Molecular epidemiology of Clostridioides difficile in patients with inflammatory bowel disease in China

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Research

Keywords: Clostridioides difficile, Inflammatory bowel disease, Epidemiology, MLST, Antibiotic resistance, Pediatric

Posted Date: December 20th, 2019

DOI: https://doi.org/10.21203/rs.2.19348/v1

Abstract

Background: *Clostridioides difficile* (*C. difficile*) infection (CDI) in inflammatory bowel disease (IBD) patients can be recurrent, resulting in poor outcomes, but the molecular characterization of *C. difficile* in IBD patients remains to be well-established in China. This study aimed to investigate the molecular epidemiology of *C. difficile* in adult and pediatric IBD patients in China.

Methods: *C. difficile* strains were isolated and identified from the fecal samples of adult and pediatric IBD patients. Toxigenic strains were typed using multilocus sequence typing (MLST), and susceptibility to 10 antimicrobials was evaluated using E-test.

Results: Among the 838 IBD patients, 96 (11.5%) patients were positive for CDI, which comprised of 53 adult (9.6%) and 43 children (14.9%) cases. Isolates positive for both toxin A and toxin B genes (A+B+) accounted for 90.2% (74/82), while the remaining 9.8% were negative for toxin A, but positive for toxin B (A–B+). These toxigenic strains were susceptible to metronidazole and vancomycin, but highly resistant to clindamycin, levofloxacin, erythromycin and ciprofloxacin. Furthermore, the isolates obtained from pediatric patients had a significantly higher resistance rate to clindamycin, when compared to isolates obtained from adult CDI (p=0.009). In addition, these toxigenic strains were categorized as 18 sequence types (STs). The dominant types consisted of ST-35 (20.7%), ST-2 (17.1%), ST-54 (13.4%) and ST-3 (13.4%) in all patients, ST-2 (19.6%), ST-35 (15.2%) and ST-54 (13.0%) in adult patients, and ST-35 (27.8%), ST-3 (19.4%), ST-2 (13.9%), ST-54 (13.9%) and ST-37(8.3%) in pediatric patients, respectively. All isolates formed three distinct clusters in the phylogenetic analysis.

Conclusions: The incidence and molecular epidemiology of *C. difficile* infection in adult IBD patients resembled CDI in the general inpatient population. A higher antibiotic resistance rate was identified among the *C. difficile* isolates obtained from pediatric IBD patients, and few STs accounted for most multidrug-resistant strains. However, the molecular genetic features of the same ST-type between these two groups remained highly correlated.

Introduction

Clostridioides difficile (C. difficile) is one of the leading pathogens of infectious and nosocomial diarrhea and pseudomembranous colitis.[1, 2] The incidence and severity of global C. difficile infection (CDI) have steadily increased over the past decades. Furthermore, CDI is no longer restricted to the nosocomial setting. High CDI frequency has been reported in the historically low-risk group and residents that include children who have not been exposed to hospital settings.[3, 4] CDI incidence in the general inpatient population in China is approximately 10.0%, which similar to that in developed countries, but differs from the ribotypes, and no outbreak of the BI/NAP/027 strain has been reported.[5–9] However, the number of C. difficile multidrug-resistant (MDR) strains continues to increase, and a much higher MDR rate has been identified among the toxigenic isolates obtained from hospitalized patients in China.[5, 8]

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic idiopathic disorder that features intermittent inflammation in the gastro-intestinal tract.[10–14] In the early 1980s, IBD was first found to be accompanied by CDI.[15] Subsequently, several studies suggested that IBD patients carried a higher CDI risk as a result of the intestinal dysbiosis related to IBD, when compared to the general population.[10, 11, 14] Once CDI complicating IBD, IBD patients tend to have higher recurrent onsets that require repeated hospitalizations, and intensified antibiotic treatment and poorer outcomes, leading gastrointestinal surgery and higher mortality, when compared to mono-IBD.[16, 17] Retrospective studies have revealed almost a two-fold higher risk for colectomy and a 4–6 fold increase in mortality risk among IBD patients with CDI.[18, 19]

The CDI incidence in IBD patients varied in different studies, reflecting the differences in both geographical regions and ethnic participants.[16, 20] The CDI incidence in IBD patients was moderate in Western countries.[10] Patients hospitalized for IBD flare also presented with CDI, which ranged from 3.7–8.0%.[16, 17] However, as high as 13.9% has been reported for CDI in Chinese IBD patients.[21, 22] The major risk factors for CDI in the general population include the excessive or non-standard use of antibiotics, older age, prior and prolonged hospitalization, and multiple comorbidities.[23] However, CDI may occur in IBD patients, regardless of the younger age and absent antibiotic exposure. CDI in IBD patients was associated with corticosteroid exposure, and CDI in the majority of hospitalized IBD patients was community-acquired.[16, 18, 24, 25]

Globally, CDI is typically more common in adults, when compared with children, and the clinical features, which include the outcomes in UC and CD patients with CDI, have been well-studied.[11, 16, 23–25] However, few studies have investigated CDI in pediatric IBD patients and characterized the molecular features of C. difficile in adult and pediatric IBD patients, especially in China. The present study aimed to investigate the molecular epidemiology of C. difficile in pediatric and adult IBD patients in China.

Methods

Definitions

IBD Patients were identified with discharge diagnoses codes, according to the International Classification of Diseases, 9th revision, Clinical Modification (ICD-9-CM).

Bacterial isolates

Unformed stool samples obtained from adult IBD patients (between June 2011 and June 2015) and pediatric IBD patients (between November 2011 and September 2017), who were suspected of CDI, were sent to the Clinical Microbiology Laboratory at the First Affiliated Hospital of Zhejiang University (Hangzhou, China). All specimens were cultured on selective media cycloserine-cefoxitin-taurocholate agar (CCFA-TA; Oxoid) supplemented with 7.0% sheep blood at 35 °C for 48 hours. Then, the C. difficile isolates were confirmed by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) analysis using the Bruker Daltonics Microflex LT system (Bruker Daltonik GmbH, Bremen, Germany).

Detection of toxin genes

Bacterial genomic DNA was extracted using the simplified alkaline lysis method. All isolated strains were tested for tcdA, tcdB and binary toxin genes by polymerase chain reaction (PCR), as previously described.[7, 8]

Multilocus sequence typing (MLST) and phylogenetic analysis

MLST was used to genotype all toxigenic isolates, as previously described.[7, 8] The allele designations were obtained through the C. difficile PubMLST batch profile query page (http://pubmlst.org/cdifficile/). The phylogenetic tree was constructed using the MLST sequences (the maximum likelihood method with MEGA X [64-bit] software, http://www.megasoftware.net). Evolutionary distances were calculated using the General Time Reversible model and the bootstrap consensus tree generated from 1,000 replicates.

Antibiotic susceptibility testing and mechanisms of resistance

The antibiotic susceptibility of all toxigenic isolates was analyzed using the E-test strips (bioMérieux, Marcy-l'Étoile, France), which were coated with drug concentrations that ranged within 0.016-256.000 mg/L for metronidazole, vancomycin, clindamycin, erythromycin, tetracycline and linezolid, and 0.002-32.000 mg/L for ciprofloxacin, moxifloxacin, levofloxacin and rifampicin. Briefly, these selected isolates were grown on Brucella agar plates that contained 1 mg/L of vitamin K, 5 mg/L of hemin and 5.0% sheep red blood cells. Then, the agar surface was covered with E-test strips and incubated in an anaerobic atmosphere for 24–48 hours at 37 °C. The minimum inhibitory concentrations (MICs) were read at the point at which the zone of complete inhibition intersected with the MIC scale with the Clinical and Laboratory Standards Institute (CLSI) guidelines. The selected resistance breakpoints included 8 mg/L for erythromycin, clindamycin, tetracycline and the fluoroquinolones, and 32 mg/L for metronidazole, according to the CLSI interpretative categories approved for anaerobic bacteria. The 4 and 16 mg/L breakpoints were selected for rifampicin and vancomycin, respectively, according to the CLSI interpretive categories approved for Staphylococcus aureus, because no values were provided for anaerobes. The breakpoint for linezolid was set at 4 mg/L. The control isolate was C. difficile ATCC 700057. An isolate that resisted ≥ 3 classes of antibiotics was called multidrug resistance strain.[26]

Data analysis

All statistical analyses were performed using SPSS 20.0. A P-value of < 0.05 was considered statistically significant.

Results

A total of 96 (11.5%) non-redundant C. difficile isolates were identified from 838 samples. Among these isolates, 53 isolates were obtained from 550 adult IBD patients, while the remaining 43 (14.9%) isolates were obtained from 288 pediatric IBD patients. Furthermore, among these 96 isolates, 82 (85.4%) isolates were toxigenic strains, while the remaining 14 (14.6%) isolates were non-toxigenic. Among the 82 toxigenic strains, 74 (90.2%) strains were positive for both toxin A and toxin B genes (A + B+), while the remaining eight (9.8%) strains were toxin A gene-negative/toxin B gene-positive (A–B+). Furthermore, three (3.7%) of the toxigenic isolates were binary toxin genes-positive (CDT+) (Table 1). A total of 46 (56.1%) toxigenic C. difficile isolates were identified in adult IBD patients, while 36 (43.9%) isolates were identified in pediatric IBD patients (Table 1).

Table 1
Sequence types of toxigenic C. difficile strains isolated from IBD patients

	Positivity (%)	Total IBD patients (n)	Adult patient (n)	Pediatric patient (n)	A + B+ (n)	A-B+ (n)	A + B+ binary+ (n)
All		82	46	36	74	8	3
ST-2	17.1	14	9	5	14	-	-
ST-3	13.4	11	4	7	11	-	-
ST-5	2.4	2	1	1	2	-	2
ST-8	6.1	5	4	1	5	-	-
ST-11	1.2	1	1	-	1	-	1
ST-33	1.2	1	1	-	1	-	-
ST-35	20.7	17	7	10	17	-	-
ST-37	8.5	7	4	3	-	7	-
ST-39	1.2	1	1	-	-	1	-
ST-42	1.2	1	1	-	1	-	-
ST-51	1.2	1	1	-	1	-	-
ST-54	13.4	11	6	5	11	-	-
ST-81	3.7	3	2	1	3	-	-
ST-99	1.2	1	1	-	1	-	-
ST-102	1.2	1	-	1	1	-	-
ST-103	2.4	2	1	1	2	-	-
ST-129	2.4	2	2	-	2	-	-
ST-139	1.2	1	1	-	1	-	-

MLST types

A total of 18 sequence types (STs) were classified for toxin producing strains (Table 1). The most prevalent type included ST-35 (20.7%, 17/82), followed by ST-2 (17.1%, 14/82), ST-3 (13.4%, 11/82), ST-54 (13.4%, 11/82), ST-37 (8.5%, 7/82) and ST-8 (6.1%, 5/82). ST-1 (NAP1/B1/027), which is a hypervirulent C. difficile strain, was negative in this cohort. ST-2 (19.6%, 9/64) was the prevalent type, followed by ST-35 (15.2%, 7/64), ST54 (13.0%, 6/64), ST3 (8.7%, 4/64), ST8 (8.7%, 4/64) and ST37 (8.7%, 4/64), in adult IBD patients. ST-35 (27.8%, 10/36) was the most common among all STs, followed by ST-3 (19.4%, 7/36), ST-2 (13.9%, 5/36), ST-54 (13.9%, 5/36) and ST-37 (8.3%, 3/36), in pediatric IBD patients. The phylogenetic tree consisted of three major (distinct) lineages (Fig. 1). One lineage only comprised of a single ST-11. The 2nd lineage consisted of ST-5, ST-39, ST-81 and ST-37, while the 3rd lineage collected all the remaining STs.

Antimicrobial resistances

The MICs of different antimicrobial agents against C. difficile isolates are presented in Table 2. A 100% resistance to ciprofloxacin was observed, followed by 92.7% to levofloxacin, 64.6% to clindamycin, 62.2% to erythromycin, 15.9% to tetracycline, 13.4% to moxifloxacin, and 3.7% to rifampicin. All isolates were sensitive to metronidazole, vancomycin and linezolid, showing that these were within a narrow range (Table 2). A significantly higher resistant rate to clindamycin ($P = 0.009$) was found in isolates obtained from pediatric patients, when compared to adult patients. No heteroresistance was observed. Each of all the tested isolates was resistant to at least one antibiotic. Specifically, 37.8% of the 82 isolates, in which 26.8% were obtained from adult IBD patients and 11.0% were obtained from pediatric IBD patients, were resistant to only one or two classes of antibiotics, respectively, and as high as 62.2% of these isolates were resistant to ≥ 3 classes of antibiotics or MDR strains. Furthermore, 14.6% of the MDR isolates, in which 12.2% were from adult IBD patients and 2.4% were from pediatric IBD patients, resisted four classes of antibiotics (i.e. clindamycin, erythromycin, tetracycline, levofloxacin and ciprofloxacin). Interestingly, the resistant rate to three classes of antibiotics in pediatric IBD patients with was nearly twice as many as that in adult IBD patients (i.e. clindamycin, erythromycin, ciprofloxacin

and levofloxacin), while the resistance to four classes of antibiotics in adult patients was almost four times higher, when compared to pediatric patients (Table 3). No isolate was resistant to all antibiotics. Multidrug-resistant isolates were distributed among the 11 different STs. Among these, ST-35 (31.4%), ST-3 (15.7%), ST-37 (13.7%), ST-54 (13.7%) and ST-2 (7.8%), ST-35 (25.0%), ST-37 (16.7%), ST-3 (12.5%), ST-54 (12.5%) and ST-2 (12.5%) were predominant in adult IBD patients, while ST-35 (37.0%), ST-3 (18.5%), ST-54 (14.8%), ST-37 (11.1%) and ST-2 (3.7%) were predominant in pediatric IBD patients (Table 4). However, no significant difference in resistant rate to antibiotic classes by the same multidrug-resistant ST strains was observed between these two groups.

Table 2
Minimum inhibitory concentrations (MICs) of the 10 antimicrobial agents against toxigenic C. difficile strains isolated from IBD patients

Antibiotics	Resistance rates (%)			MIC (μg/ml)								
				MIC50			MIC90			Range		
	Total	Adult patient	Pediatric patient	Total	Adult patient	Pediatric patient	Total	Adult patient	Pediatric patient	Total	Adult patient	Pediatric patient
Metronidazole	0	0	0	0.023	0.023	0.032	0.047	0.047	0.047	0.016–0.32	0.016–0.094	0.016–0.32
Linezolid	0	0	0	0.5	0.5	0.5	1	1	1	0.016-2	0.016-1.5	0.25-2
Vancomycin	0	0	0	0.38	0.38	0.38	0.5	0.5	0.5	0.016-3	0.016–0.75	0.19-3
Clindamycin	64.6	56.5	75.0	256	32	256	256	256	256	0.5–256	0.5–256	2-256
Rifampicin	3.7	4.4	2.8	0.002	0.002	0.002	0.002	0.002	0.002	0.002-32	0.002-32	0.002-32
Levofloxacin	92.7	91.3	94.4	32	32	32	32	32	32	0.012-32	0.012-32	0.125-32
Erythromycin	62.2	52.2	75.0	256	256	256	256	256	256	0.094-256	0.094-256	0.5–256
Moxifloxacin	13.4	10.9	16.7	1	1	1	32	32	32	0.25-32	0.25-32	0.38-32
Tetracycline	15.8	21.7	8.3	0.047	0.047	0.032	8	8	6	0.016-256	0.016-256	0.016-12
Ciprofloxacin	100	100	100	32	32	32	32	32	32	32–32	32–32	32–32

Table 3
The resistant rates of each class of antibiotic in IBD patients

Classes	No. of resistant patients		Resistance rates (%)	
	Adult patient	Pediatric patient	Adult patient	Pediatric patient
One class of antibiotic	18	8	39.1	22.2
Two classes of antibiotics	4	1	8.7	2.8
Three classes of antibiotics	14	25	30.4	69.4
Four classes of antibiotics	10	2	21.8	5.6
Total	46	36	100	100

Table 4
Sequence types of C. difficile multidrug-resistant (MDR) strains isolated from IBD patients

ST	NO			NO rates (%)		
	Total	Adult patient	Pediatric patient	Total	Adult patient	Pediatric patient
2	4	3	1	7.8	12.5	3.7
3	8	3	5	15.7	12.5	18.5
35	16	6	10	31.4	25.0	37.0
37	7	4	3	13.7	16.7	11.1
39	1	1	0	2.0	4.2	0
42	1	0	1	2.0	0	3.7
54	7	3	4	13.7	12.5	14.8
81	3	2	1	5.9	8.3	3.7
102	1	0	1	2.0	0	3.7
103	1	0	1	2.0	0	3.7
129	2	2	0	3.9	8.3	0

Discussion

In the present study, the detected incidence of CDI in adult IBD patients was lower than that reported in China, but comparable to the CDI frequency reported in non-Chinese IBD patients.[16, 17, 21, 22] The different compositions in the general inpatient population in different hospitals may have tilted the CDI frequency one way or the other. It has been suggested that the higher CDI in IBD patients could be partially explained by the increased CDI incidence in the general inpatient population.[11, 16] It was also found that the CDI incidence in pediatric IBD patients was higher than that in adult IBD patients. As reported, IBD children had higher intermittent CDI, when compared to IBD adults.[27, 28] It is known that 3.0–70.0% of children may carry an asymptomatic C. difficile.[29, 30] It was no surprise to detect a higher CDI in IBD children due to the higher CD carriage background in this population. In addition, dynamic changes in microbiome in the gut of children represent another risk factor for CDI in pediatric IBD patients.[27]

Previous studies have categorized ST-54, ST-35 and ST-37 as the top three genotypes isolated from inpatients with confirmed hospital-acquired CDI in China.[6, 7, 9] However, ST-2, ST-35 and ST-54 ranked as the top three genotypes among the toxigenic C. difficile strains in adult IBD patients in the present study, while ST-35, ST-3, ST-54 and ST-2 were dominant in pediatric IBD patients. The ST-3 carrier rate in healthy infants and adults has been reported to be 32.7% and 11.0%, respectively.[31] ST-35 and ST-3 were frequently detected in children patients with community-acquired CDI, while ST-54 was detected in adults in southwest China.[32] McFarland LV et al.[33] suggested that CDI in children was more frequently community-acquired, when compared to adult CDI cases. However, it remains conceivable that CDI in pediatric IBD patients is mainly community-acquired, and that CDI in adult IBD patients is more likely hospital-acquired. However, further studies are required to ascertain the two different transmission sources of C. difficile responsible for CDI in children and adults. In addition, although there was no significant difference in the same STs based on the results of the phylogenetic analysis, different ST distributions were noted between adult IBD patients and pediatric IBD patients, but the difference was insignificant. The differences in transmission source and age may have contributed to the different ST distributions. After all, the molecular epidemiology of C. difficile may be subjected to random, but dynamic changes.[34]

Antibiotic use has been considered an important cause for CDI increase. Several classes of antimicrobials have been linked with high CDI risk. For instance, fluoroquinolones have been referred as a common cause of CDI.[35] Multiple antibiotics were suggested to facilitate C. difficile spore germination and toxin production.[23] Antibiotic exposure is not essential in CDI occurrence in IBD patients.[11] However, antibiotic pressure may prompt C. difficile to acquire resistance to antibiotics, and confer survival properties and enhanced virulence. The newly acquired abilities by C. difficile may explain recurrent course and poorer outcomes in IBD patients.[16, 17] All isolates in the present study resisted ciprofloxacin, and nearly all of these also resisted levofloxacin with the highest MICs ($\geq 32\ \mu g/ml^{-1}$), resembling the hospital-acquired CDI in China.[5–7, 9] However, merely 13.4% of isolates in IBD patients exhibited a resistance to moxifloxacin, which was significantly lower, when compared with the hospital-acquired CDI in China. The relatively low resistance was likely the result of the infrequent prescription of moxifloxacin for IBD patients. Meanwhile, it was found that the resistance to clindamycin in pediatric IBD patients was significantly higher than that in adult IBD patients (75% vs. 56.5%, P = 0.009). This difference likely resulted from the frequent prescription of clindamycin for children, reflecting a fact that a greater number of

children catch flu with lung inflammation, when compared to adults. In addition, ST-3, ST-35, ST-37 and ST-54 were the prevalent types responsible for clindamycin resistance in pediatric patients. Similar resistant genotypes were identified, and these included nearly 70.0% of the isolates that were resistant to clindamycin, including ST-35 (90.9%), ST-54 (89.0%), ST-37 (85.5%) and ST-3 (77.1%), in the previous studies conducted by the investigators.[7, 8]

In the present study, the MDR strains in adult IBD patients was 52.2%, which was similar to the previously reported 47.1%-58.6% in the inpatient population in China, but significantly lower than the reported 75.0% in pediatric IBD patients.[7, 8] The MDR strains resistant to the three classes of antibiotics of clindamycin, erythromycin and fluoroquinolones accounted for 69.4% of all MDR strains identified in pediatric IBD patients, which was significantly higher than that in the adult IBD patients in the present study. Thus, clindamycin, fluoroquinolones and cephalosporins have been identified as the greatest risk for CDI in children, since many children with community-acquired CDI were exposed to multiple classes of antibiotics in the 12 weeks preceding CDI.[28, 36] The MDR rate in all STs and RTs in CDI patients in eastern China was high, which was 14.9–19.6% for ST-2/ST-3 and 17.0% for RT001.[5] As shown by the present study, ST-35 is an additional type of toxigenic C. difficile MDR strain in IBD patients in eastern China.

Conclusion

According to the results, the incidence and molecular epidemiology features of CDI in adult IBD patients resembled in the general inpatient population. There were significant differences in the distribution of STs and antibiotic resistance rates between adult IBD patients and pediatric IBD patients. C. difficile isolates in pediatric IBD patients had a relatively higher resistance. However, the MDR strains were limited, regardless of the comparable phylogenetic features of C. difficile between the two groups. The high resistance rate accompanied by the dominant MDR strains in pediatric IBD patients call for more effective measures to reduce CDI in children.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that there are no conflicts of interest.

Funding

This study was funded by a grant from the Major Infectious Diseases Such as AIDS and Viral Hepatitis Prevention and Control Technology Major Projects (2018ZX10712-001) and the Zhejiang Provincial Natural Science Foundation of China under Grant No.LGC20C010002.

Author's contributions

LjL contributed in the experimental studies, and drafting the work, YbC and PS contributed in the conception of design, LsZ contributed in the acquisition of the data. TL, and YbC contributed to the conception of design and revising the draft, TW contributed to the analysis of the data. All authors read and approved the final manuscript

Acknowledgements

The authors wish to thank Dr K. Y. Yuen and W. C. Yam of the Department of Microbiology, Queen Mary Hospital, Faculty of Medicine, University of Hong Kong for providing the Clostridium difficile PCR ribotype 027 strain.

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Figures

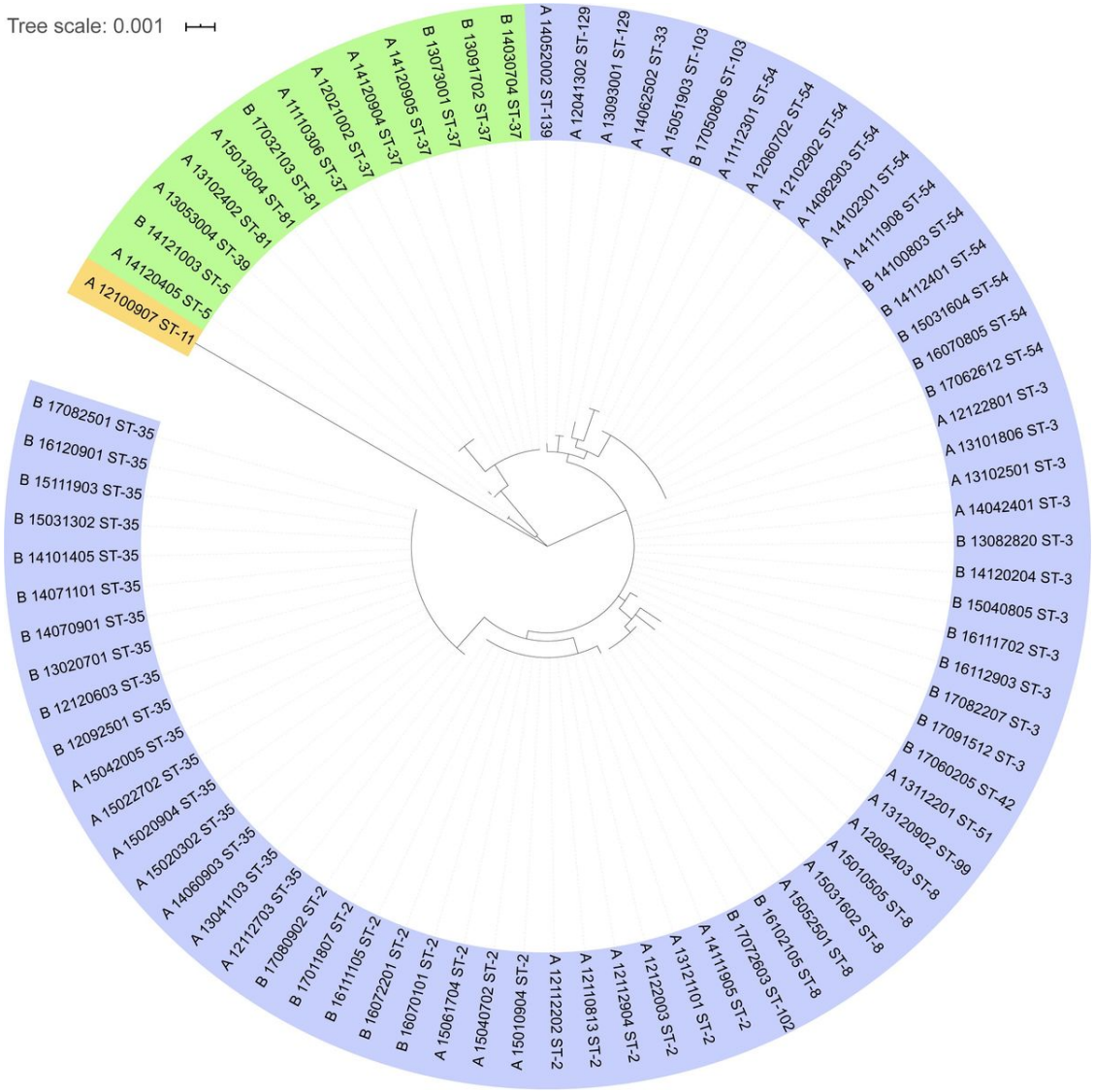

Figure 1

The phylogenetic tree of toxigenic C. difficile strains isolated from IBD patients. A: The strains obtained from adult IBD patients. B: The strains obtained from pediatric IBD patients.