in multiple microarray-based studies, with WGS methods, phylogeny and transmission have continuously studied in great detail. The *C. difficile* species presently assembled into six different phylogenetic clades determined from one to five and C-I. The illnesses causing isolates have invented in all clades; as well as, higher virulent strains with increased epidemic and virulence potent are invent in three of five toxigenic clads. Up to date, *C. difficile* is thought-out a significant nosocomial pathogen, and comparative genomics supports the looking which a huge proportion of *C. difficile* infections originate from non-hospital sources. Comment: However, this does not explain why CDI cases and their associated morbidity are rising.

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AUTHORS CONTRIBUTION

All the authors have contributed in terms of technical knowledge in framing the article.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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