Detection of *Clostridioides Difficile* Toxin B Gene: Benefits of Identifying Gastrointestinal Pathogens by mPCR Assay in the Diagnosis of Diarrhea in Pediatric Patients

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Abstract

In the pediatric population, severe *Clostridioides difficile* infection sometimes occurs, but most cases are asymptomatic. Since the asymptomatic carriage rate is reportedly high in pediatric populations, diagnosis of CDI is difficult. Here, we analyzed 960 results of gastrointestinal pathogen multiplex PCR to estimate the positive rate of toxigenic *C. difficile* in pediatric populations aged between 0 and 18 years. The overall rate of *C. difficile* toxin B positivity was 10.1% in the stool samples. The positive rate peaked in 1-year-old infants (29/153, 19.0%), and decreased continually thereafter. The positive rate we observed was lower than the rates described in the literature. Remarkably, no *C. difficile* was detected in neonates. Antibiotic usage was inversely related to the positive rate, especially in infants < 2 years of age. The odds ratio of antibiotics was 0.44 (95% confidence interval (CI) 0.28–0.68; $P < 0.001$). The presence of concomitant gastrointestinal pathogens was not associated with toxigenic *C. difficile* positivity. Even though toxigenic *C. difficile* infection is neither an important nor a common cause of pediatric diarrhea, children can spread it to adults who are at risk of developing CDI. Pediatric population can act as hidden reservoirs for pathogenic strains in the community.

1. Introduction

Clostridioides difficile, formerly known as *Clostridium difficile*, is a spore-forming, obligate anaerobic, gram-positive bacillus that is acquired either from the environment or by the fecal-oral route [1]. It is known to cause a wide range of symptoms, from mild diarrhea to severe life-threatening complications such as toxic megacolon [2]. The major virulence factors of *C. difficile* are large clostridial toxins, toxin A and toxin B, which are encoded by *tcd*A and *tcd*B [3]. *C. difficile* infection (CDI) mainly occurred in healthcare-associated cases and in adults. However, over the last decade, CDI has emerged as an important community-associated infection both in adults and children [4]. Approximately 4–5% of non-hospitalized healthy adults carry the pathogen in their intestinal flora [5] and varying positive rates of up to 70% have been reported in healthy newborns [6]. In children, the carrying capacity decreases with age, reaching adult levels of approximately 5 % by the age of 2 [7]. According to the guidelines for pediatric consideration, because of the high prevalence of asymptomatic carriage of toxigenic *C. difficile*, testing for CDI should not be routinely performed in children with diarrhea who are under 12 months of age [1, 8]. If they have rare and severe symptoms of pseudomembranous colitis, toxic megacolon, or clinically significant diarrhea, *C. difficile* testing should be performed. In children aged between 1 and 3 years, diagnostic workup for other diarrheal causes should be performed first, while *C. difficile* testing can be considered at later stages. Due to the unclear role of *C. difficile* in children with diarrhea, there are few reports on the positivity of toxigenic *C. difficile* in the pediatric population. We therefore paid attention on multiplex PCR (mPCR) was increasingly being applied to detect gastrointestinal pathogens and provided the information of *C. difficile* toxin B gene (*tcd*B) additionally in pediatric patients. In this study, we estimated the positive rate of *C. difficile tcd*B and analyzed the meaning of results through electronic medical record review.

2. Material And Methods

We reviewed 960 non-duplicated stool mPCR (Seeplex Diarrhea-B1/2 and V ACE Detection, Seegen, Korea) results obtained from pediatric patients up to the age of 18 [9] collected over a 39-month period (October 2014–December 2017) and submitted to a tertiary referral hospital in Seoul. The mPCR included *C. difficile tcd*B and other 13 diarrhea causing pathogens (*Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Campylobacter* spp., *Escherichia coli* O157:H7, *Clostridium perfringens, Yersinia enterocolitica, Aeromonas* spp., Verocytotoxin-producing *E. coli*, rotavirus group A, norovirus, astrovirus, and enteric adenovirus). The electronic medical records of the patients were reviewed to acquire information regarding age, length of hospital stay, underlying diseases (malignant neoplasm, hematology/immunology, endocrinology, cardiovascular, respiratory, digestive, inflammatory bowel diseases, and genitourinary disorder), previous history of antibiotics, and clinical diagnosis of *C. difficile* enterocolitis during the entire period of hospitalization with or without metronidazole or oral vancomycin treatment.

Unpaired *t*-test or Mann-Whitney U test were used for continuous data. Pearson's chi-squared test or Fisher's exact test were used for categorical data. The odds ratios of the antibiotic-treated versus naive groups were calculated. All statistical analyses were performed using MedCalc Statistical Software version 18 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2018).

3. Results

*1. Clinical characteristics of C. difficile tcd*B-positive and *tcd*B-negative

The overall positivity of *C. difficile tcd*B, as determined by mPCR, was 10.1% (97/960). The median age of patients with positive results were younger (median age 1.6 years) than that of those with negative results (3.8 years) ($P < 0.01$) (Table 1).

Table 1
Baseline characteristics of 960 pediatric patients with diarrhea based on *Clostridioides difficile* toxin B (*tcd*B) detected by multiplex PCR

		***tcd*B Positive (N = 97)**	***tcd*B Negative (N = 863)**	***P***
Age, median (95% CI[a])		1.6(1.4–2.2)	3.8 (3.3–4.5)	< 0.01
Age group (N)				
0 (219)	Inpatient	12	140	0.09
	Outpatient	11	56	
	Subtotal	23	196	
1 (153)	Inpatient	18	95	0.16
	Outpatient	11	29	
	Subtotal	29	124	
2–6 (265)	Inpatient	11	204	< 0.01
	Outpatient	15	61	
	Subtotal	26	239	
7–12 (171)	Inpatient	7	137	0.42
	Outpatient	3	34	
	Subtotal	10	161	
13–18 (152)	Inpatient	9	125	0.36
	Outpatient	0	27	
	Subtotal	9	143	
Total (960)	Inpatient	57	674	< 0.01
	Outpatient	40	189	
	Subtotal	97	863	
Sex				0.64
Male		54	502	
Female		43	361	
Length of stay at testing, median (95% CI)		5 (3.6-6.0)	4 (4.0–5.0)	0.807

[a]Confidence interval (CI)

		*tcd*B Positive (N = 97)	*tcd*B Negative (N = 863)	*P*
30-day mortality		2	10	0.346
other gastrointestinal pathogens	detected	30	295	0.52
	not detected	67	568	

[a]Confidence interval (CI)

No *tcd*B was detected in neonates (0/13); however, they were admitted to the neonatal intensive care unit (NICU) and administered antibiotics. Their mean length of stay was 5.0 days from the day of testing. No other diarrheal pathogens were detected in these neonates. While the youngest *tcd*B-positive infant was a 4-month-old, the *tcd*B-positive rate among infants aged 1 month to 1 year was 11.2% (23/206).

The *tcd*B-positivity peaked at 1 year of age (29/153, 19.0%) and is inversely correlated with age. In children aged 2–6 years, the positivity rate drops to 9.8%, and this incidence decreases in the group aged 7–12 years (5.8%) and 13–18 years (5.9%) (Fig. 1). The *tcdB*-positive rates are higher in outpatients than that of inpatients, except in the 13–18 years group ($P < 0.001$).

Among inpatients, the difference in hospital stay length was not statistically significant. Sex and 30-day mortality were not related to *tcd*B positivity. None of the underlying diseases were related to *tcd*B positivity. We categorized the underlying diseases into eight groups, and none of their odds ratio confidence intervals reached statistical significance (data not shown).

2. Presence of concomitant gastrointestinal pathogens

Other gastrointestinal pathogens were detected in the stool samples from 325 patients (33.8%) by mPCR. *Clostridium perfringens* 32.6%, norovirus 20.9%, *Campylobacter* spp. 14.5%, and *Salmonella* spp. 10.2% were detected. Among them, 30 (9.2%) had simultaneously *C. difficile* and other pathogens. In 635 patients, no proven etiology of diarrhea was detected. Among them, 67 patients (9.0%) were *tcd*B *positive*. Altogether, the presence of concomitant gastrointestinal pathogens did not affect the *tcd*B positive rate ($P = 0.52$).

3. Antibiotic exposure

Antibiotic exposure did not increase the *tcd*B positivity. The odds ratio of antibiotics in the antibiotic-treated group (N = 541) compared to that in the antibiotic-naïve group (N = 419) is 0.44 (95% confidence interval–CI: 0.28–0.67; $P < 0.001$). Interestingly, when we stratified the groups by age, the positivity of *tcd*B is inversely proportional in those under 7 years of age. The odds ratios of the groups over 7 years of age are not statistically significant (Table 2).

Table 2
Odds ratio of antibiotics exposed group compared to antibiotics naïve group stratified with age

Age (years)	Odds ratio	95% CI	*P* value
0	0.24	0.08–0.66	< 0.01
1	0.35	0.14–0.79	0.01
2–6	0.38	0.17–0.87	0.02
7–12	1.35	0.34–5.41	0.67
13–18	1.23	0.32–4.78	0.76
Total	0.44	0.28–0.68	< 0.01

[a]Confidence interval (CI)

4. Diagnosis and treatment of C. difficile infection

A total of 22 patients were clinically diagnosed with CDI and treated with metronidazole or vancomycin, but nine had no proven existence of *C. difficile tcd*B (data not shown).

4. Discussion

In neonates, *C. difficile* frequently colonizes the gastrointestinal tract without causing the disease, considering that colonization rates are reportedly 25–36% at 1 month of age [7]. Moreover, studies reported in the 1980s showed that the isolation rate of *C. difficile* was very high. Al-Jumaili *et al.* [10] found that the isolation rate of toxigenic *C. difficile* increased progressively with infant age, from 7% at birth to 100% by 26–35 days. They detected 66% (61/94) of toxigenic strains from neonates aged 1 to 35 days in the special care baby unit. Donta *et al.* [11] reported that *C. difficile* toxin was detected in the feces, 10.5% in normal newborn infants, and five times higher (55%) in neonates in the NICU [11]. Vaginal delivery and breastfeeding were associated with increased rates of toxin carriage. A study performed three decades later found that 45% of infants carried *C. difficile*, with 13% carrying a toxigenic isolate among healthy infants (age, 0–3 years). Toxigenic strains appeared after four months in 40% of the infants under evaluation [12].

Unlike previous reports, we did not find any toxigenic *C. difficile* in newborns and infants under 4 months of age. In this study, antibiotics were administered to all 13 patients in the NICU and 74% of patients under 4 months of age. In neonates, antibiotic administration has been reported to delay *C. difficile* colonization for at least two months [13]. This may explain why neither neonates nor infants of up to 3 months of age had detectable *C. difficile* in this study. The odds ratio, which was statistically significant in age group 0, including neonates, indicated that antibiotic usage does indeed delay *C. difficile* colonization (Table 2).

Larson *et al.* [14]surveyed three postnatal wards and reported a positivity rate of 2–52%, and their difference was statistically significant [14]. They also found epidemiological clusters in ward environments. They suspected a nosocomial spreading, which caused the high prevalence in previous studies. Hospitals systematically developed many infection control measures, such as hand hygiene and standard precautions, which may result in a lower acquisition rate in neonates. Rousseau *et al.* [12] divided the acquisition period into the neonatal phase (early) and infant stage (4–6 months, late). Our youngest toxigenic *C. difficile tcd*B-positive infant was 4 months old; therefore, the subject would have been included in the "late acquisition" group in Rousseau's study. Late acquisition has been reported to be caused by modifications in the gut microbiota composition during a variable food trial.

Figure 1 shows that positivity is the highest in the age group of 1 year in both outpatients and inpatients, and since then, the positivity decreases continually. The high colonization rate of *C. difficile* in infants could result from the commensal microbiota in the pre-weaning period, which is dominated by *Bifidobacterium* spp. and *Lactobacillus* spp., which is more permissive to colonization [15]. After solid food intake, the microbiota is similar to that of adults dominated by *Bacteroidetes* and *Firmicutes* spp. According to a longitudinal observation of the gut microbiome analyzed by 16S rRNA gene sequencing from an infant, the introduction of solid food at around 4 months resulted in a huge change in the microbiome, and the diversity of intestinal microbiota was related to *C. difficile* disappearance [15]. During the observation period, *C. difficile* counts varied with fluctuations of more than 10^5 and eventually disappeared at 12 months. This may explain our first detection of *C. difficile tcd*B in a 4-month-old infant.

We observed that antibiotic usage within 30 days did not increase the positive rate of *C. difficile* infection (Table 1). The odds ratios of the age groups 1, 2–6 and total indicated that antibiotic usage is inversely related to the *tcd*B positive rate. Antibiotic use is a major risk factor for adult CDI, and research by Donta and Myers showed that *C. difficile* toxin could be found in 85% of infants after 14 days of exposure to antibiotics, even when the toxin was not detected during antibiotic therapy [11]. However, only 5% (3/53) were positive for *C. difficile tcd*B 14 days after antibiotic therapy in our study (data not shown). Similar to our results, found antibiotic exposure prior to *C. difficile* detection not different between positive patients and the overall population [12].

Considering the process by which toxigenic *C. difficile* is acquired in these age groups, our study suggests that multiple factors beyond antibiotic usage might affect the positive rate. Our study was based on a molecular method using fresh stool specimens to detect the *C. difficile tcd*B gene. Molecular testing has a higher sensitivity than other methods, which used cell culture with frozen stool samples. Although non-toxigenic *C. difficile* was not included in this study, toxic *C. difficile* detection is important in clinical settings. Although molecular testing alone is too sensitive and not specific for diagnosing CDI [8], it is an appropriate test to estimate the presence of low concentrations, not infection status by *C. difficile* overgrowth, and production of abundant toxin.

In addition, *C. difficile tcd*B was detected in 10.1% of patients with diarrhea in the population under study (0–18 years of age). In a previous study, *C. difficile* was cultured in 7.0% of patients with diarrhea and

14.8% of patients without diarrhea between 2 weeks and 16 years of age. Therefore, the *C. difficile* isolation rate in patients without diarrhea was more than 50% higher than that in patients with diarrhea among outpatients [16]. Another study showed no correlation between diarrhea and *C. difficile* colonization rates in infants [17]. Further, a group aged over 8 years had an infection rate of approximately 5%, which was similar to that of healthy adults [5]. Among children under 15 years of age, Kim *et al.* [18] reported that 15.6% of the group with diarrhea and 6.7% of the control group had *C. difficile* toxin by cytotoxicity neutralization assay, indicating a higher positive rate in those with diarrhea. In the group with diarrhea, the possibility of *C. difficile* infection should be considered for some positive patients.

We observed that the *C. difficile tcd*B-positive group was younger than the negative group. More *tcd*B-positives were found in outpatients than that of inpatients, and their length of stay was shorter than that of the negative group. Underlying diseases such as neoplasm, hematologic, respiratory, genitourinary disorder, and inflammatory bowel disease were not statistically related to *tcd*B positivity, which was contrary to adult CDI. Although severe infection sometimes occurs, most cases in this age group are asymptomatic [19]. It has been suggested that *C. difficile* cannot play a pathogenic role, since immature intestinal epithelial cells of neonates and infants lack receptors that enable the invasion of *C. difficile* toxin A in a study of rabbit ileum [20].

The pediatric patients included in this study were not entirely healthy, since they had diarrhea that required hospital visits, during which stool samples were collected and tested for the presence of diarrhea-causing pathogens. Therefore, our study included both patients with CDI and carriers with non-CDI diarrhea etiology. The limitation of our study is that patients with CDI and carriers cannot be accurately discriminated.

Clinically significant diarrhea can be defined as frequent passage of loose stool. When a child has clinically significant diarrhea with proven toxigenic *C. difficile* presence and without other gastroenteric pathogens, it can be CDI [8]. In clinical practice, even when a diarrheal patient with toxigenic *C. difficile* is positive, it is challenging to distinguish true *C. difficile* infection from colonization.

In our study, 22 patients were clinically diagnosed with CDI and treated with metronidazole or vancomycin, yet nine had no proven existence of *C. difficile tcd*B (data not shown). Moreover, 30.9% (30/97) of *C. difficile tcd*B-positive patients showed positive gastrointestinal pathogens at the same time. This is in accordance with another study which reported a simultaneous positive rate of > 50% with *C. difficile* [21]. We cannot define the remaining 70% as CDI because we did not exclude all other etiologies.

We noticed that *C. difficile tcd*B positivity was not affected by concomitant gastrointestinal pathogens. This result suggests that most *C. difficile tcd*B positive cases are more likely to be colonization and not CDI. The clinical factors that were known as risk factors for CDI, such as underlying disease, antibiotic exposure, and hospital administration, did not increase the CD positive rate in this study. CDI cases were certainly included, but the rate did not appear to be substantial. Therefore, we may cautiously draw a sketch of pediatric CD colonization with this positive rate rather than CDI.

5. Conclusions

Clostridioides difficile is thought to be a hospital-associated infectious pathogen. The acquisition in the community seems to have prevailed due to improvements in individual hygiene levels and hospital infection control at least, in pediatric population. This study demonstrated lower *C. difficile* positivity in the pediatric population than previously reported. Although toxigenic *C. difficile* infection is neither an important nor a common cause of pediatric diarrhea, children can spread it to adults who are at risk of developing CDI. Therefore, children can act as hidden reservoirs for pathogenic strains in the community. Monitoring of toxigenic *C. difficile* positivity in the pediatric population should be approached as an infection control measure, as well as individual diagnosis.

In stool samples of patients with diarrhea aged 0–18 years, *C. difficile* toxin B positivity by gastrointestinal pathogen multiple PCR was 10.1%. The presence of concomitant gastrointestinal pathogens was not associated with toxigenic *C. difficile* positivity, therefore, it is difficult to determine whether *C. difficile* is the cause of diarrhea in the pediatric population. It is thought that the risk of transmission of CDI along with the difficulty of diagnosis should be noted.

Declarations

- **Ethics approval and consent to participate:** This study was approved by the Institutional Review Board of Severance Hospital (2018-0281-001).
- **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 they have no competing interests
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- **Authors' contributions:** All authors have accepted their responsibility for the entire content of this manuscript and have approved its submission.
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References

1. Schutze GE, Willoughby RE. *Clostridium difficile* infection in infants and children. Pediatrics. 2013;131(1):196-200.
2. Lessa FC, Mu Y, Bamberg WM, Beldavs ZG, Dumyati GK, Dunn JR, et al. Burden of *Clostridium difficile* infection in the United States. N Engl J Med. 2015;372(9):825-34.
3. Spigaglia P, Mastrantonio P. Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. J Clin Microbiol. 2002;40(9):3470-5.

4. Chitnis AS, Holzbauer SM, Belflower RM, Winston LG, Bamberg WM, Lyons C, et al. Epidemiology of community-associated *Clostridium difficile* infection, 2009 through 2011. JAMA Intern Med. 2013;173(14):1359-67.
5. Miyajima F, Roberts P, Swale A, Price V, Jones M, Horan M, et al. Characterisation and carriage ratio of *Clostridium difficile* strains isolated from a community-dwelling elderly population in the United Kingdom. PLoS One. 2011;6(8):e22804.
6. Jangi S, Lamont JT. Asymptomatic colonization by *Clostridium difficile* in infants: implications for disease in later life. J Pediatr Gastroenterol Nutr. 2010;51(1):2-7.
7. Lees E, Miyajima F, Pirmohamed M, Carrol E. The role of *Clostridium difficile* in the paediatric and neonatal gut—a narrative review. Eur J Clin Microbiol Infect Dis. 2016;35(7):1047-57.
8. McDonald LC, Gerding DN, Johnson S, Bakken JS, Carroll KC, Coffin SE, et al. Clinical Practice Guidelines for *Clostridium difficile* Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). Clin Infect Dis. 2018;66(7):e1-e48.
9. Sawyer SM, McNeil R, Francis KL, Matskarofski JZ, Patton GC, Bhutta ZA, et al. The age of paediatrics. The Lancet Child & Adolescent Health. 2019;3(11):822-30.
10. Al-Jumaili I, Shibley M, Lishman A, Record C. Incidence and origin of *Clostridium difficile* in neonates. J Clin Microbiol. 1984;19(1):77-8.
11. Donta ST, Myers MG. *Clostridium difficile* toxin in asymptomatic neonates. J Pediatr. 1982;100(3):431-4.
12. Rousseau C, Poilane I, De Pontual L, Maherault A-C, Le Monnier A, Collignon A. *Clostridium difficile* carriage in healthy infants in the community: a potential reservoir for pathogenic strains. Clin Infect Dis. 2012;55(9):1209-15.
13. Holton A, Hall M, Lowes J. Antibiotic exposure delays intestinal colonization by *Clostridium difficile* in the newborn. J Antimicrob Chemother. 1989;24(5):811-7.
14. Larson H, Barclay F, Honour P, Hill I. Epidemiology of *Clostridium difficile* in infants. J Infect Dis. 1982;146(6):727-33.
15. Davis MY, Zhang H, Brannan LE, Carman RJ, Boone JH. Rapid change of fecal microbiome and disappearance of *Clostridium difficile* in a colonized infant after transition from breast milk to cow milk. Microbiome. 2016;4:53.
16. Boenning DA, Fleisher GR, Campos JM, Hulkower CW, Quinlan RW. *Clostridium difficile* in a pediatric outpatient population. Pediatr Infect Dis. 1982;1(5):336-8.
17. Holst E, Helin I, Mårdh P-A. Recovery of *Clostridium difficile* from children. Scand J Infect Dis. 1981;13(1):41-5.
18. Kim K, Suh I-S, Kim JM, Kim CW, Cho Y-J. Etiology of childhood diarrhea in Korea. J Clin Microbiol. 1989;27(6):1192-6.

19. Enoch DA, Butler MJ, Pai S, Aliyu SH, Karas JA. *Clostridium difficile* in children: colonisation and disease. J Infect. 2011;63(2):105-13.
20. Eglow R, Pothoulakis C, Itzkowitz S, Israel EJ, O'Keane CJ, Gong D, et al. Diminished *Clostridium difficile* toxin A sensitivity in newborn rabbit ileum is associated with decreased toxin A receptor. J Clin Invest. 1992;90(3):822-9.
21. Valentini D, Vittucci A, Grandin A, Tozzi A, Russo C, Onori M, et al. Coinfection in acute gastroenteritis predicts a more severe clinical course in children. Eur J Clin Microbiol Infect Dis. 2013;32(7):909-15.

Figures

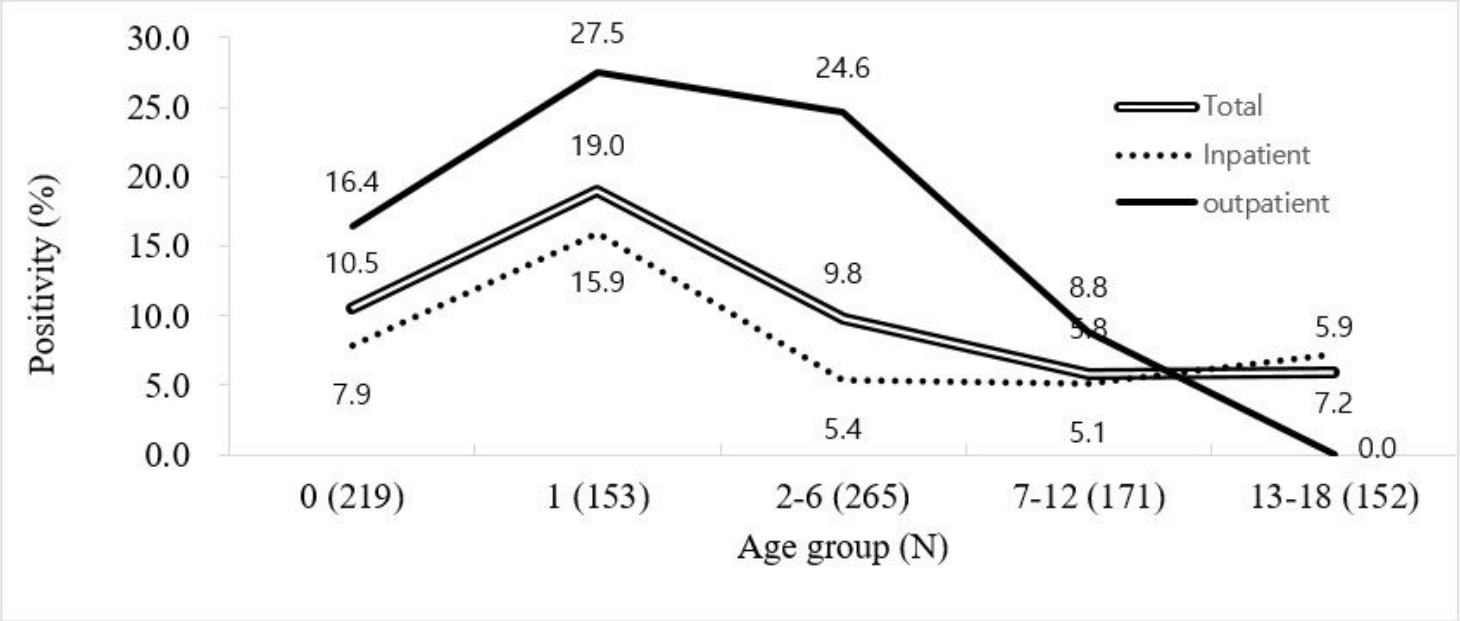

Figure 1

Clostridioides difficile toxin B positivity detected by multiplex PCR of indicated age groups.