Original Article

Analysis of risk factors for community-acquired *Clostridioides difficile* diarrhea in children: a case-control study in Chenzhou, China

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Background: Most previous studies on *Clostridium difficile* infection (CDI) mainly focused on adults with underlying diseases or critical illnesses. However, the number of CDI cases in children has also significantly increased, especially the growth of community-acquired CDI, which has attracted attention. This study was conducted to examine the toxin gene characteristics and the risk factors associated with community-acquired CDI (CA-CDI) in children with diarrhea.

Methods: Children with diarrhea before admission or within 48 hours of hospitalization were included in the study. Stool samples were collected from children with community-acquired diarrhea who were treated at the Children's Hospital of the First People's Hospital of Chenzhou, China from June of 2021 to June of 2022. Fluorescence real-time polymerase chain reaction was utilized to detect *Clostridioides difficile* (CD) toxins A (*tcdA*) and B (*tcdB*) genes as well as binary toxin gene A (*cdtA*) and B (*cdtB*) in the specimens cultured for CD. Each child with CA-CDI was matched with four control children of the same sex, age, and place of residence. Necessary clinical data were extracted from the hospital's electronic medical record system. Then, a multivariate conditional logistic regression analysis was applied to identify potential risk factors for CA-CDI.

Results: Sixteen (8.3%) of the 193 stool specimens who tested positive for CD were selected for the case group, and their matching 64 control patients were in the study cohort. The breakdown of the CD genotypes of the 16 positive cases were follows: 14 ($tcdA^+$ and $tcdB^+$) (7.25%) and 2 ($tcdA^+$ and $tcdB^-$) (1.04%). The cdtA and cdtB binary toxin genes were negative in all. The results of multivariate conditional logistic regression analysis identified antibiotic use within the previous month [odds ratio (OR) =5.13; 95% confidence interval (CI): 1.65–15.91] and non-breastfeeding (OR =4.89; 95% CI: 1.11–21.53) as independent risk factors for CDI in pediatric patients experiencing community-acquired diarrhea.

Conclusions: Children who had been treated with antibiotics and not breastfed were more susceptible to CDI. Therefore, in order to prevent and to control the spread of CD infection, being prudent to the aforementioned high-risk factors is strongly advocated in clinical practice.

Keywords: Children; *Clostridioides difficile* (CD); community-acquired infection; diarrhea; case-control study

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Introduction

Clostridioides difficile (CD), previously known as *Clostridium difficile*, is the most common pathogen known to cause diarrhea in the healthcare setting (i.e., nosocomial infection), which is associated with significant morbidity and mortality. The 2017 US Centers for Disease Control (CDC) reported nearly 12,000 deaths among 230,000 CD infection (CDI) cases per year in Americans, accounting for over 1 billion in the disease-related economic burden. Therefore, CDI is considered a serious antibiotic-induced urgent public health threat, and a difficult to manage antibiotic-resistant infection (https://www.cdc.gov/drugresistance/biggest-threats.html), and the recurrence rate was high when treated with antibiotics alone (1).

CD is a gram-positive, spore-forming, obligate anaerobic bacterium ubiquitously present in the environment. CD is known as a conditional pathogen within human and animal gastrointestinal (GI) tracts and is implicated in causing antibiotic-associated diarrhea in susceptible individuals (2,3). The spectrum of clinical presentation of CDI is broad, from asymptomatic colonization to diarrhea, accompanied by nausea, abdominal pain or tenderness, loss of appetite, and fever. In severe CDI cases, pseudomembranous colitis and megacolon can develop which can be fatal (4).

The chief virulence factors of CD include enterotoxin A (tcdA) and cytotoxin B (tcdB) which are encoded by the *tcdA* and *tcdB* genes in the pathogenicity locus (PaLoc), respectively. Some CD strains can also produce cytolethal distending toxin (CDT), encoded by the *cdtA* and *cdtB* genes in the CDT locus (CdtLoc). *cdtB* mediates *cdtA* entry into cells, and the CDT complex disrupts the actin cytoskeleton, and causes cell death (5-7).

The incidence and mortality rates of CDI in the United States, Canada, and Europe have significantly increased over the past 20 years, despite improved surveillance, intervention, and treatments (3). Although many countries have established guidelines for CDI diagnosis, treatment, prevention, outbreak control, and management of antibiotic use, studies on CDI in China have remained limited. With advances in detection, an increasing number of cases of CDI are being reported outside of medical institutions. Data from Europe and the United States show that approximately 20–27% of CDIs are community-acquired, and the incidence is about 20–30 per 100,000 (8,9), and the prevalence of CDI as a community-acquired infection has risen steadily in recent years (10).

CD has become an inexorable nosocomial pathogen (11), and the majority of previous studies on CDI were focused on adults, particularly on older patients with an underlying disease or who are critically ill. However, there is an alarming increase in the number of CA-CDI cases globally in the pediatric population (12,13). Compared to the hospital-acquired CDI (HA-CDI), there seems to be a trend for younger patients to contract community-acquired CDI (CA-CDI). A Study had shown that children under 2 years old were mostly colonized (14), possibly due to immature intestinal function, which means that epithelial cells lack mature toxin binding receptors and were not sensitive to *Clostridium difficile* toxins. However, asymptomatic individuals could also serve as hosts for Clostridium difficile (15), and when the gut microbiota status was disrupted, CDI could occur (16). Despite this increasing trend, there have only been a few published studies on pediatric patients, and in China, there are very limited data on children. Therefore, we have decided to examine the toxin gene characteristics in pediatric patients with community-acquired diarrhea and to identify the risk factors for CA-CDI in Chenzhou, Hunan Province to provide supportive data for future studies. We present this article in accordance with the STROBE reporting checklist (available at https://tp.amegroups.com/article/

Highlight box

Key findings

- Antibiotics use and non-breastfeeding were identified as independent risk factors for community-acquired *Clostridioides difficile* infection (CA-CDI) in children in Chenzhou, China.

What is known and what is new?

- *Clostridioides difficile* (CD) is a well-known hospital pathogen for nosocomial infection in adults, but less is known about factors influencing CDI in children, due to scarcity of available data, especially on CA-CDI in children in China.
- The prevalent toxin genotype of CD was characterized and used to identify patients for a case-control study to identify high-risk factors associated with contracting CDI among pediatric patients who were diagnosed with community-acquired diarrhea in Chenzhou, China.

What is the implication, and what should change now?

- In clinical practice, more attention should be paid to the aforementioned high-risk factors to prevent and to control the spread of CD infection and CA-CDI in pediatric patients.

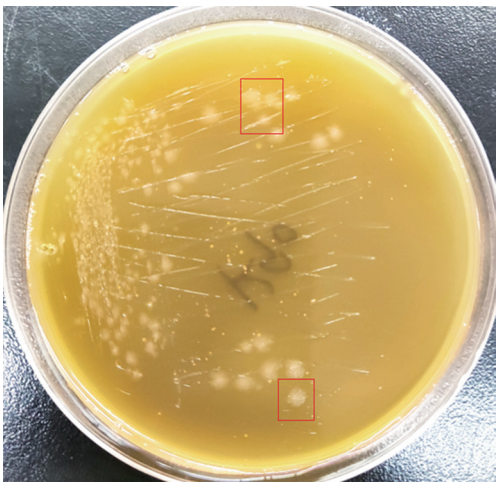

Figure 1 CD colonies cultured on cycloserine-cefoxitin fructose agar medium. The two red boxes represent typical CD colonies on the culture medium. CD, *Clostridioides difficile*.

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Methods

Study participants

The study participants were pediatric patients treated at Children's Hospital of the First People's Hospital of Chenzhou, an affiliated hospital of Xiangnan University, from June 2021 to June 2022.

A meta-analysis showed that the average colonization rate of children was around 35% (17), so the sample size estimation method for this study was: $n=4p(1-p)/d^2=186$, p=0.35, d=0.2p. And a 1:4 case-control design was employed for exploring risk factors. The case group consisted of sixteen pediatric patients who were positive for CD toxin gene analysis, and every test-positive patient was matched with 4 test-negative patients of the same sex, age, and place of residence, totaling 64 patients in the control group.

The onset of diarrhea occurring prior to admission or within 48 hours of hospitalization is used as the definition of CDI. The inclusion criteria were: (I) age <12 years, (II) having ≥3 diarrheal stools per day, (III) stools with watery or unformed consistencies. The exclusion criteria were patients with (I) diarrhea confirmed to be caused by non-CD pathogens; (II) diarrhea caused by food poisoning; (III) recent treatment with diarrheal medications or GI drugs; (IV) a history of digestive system diseases such as enteritis, GI tumors, irritable bowel syndrome, or gastrojejunostomy; and (V) a serious comorbidity such as heart, lung, brain, liver, kidney, blood, or immune disorder. This study was conducted in accordance with the Helsinki Declaration (as revised in 2013). This study was approved by ethics board of Xiangnan University (No. XNXY2020023) and has obtained informed consent from the patients' parents or legal guardians.

Main reagents and equipment

A probe qPCR assay kit (cat no. RR390A; Takara Bio, Otsu, Japan), a refrigerated centrifuge (Thermo Fisher Scientific, Waltham, USA), and a fluorescence-based 7500 Real-Time PCR System machine (Applied Biosystems, Waltham, USA, Thermo Fisher Scientific) were utilized in the study.

Collection of stool samples

Fresh stool samples were collected and capped in a sterilized container immediately. The specimens were void of any urine or wastewater contamination and sent for testing within 1 hour of collection. Each stool sample was divided into two aliquots: one for culture and testing, and the other storage at –80 ℃ for later use if needed.

CD culture and microscopy

Stool samples were mixed with absolute ethanol and vortexed to allow thorough mixing. The solution was then left to stand at room temperature for 60 minutes and centrifuged at 3,000 rpm for 10 minutes. The formed pellet was collected, and then, poured and spread evenly onto a dried cycloserine-cefoxitin fructose agar as selective culture medium for CD. The plates were placed in anaerobic bags and cultured at 37 ℃ for 48 hours. Typical CD colonies were grayish white or pale yellow, flat, and dry, coarse edges, and "omelet-like", in appearance with a characteristic foul smell and fluoresced under ultraviolet light (*Figure 1*). CD were observed as typical Gram-positive thick rods with oval spores located near the poles, resembling a classic matchstick-like appearance under the microscope (*Figure 2*).

Detection of CD toxin A/B and binary toxin genes cdtA and cdtB

A bacterial DNA extraction kit (Tiangen Biochemical Technology Co., Ltd. Beijing, China) was used to extract CD DNA from suspected positive strains after anaerobic

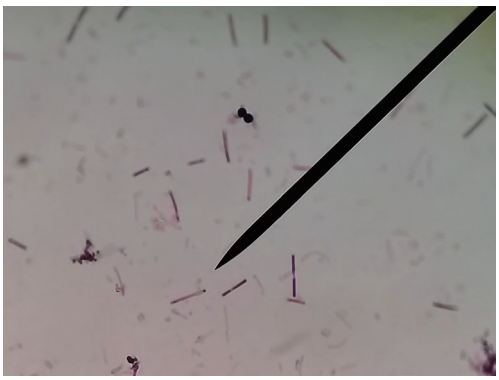

Figure 2 CD under microscope (×1,000): gram-positive Bacillus. CD, *Clostridioides difficile*.

cultures, and fluorescence real-time PCR was utilized to amplify *tcdA* and *tcdB* and binary toxin genes, *cdtA* and *cdtB*. Primers and probes were synthesized (per instruction provided by the Chinese Center for Disease Control and Prevention), and detection was performed as previously described (18).

The reaction system was set up according to the assay kit instruction (cat no. RR390A; Takara Bio) which included: 10 μL of Premix Ex Taq (Probe qPCR) 2× (Takara RR390A), 0.4 μL of PCR forward primer (10 μM), 0.4 μL of PCR reverse primer (10 μM), 0.8 μL of probe (10 μM), 0.2 μL ROX reference dye II, 2 μL of DNA template, and 6.2 μL of ddH_2O. Reactions were run under the following conditions: one cycle at 95 ℃ for 20 seconds, then sat idle at 95 ℃ for 3 seconds, and finally, 40 cycles at 58 ℃ for 30 seconds per cycle; the controls were arranged, simultaneously. Samples showed a typical amplification curve, with a Ct value was <35, indicating a positive result, while without a typical amplification curve, indicating a negative result.

Data collection

The hospital's electronic medical record system was utilized to collect clinical data such as age, sex, place of residence, history of preterm birth, breastfeeding up to 1 year, sharing utensils, history of other diseases prior to admission, past hospitalization and surgery, antibiotic use within the previous month, antibiotic use ≥1 week, type of antibiotic prescribed, glucocorticoid, antacid or nonsteroidal anti-inflammatory medication use within the previous month, clinical symptoms (abdominal pain, watery stools, vomiting, or fever), and laboratory test results (hemoglobin, white blood cell count, serum albumin, and serum C-reactive protein).

Statistical analysis

The SPSS 25.0 software (IBM Corp. New York, USA) was used for statistical analysis. Qualitative data were expressed as the percentage (%), and the χ^2 test was applied for intergroup comparisons. Normally distributed continuous variables were expressed as mean ± standard deviation, and the *t*-test was used for intergroup comparisons. Nonnormally distributed data were expressed as median (P_{25}–P_{75}). After χ^2 test/*t*-test, the possible risk factors which statistically significant variables were included in the conditional logistic regression model for stepwise regression analysis. A conditional logistic regression analysis was applied to analyze possible risk factors, with a significance level of two sides α=0.05.

Results

CA-CDI in children and toxin gene test results

A total of 193 children with community-acquired diarrhea were recruited. Sixteen (8.3%) of these children were identified to have CDI, and 10 of them were males. The children with CDI had a mean age of 1.4 years (1.0–3.1 years), with a range from 1 month to 5.5 years. Fourteen of the 16 children had $tcdA^+$ and $tcdB^+$ CDI strain, while two had $tcdA^+$ and $tcdB^-$ CDI strain. The *cdtA* and *cdtB* binary toxin genes were not detected in any of the children.

Univariate analysis of CA-CDI

The univariate analysis of 21 factors identified non-breastfeeding, sharing utensils, and antibiotic use within the previous month as the primary risk factors for CA-CDI (*Table 1*).

Multivariate conditional logistic regression analysis

The three principal risk factors from the univariate analysis were selected as independent variables, and the children with CDI were used as the dependent variable for a conditional logistic regression analysis. Antibiotic use within the previous month [odds ratio (OR) =5.13; 95% confidence interval (CI): 1.65–15.91] and non-breastfeeding (OR =4.89; 95% CI: 1.11–21.53) were both categorized as independent risk factors for CDI (P<0.05) (*Table 2*).

Table 1 Univariate analysis of CA-CDI in children in Chenzhou

Factors	Case group (n=16)	Control group (n=64)	χ^2/t	P
Delivery status			0.848	0.654
Preterm birth	15 (93.8)	56 (87.5)		
Full-term birth	1 (6.2)	5 (7.8)		
Post-term birth	0 (0.0)	3 (4.7)		
Delivery method			2.201	0.138
Vaginal delivery	7 (43.8)	41 (64.1)		
Cesarean section	9 (56.2)	23 (35.9)		
Feeding patterns			7.937	0.005
Breastfeeding up to 1 year	2 (12.5)	33 (51.6)		
Non-breastfeeding	14 (87.5)	31 (48.4)		
Sharing utensils			7.584	0.006
Yes	11 (68.7)	20 (31.3)		
No	5 (31.3)	44 (68.7)		
Other diseases before admission			0.317	0.573
Yes	10 (62.5)	35 (54.7)		
No	6 (37.5)	29 (45.3)		
Surgical history			—	—
Yes	0 (0.0)	0 (0.0)		
No	16 (100.0)	64 (100.0)		
Previous hospitalization history			2.179	0.140
Yes	4 (25.0)	29 (45.3)		
No	12 (75.0)	35 (54.7)		
Use of antibiotics within the previous month			14.066	0.000
Yes	12 (75.0)	16 (25.0)		
No	4 (25.0)	48 (75.0)		
Antibiotic treatment ≥1 week			0.583	0.445
Yes	5 (41.7)	9 (56.3)		
No	7 (58.3)	7 (43.7)		
Type of antibiotics			4.654	0.199
β-lactam antibiotics	8 (66.7)	15 (93.8)		
Lincosamides	1 (8.3)	0 (0.0)		
Macrolides	2 (16.7)	0 (0.0)		
Vancomycin	1 (8.3)	1 (6.2)		
Use of nonsteroidal anti-inflammatory drugs within the previous month			2.124	0.145
Yes	10 (62.5)	27 (42.2)		
No	6 (37.5)	37 (57.8)		

Table 1 (*continued*)

Table 1 (*continued*)

Factors	Case group (n=16)	Control group (n=64)	χ^2/t	P
Use of glucocorticoids within the previous month			—	—
Yes	0 (0.0)	0 (0.0)		
No	16 (100.0)	64 (100.0)		
Use of antacid use within the previous month			0.364	0.555
Yes	1 (6.3)	2 (3.1)		
No	15 (93.2)	62 (96.9)		
Clinical presentation				
Abdominal pain	3 (18.8)	8 (12.5)	0.422	0.516
Watery stools	7 (43.8)	16 (25.4)	2.083	0.149
Vomiting	4 (25.0)	18 (28.1)	0.063	0.802
Fever	8 (50.0)	20 (31.3)	1.978	0.160
Laboratory tests				
Hemoglobin (g/L)	118.38±13.76	113.70±14.59	1.158	0.250
White blood cell count ($\times10^9$/L)	10.70±4.02	8.92±3.80	1.647	0.104
Serum albumin (g/L)	44.88±4.85	42.89±5.25	0.957	0.342
Serum C-reactive protein (mg/L)	5.21±2.66	4.96±3.83	0.253	0.801

Data are shown as n (%) or mean ± standard deviation. CA-CDI, community-acquired *Clostridioides difficile* infection.

Table 2 Multivariate logistic regression analysis of CA-CDI in children in Chenzhou (16 cases and 64 controls)

Factor	β	SE	Wald χ^2	OR	95% CI	P
Use of antibiotics within the previous month	1.635	0.578	8.004	5.13	1.65–15.91	0.005
Non-breastfeeding	1.587	0.757	4.397	4.89	1.11–21.53	0.036
Sharing utensils	0.798	0.554	2.079	2.22	0.75–6.56	0.149

CA-CDI, community-acquired *Clostridioides difficile* infection; SE, standard error; OR, odds ratio; CI, confidence interval.

Discussion

CD research has gradually expanded in China in recent years. With a better understanding of CDI, more investigators have reported on the incidence and severity of CA-CDI (19). The recently published meta-analysis results indicated that the detection rate of *CD* in diarrhea patients is approximately 14% (20). In addition, a retrospective analysis revealed that the nucleic acid detection rate of toxin-producing CD in patients with community-acquired diarrhea in southwestern China was approximately 14% (21). However, these CDI studies have focused on older or critically ill patients with HA-CDI, and only a few studies have included children with CA-CDI.

In our study, 193 pediatric patients with community-acquired diarrhea were evaluated, and CDI was detected in 16 patients with the detection rate of 8.29%, below the reported rate. Our CD genotype analysis revealed that the majority (14/16) were *tcdA*$^+$, *tcdB*$^+$, *cdtA*$^-$, and *cdtB*$^-$ strain, indicating that the A/B toxin (*tcdA*$^+$ and *tcdB*$^+$) without the binary toxin strain was the main type of CA-CDI in the Chenzhou region, which was consistent with the results from other geographical regions of China (22,23).

The conditional logistic regression analysis identified that intake of antibiotics within the previous month and non-breastfeeding as independent risk factors for CA-CDI

in our cohort; indeed, an antibiotic exposure has been a well-documented independent risk factor for CDI (24,25). One study has found that a use of antibiotics within three months increased the risk of CDI by more than 3-folds (OR =3.68; 95% CI: 2.04–6.62) (26); in patients with ulcerative colitis, a use of antibiotics within one month increased the risk of CDI by 12-folds (27). Although children have not been the focus of previous studies in CDI, a misuse or overuse of broad-spectrum antibiotics seemed to have resulted in more CDI cases in children. This is reflected in a steady increase in the incidence of HA-CDI and CA-CDI reported in this age group (24,28).

Our investigation has also shown that the use of antibiotics in the previous month increased the risk of contracting CDI and has resulted in more cases of community-acquired diarrhea in children (OR =5.13; 95% CI: 1.65–15.91), similar to the other study findings (24,29). Although cephalosporin and penicillin antibiotics had been the antibiotics implicated to result in CDI (30,31), our results did not uncover any statistical differences between the types of antibiotics though β-lactam being the most common.

One study reported the colonization rates in breastfed and mixed-fed infants were 3% and 14%, respectively, while the colonization rate in formula-fed infants was 33% (32), significantly higher, implicating that the type of milk might dictate the colonization pattern of CD in children. Our study results showed that the percentage of non-breastfed pediatric patients in the case group was 87.5%, which is a significantly higher proportion than that in the control group, demonstrating a higher risk for CA-CDI in non-breastfed patients (OR =4.89).

It is well-documented that breastfeeding has many benefits, beyond providing nutrients. A significant difference in gut microbiota between formula-fed and breastfed infants has been reported, and a relative low abundance of *Bifidobacteria* in the intestines of non-breastfed infants was noted (33). This may be explained by the fact that breastmilk has a slightly lower pH than plasma and facilitates the growth of *Bifidobacteria*, which contain certain microbiota that are essential for the early and stable establishment of gut microbiome environment (34). This initial colonization sets a healthy balance for the host's immune system and lays a protective barrier against harmful pathogen from colonizing, including CD. Immunoglobulin A, in particular, transferred among maternal immunoglobulins contained in breastmilk, can neutralize CD toxin A (35), thereby mitigating its toxic effects on the intestinal cells.

Although the current study was conducted with the well-defined processes on a prospectively recruited group of children with the rigorous inclusion and exclusion criteria, our results at a single study site may not be generalizable to patients in other geographical regions. Due to the low incidence of CD in our cohort and the relatively low number of participants, presence of bias in our results cannot be discounted. In the past, a few studies have identified a history of glucocorticoid and gastric acid-inhibitor use (including proton-pump inhibitors and H2-receptor blockers) as risk factors for CDI (36,37). However, our study did not demonstrate their association with the disease; this might have been because infants and toddlers were less likely to be exposed to these drugs.

Therefore, there is a need for a multicenter comprehensive study with a larger sample size to be conducted in the future. Establishment of biorepositories for clinical studies may be necessary (38) for in-depth analysis of various factors and novel discovery of differences in certain factors which may also be important in managing the risk of CDI. Regardless, our study results enhanced our understanding of the genetic characteristics of the CD strain and the contributing risk factors of CD in the pediatric patients in the Chenzhou area.

Conclusions

Our current study has demonstrated that antibiotics exposure and lack of breastfeeding up to 1 year were the critical risk factors for CA-CDI in children in Chenzhou, China. These two factors greatly increased the risk of children developing CA-CDI diarrhea by 5.13 times and 4.89 times, respectively. Despite the limitations of this study, these findings can facilitate improving public health and modifying clinical decision-making. Furthermore, our study can serve as a reference study, upon which multicenter studies with larger cohorts of children for further in-depth analyses of various factors that influence CDI and toxin gene characterization to be conducted in China.

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Footnote

Reporting Checklist: The authors have completed the STROBE reporting checklist. Available at https://tp.amegroups.com/article/view/10.21037/tp-23-448/rc

Data Sharing Statement: Available at https://tp.amegroups.com/article/view/10.21037/tp-23-448/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-23-448/coif). A.S.D. received speaking fees and travel support for the above speaking event from AbbVie and serves as the Member of Executive (Secretary) of NZ Gastroenterology Society. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Helsinki Declaration (as revised in 2013). This study was approved by ethics board of Xiangnan University (No. XNXY2020023) and has obtained informed consent from the patients' parents or legal guardians.

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