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ORIGINAL RESEARCH

Prevalence and Genetic Diversity of *Clostridium perfringens* Isolates in Hospitalized Diarrheal Patients from Central China

Baoya Wang[1]
Wenjuan Dong[2]
Liyan Ma[3]
Yonghui Dong[4]
Shanmei Wang[1]
Youhua Yuan[1]
Qiong Ma[1]
Junhong Xu[1]
Wenjuan Yan[1]
Jing Nan[1]
Qi Zhang[1]
Wenbo Xu[1]
Bing Ma[1]
Yafei Chu[1]
Jiangfeng Zhang[1]
Li Li[1]
Yi Li[1]

[1]Department of Clinical Microbiology, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, and People's Hospital of Henan University, Zhengzhou, 450003, Henan, People's Republic of China; [2]Department of Clinical Microbiology, Xicheng District Pingan Hospital, Beijing, 100035, People's Republic of China; [3]Department of Clinical Microbiology, Beijing Friendship Hospital, Capital Medical University, Beijing, 100050, People's Republic of China; [4]Department of Osteology, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, and People's Hospital of Henan University, Zhengzhou, 450003, Henan, People's Republic of China

Objective: This study aimed to investigate the prevalence, genetic diversity and clinical characteristics of *Clostridium perfringens* isolates from hospitalized clinical diarrheal patients.

Methods: A prospective study was conducted on 1108 patients with diarrhea during hospitalization. Stool samples were cultured for *C. perfringens*, and the toxin genes were detected by PCR. The available clinical data of 112 patients were analyzed to study the clinical features of various isolates. Multi-locus sequence typing (MLST) was performed to assess phylogenetic relationship between different isolates.

Results: A total of 153 (13.8%) isolates were obtained from patients' stools. *C. perfringens* type F (49.0%) was the major toxin type in the isolates, followed by type A (n = 59, 38.6%) and type C (n = 14, 9.2%). Patients older than 50 years and those with underlying diseases of cancer, hepatobiliary system, and ulcerative colitis (UC) were more predisposed to *C. perfringens* type F and type A infection than to type C. The patients infected with type C experienced more severe clinical symptoms compared to those with type A infection. There was a significant association between type F^C and foodborne gastrointestinal (GI) diseases ($p = 0.018$), between type F^P and antibiotic-associated diarrhea (AAD) ($p < 0.001$), and between type A and sporadic diarrhea (SD) ($p < 0.001$). Phylogenetic analysis indicated that type F isolates carrying a chromosomal *cpe* gene mainly belonged to ST77 (6/15 isolates). Type F isolates with *cpe* gene on a plasmid exhibited high genetic diversity.

Conclusion: High prevalence and considerable genetic diversity of *C. perfringens* type F were found in clinical diarrheal patients. Elderly people and patients with cancer, hepatobiliary diseases or UC, or suspected of having food poisoning (FP) may be targeted for routine testing of *C. perfringens* toxin genes and may benefit from early detection of *C. perfringens* type C isolates that cause more severe clinical symptoms.

Keywords: *Clostridium perfringens*, toxin type, clinical characteristics, prevalence, MLST

Correspondence: Yi Li; Li Li
Department of Clinical Microbiology, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, and People's Hospital of Henan University, Weiwu Road 5, Zhengzhou, 450003, Henan, People's Republic of China
Tel +8637165580484
Fax +8637187160318
Email liyilabmed@henu.edu.cn; hnykylili@126.com

Introduction

Clostridium perfringens (*C. perfringens*) is an important pathogen, causing human gastrointestinal (GI) diseases, particularly in the hospital environment. Recently, subtypes of *C. perfringens* have been classified into seven toxin types (A through G) based on their ability to produce the major lethal toxins, alpha, beta, epsilon, iota, enterotoxin (CPE) and NetB.[1] CPE encoded by *cpe* gene, is a 35-kDa polypeptide that binds to claudin receptors on enterocytes to form pores, disrupting the intercellular claudin tight junctions and causing intestinal disease symptoms.[2] CPE-positive *C. perfringens*

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subtypes are responsible for causing food poisoning (FP) and non-foodborne gastrointestinal (GI) diseases such as AAD and SD.[3–5] Type F represents the formerly called CPE-positive isolates of *C. perfringens* type A and produces CPA and CPE toxins. Recently, Azimirad et al reported that 13.3% of patients with AAD carried type F isolates, demonstrating the importance of type F in the development of AAD.[4] Additionally, type F was reported as an essential infection source for asymptomatic carriers and foodborne diseases.[6] *Clostridium perfringens* type C and type D also express CPE,[1] and type C can cause enteric diseases characterized by vomiting, diarrhea, and abdominal cramps. Type C can also cause fatal intestinal necrosis in humans and other animals.[7,8] Apart from CPE, beta2 toxin encoded by the *cpb2* gene has been associated with enteric disease in humans and necrotic enteritis in chickens and many other animal species.[7,9,10] A recent study suggested that beta2 toxin was significantly associated with children suffering from autism spectrum disorders (ASD).[11]

Multi-locus sequence typing (MLST) is a commonly used technique for the typing of human, animal and foodborne pathogens. An MLST scheme for *C. perfringens* was developed by Xiao et al, comparing nucleic acid sequences of eight housekeeping genes of *C. perfringens* isolates and analyzing phylogenetic relationship between different species.[12] Two studies have shown that different toxin types of *C. perfringens* exhibited distinct genetic characteristics.[6,13]

C. perfringens is well known in many countries as the causative agent of several forms of enteric disease;[4,14–16] however, the prevalence of enterotoxigenic *C. perfringens* isolates has not been systematically studied in hospitals in China. This study aimed to determine the prevalence, clinical characteristics, and molecular epidemiology of *C. perfringens* in hospitalized patients suffering from gastroenteritis, including foodborne GI diseases, AAD and SD, in the central region of China.

Materials and Methods

Sample and Clinical Data Collection

Fecal samples were collected from 1108 patients suffering from diarrhea and admitted to Henan Provincial People's Hospital, Zhengzhou, China between Oct 2018 and Oct 2019. The samples were tested for *C. difficile* and *C. perfringens*. *C. difficile* toxin genes (*tcdB, cdt*, and *tcdc* deletion at nucleotide 117) from fecal samples were detected by GeneXpert *C. difficile* PCR assay (Cepheid Inc., USA). The available medical records of 112 patients with fecal samples positive for *C. perfringens*, were reviewed for age, gender, clinical profile, underlying disease, procedures performed and results of laboratory tests.

Culture and Identification of *C. perfringens* Isolates

The freshly collected stool specimens were delivered within 2 h of collection to a clinical microbiology laboratory to test for the presence of *C. perfringens*. After shock treatment with 95% alcohol, stool specimens were cultured anaerobically on 5% sheep blood agar plates at 37°C for 24 h. The colonies suspected to be belonging to *C. perfringens* were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Bruker Daltonics GmbH, Billerica, MA, USA). Isolates were maintained in cooked-meat medium with glycerol (30%) at −80°C for further studies.

PCR Amplification of Toxin Genes

Bacterial genomic DNA was extracted from the colonies growing on 5% sheep agar plates using a Bacterial Genomic DNA Extraction Kit (Cat. No. 9763, Takara, Japan). Plasmid DNA was purified from the pure bacterial cultures using E.Z.N.A. Plasmid Mini Kit I (Cat. No. D6942, Omega, USA). The genomic and plasmid DNA were stored at −20°C till used for PCR experiments.

The toxin genes *cpa, cpb, cpb2, etx, iap, cpe* and *netB* were amplified by PCR using specific primer pairs[1,17] (Table 1). DNA amplification by PCR was carried out in a reaction volume of 25 μL with 3 μL of template DNA (200 ng/μL), 1.5 μL each of 10 pmol/μL forward and reverse primers, 6.5 μL of water, and 12.5 μL of 2× Multiplex PCR Mix. The PCR was performed using a Bio-Rad T100 system (USA) using the following program: Initial denaturation step of 5 min duration at 95°C, followed by 35 cycles of 30 s at 94°C, 1 min at 50 °C, and 1 min at 72°C. The final extension was performed for 10 min at 72°C. The PCR products (4 μL) were visualized by electrophoresis on a 2% agarose gel with a 2000-bp ladder (DL2000, Takara, Japan) as the molecular size marker. The type B isolate *C. perfringens* ATCC 3626 was used as a positive control for *cpa, cpb* and *etx* genes. Isolates SM101 and ATCC 13124 were used as positive and negative controls, respectively, for *cpe* gene.

Table 1 Primers Used for PCR Amplifications in This Study

Target Genes	Primers	Sequence (5′–3′)	Size	Reference
cpa	CPA5L CPA5R	AGTCTACGCTTGGGATGGAA TTTCCTGGGTTGTCCATTTC	900bp	[17]
cpb	CPBL CPBR	TCCTTTCTTGAGGGAGGATAAA TGAACCTCCTATTTTGTATCCCA	611bp	[17]
cpe	CPEL CPER	GGGGAACCCTCAGTAGTTTCA ACCAGCTGGATTTGAGTTTAATG	506bp	[17]
etx	CPETXL CPETXR	TGGGAACTTCGATACAAGCA TTAACTCATCTCCCATAACTGCAC	396bp	[17]
iap	CPIL CPIR	AAACGCATTAAAGCTCACACC CTGCATAACCTGGAATGGCT	293bp	[17]
cpb2	CPB2L CPB2R	CAAGCAATTGGGGGAGTTTA GCAGAATCAGGATTTTGACCA	200bp	[17]
netB	JRP6656 JRP6655	CTTCTAGTGATACCGCTTCAC CGTTATATTCACTTGTTGACGAAAG	738bp	[1]
colA	colA-F colA-R2	ATTAGAAAGTTTATGTACAATAGGTG AAGACATTCTATTATTTCTATCGTAAGC	681bp	[12]
groEL	groEL-F groEL-R	TACAAGATTTATTACCATTACTTGAG CATTTCTTTTTCTGGAATATCTGC	685bp	[12]
sodA	sod-F sod-R	CAAAAAAAGTCCATTAATGTATCCAG TTATCTATTGTTATAATATTCTTCAC	554bp	[12]
plc	plc-F pgk-R	AGGAACTCATGATTGTAACTC GGATCATTACCCTCTGATACATCGTG	671bp	[12]
gyrB	gyrB-F gyrB-R	ATTGTTGATAACAGTATTGATGAAGC ATTTCCTAATTTAGTTTTAGTTTGCC	735bp	[12]
sigK	sigK-F sigK-R	CAATACTTATTAGAATTAGTTGGTAG CTAGATACATATGATCTTGATATACC	589bp	[12]
pgk	pgk-F pgk-R	GACTTTAACGTTCCATTAAAAGATGG CTAATCCCATGAATCCTTCAGCGATG	681bp	[12]
nadA	nadA-F nadA-R	ATTAGCACATTATTATCAAATTCCTG TTATATGCCTTTAATCTTAAATCCTC	689bp	[12]

MLST Analysis

MLST typing was performed as described previously.[12] In brief, eight loci (*colA, groEL, sodA, plc, gyrB, sigK, pgk* and *nadA*) were amplified by PCR and the PCR products were sequenced by using a 3730 XL DNA Analyzer (Applied Biosystems, USA). DNA sequences were submitted to a public *C. perfringens* MLST database (https://pubmlst.org/organisms/clostridium-perfringens) to obtain the sequences type (ST). New alleles and STs were deposited in the *C. perfringens* MLST database. The phylogenetic trees were constructed from the concatenated sequences by the maximum likelihood method, using a Tamura-Nei model in MEGA version 7.0 software.[18]

Statistical Analysis

Data were excluded from analyses if complete data sets were not obtained for a particular case. Statistical analyses were performed by IBM SPSS Software Version 20.0 (IBM, Armonk, NY). Clinical data were analyzed by the chi-square or Fisher's exact test. A p-value of less than

Table 2 Prevalence of *C. Perfringens* Isolates and Toxin Gene Profiles

Toxin Type	Total Strains (n=153)		Toxin Genes						
	No.	Prevalence	*cpa*	*cpb*	*etx*	*iap*	*cpe*	*netB*	*cpb2*
A	59	38.6%	+	–	–	–	–	–	–
C-1	10	6.5%	+	+	-	-	+	-	-
C-2	4	2.6%	+	+	-	-	-	-	-
F	75	49.1%	+	-	-	-	+	-	
H1	2	1.3%	+	-	-	-	+	-	+
H2	3	1.9%	+	-	-	-	-	-	+
Total	153	100.0%	153 (100%)	14 (9.2%)	0	0	85 (55.6%)	0	5 (3.3%)

Abbreviations: C-1, type C isolates carry *cpa, cpb*, and *cpe* genes; C-2, type C isolates carry *cpa* and *cpb* genes.

Table 3 Disease Caused by Different Toxin Types of *C. Perfringens* Isolates

No. of Different Toxin Genotype	No. of Isolates	Foodborne Gastroenteritis (n=18)	AAD (n=48)	Sporadic Diarrhea (n=87)	P value
F^C	15	5 (27.8%)	5 (10.4%)	5 (5.7%)	0.018
F^P	60	7 (38.9%)	30 (62.5%)	23 (26.4%)	<0.001
C	14	3 (16.7%)	5 (10.4%)	6 (6.9%)	0.338
A	59	2 (11.1%)	7 (14.6%)	50 (57.5%)	<0.001
H1	2	0	0	2 (2.3%)	0.641
H2	3	1 (5.5%)	1 (2.1%)	1 (1.1%)	0.344

Abbreviations: F^C, type F isolates with *cpe* gene on a chromosome; F^P, type F isolates with *cpe* gene on a plasmid.

0.05 was considered as statistically significant. Categorical variables were reported as frequencies and percentages, normally distributed continuous variables were reported as means and standard deviations (SDs) and non-normally distributed continuous variables as medians and interquartile ranges (IQRs).

Results

Prevalence of Toxin Genes and Toxin Types

A total of 153 *C. perfringens* isolates were collected from 1108 diarrheal stool samples. *C. difficile* pathogens were also detected in six specimens (3.9%) positive for *C. perfringens*. A total of 132 toxigenic *C. difficile* isolates were detected out of all stool samples by the GeneXpert *C. difficile* PCR assay. The *cpa* gene was detected in all isolates, *cpe* in 55.6% (85/153), *cpb* in 9.2% (14/153), and *cpb2* in 3.3% (5/153) of the positive samples. The genes *etx, iap*, or *netB* were not detected in any of the isolates. *C. perfringens* type F (positive for *cpa* and *cpe genes*) was the main toxin type, accounting for 49.5% (75/153) of all isolates, followed by type A (*cpa*-positive), which was found in 38.6% (59/153) of the isolates. The type C isolates were grouped into two subtypes, type C-1 and type C-2, based on their toxin genes. Ten isolates carrying *cpa, cpb* and *cpe* genes were assigned to type C-1, and four isolates carrying *cpa* and *cpb* genes were assigned to type C-2. In addition, two isolates (positive for *cpa* and *cpb2* genes) belonged to type H1, and three isolates (positive for *cpa, cpe* and *cpb2* genes) were classified as type H2[13] (Table 2).

Association of Toxin Types of *C. perfringens* with GI Diseases

The 153 patients positive for *C. perfringens* infection suffered from three types of gastroenteritis (GI) diseases: foodborne GI diseases, AAD and SD. The food borne GI diseases were caused by type F (12/18), type C (3/18), type A (2/18) and type H2 (1/18) isolates. The 12 type F isolates could be further divided into five F^c (*cpe* gene on chromosome) and seven F^p (*cpe* gene on plasmid) types. AAD was mainly caused by type F^P isolates (30/48 cases), but a small number of patients were also found to be infected with type F^C (5/48), type C (5/48), type A (7/48) and type H2 (1/48). SD was caused mainly by types A (50/87), and F^p (23/87); however, a small number of patients were also found to be infected with type F^c (5/

87), type C (6/87) and type H1 (3/87). Type F^P was found at higher frequency in AAD, compared to foodborne GI diseases and SD (62.5% versus 38.9% and 26.4%, $p < 0.001$), and type A was more prevalent in SD, compared to food borne GI diseases and AAD (57.5% versus 11.1% and 14.6%, $p < 0.001$). Type F^C had a higher rate in foodborne GI diseases, compared to AAD and SD (27.8% versus 10.4% and 5.7%, $p < 0.001$), There was no significant association between other types (type C, type H1 and type H2) and the three GI diseases ($p > 0.05$) (Table 3).

Clinical Features of Toxin Types of Infected *C. perfringens* Patients

The data for age, gender, length of hospitalization, underlying diseases and results of laboratory investigations were obtained from the available medical records of 112 patients. *C. perfringens* infection was more common in the elderly patients and the patients older than 50 years were mainly infected with *C. perfringens* type F (39/60, 65.0%), type C (7/11, 63.6%) and type A (30/41, 73.2%). The age range of patients infected with type F (1–83 years) and type A (8–90 years) was broader as compared to that of type C (20–79 years). The ratio of male to female patients and the average period of hospitalization in type F, type C and type A infected patients were not different. Type F patients mainly suffered from malignancy (19/60, 31.7%), hepatobiliary disease (13/60, 21.7%) and ulcerative colitis (UC) (11/60, 18.3%). The main underlying diseases in type A patients were malignancy (16/41, 38.1%), hepatobiliary disease (10/41, 23.8%), cerebral infarction (7/41, 16.7%) and cardiovascular diseases (7/41, 16.7%). The numbers of type C patients were relatively small, the clinical course of type C patients was

Table 4 Demographic and Clinical Characteristics in Different Toxin Types of *C. Perfringens* Groups

Patient Characteristics	Type F Group (n=60) Median (P25, P75)	Type C Group (n=11) Median (P25, P75)	Type A Group (n=41) Median (P25, P75)	P value
Age Median (P25, P75)	62 (53, 68)	64 (57, 68)	64 (54, 72)	0.45
Range	1–83	20–79	8–90	
1–10	3 (5.0%)	0	1 (2.4%)	
11–20	5 (8.3%)	1 (9.1%)	1 (2.4%)	
21–30	4 (6.7%)	0	1 (2.4%)	
31–40	2 (3.3%)	2 (18.2%)	3 (7.3%)	
41–50	7 (11.7%)	1 (9.1%)	5 (12.2%)	
>50	39 (65.0%)	7 (63.6%)	30 (73.2%)	
Male, %	38 (63.3%)	5 (45.5%)	29 (69.1%)	0.35
Length of hospital stay (days) Median (P25, P75)	20 (11, 33)	19 (9, 26)	20 (12, 30)	0.06
Underlying diseases				
Ulcer colitis (UC)	11 (18.3%)	0	5 (11.9%)	0.25
Respiratory disease	7 (11.7%)	1 (9.1%)	2 (4.7%)	0.48
Renal disease	3 (4.3%)	1 (9.1%)	1 (2.3%)	0.38
Hepatobiliary disease	13 (21.7%)	1 (9.1%)	10 (23.8%)	0.546
Malignancy	19 (31.7%)	2 (18.2%)	16 (38.1%)	0.40
Cerebral infarction	10 (16.7%)	2 (18.2%)	7 (16.7%)	0.99
Cardiovascular diseases	10 (16.7%)	2 (18.2%)	7 (16.7%)	0.99
Diabetes mellitus	7 (10.1%)	2 (18.2%)	2 (4.8%)	0.23
Abdominal surgery, yes	15 (25.0%)	1 (9.1%)	5 (11.9%)	0.17
Temperature >38.3°C	20 (30.0%)	6 (54.5%)	7 (16.7%)	0.03[a]
WBC count	7.4 (4.5, 10.9)	7.8 (5.7, 13.5)	8.0 (5.8, 10.0)	0.94
C-reactive protein (ng/ul)	28.7 (4.5, 88.9)	56.4 (23.4, 185.6)	9.0 (3.7, 56.2)	<0.01[b, c]
Procalcitonin (ng/mL)	0.43 (0.05, 2.87)	1.2 (0.24, 8.4)	0.1 (0.04, 0.48)	<0.01 [b, c]
Presence of fecal occult blood	24 (40.0%)	5 (45.5%)	14 (33.3%)	0.69

Notes: [a]Type C VS Type A, p=0.03; [b]Type F VS Type A, p<0.01; [c]Type F VS Type A, p < 0.01.

more severe. In type F and type C patients, C-reactive protein and Procalcitonin (ng/mL) were higher than type A patients ($p < 0.05$) (Table 4). Type C patients also had a higher incidence of fever (6/13, 54.5%), compared to type F (20/20, 30%) and type A 7/41, 16.6%) patients ($p < 0.05$). WBC count and fecal occult blood were similar in the three groups (Table 4). The mean length of hospitalization of patients suffering from foodborne GI disease was shorter (5.7± 2.3 days) than for those with AAD (34 ±15.6 days) or SD (11 ± 7.6 days) ($p < 0.01$). Similarly, patients suffering from foodborne GI disease and AAD had more serious *C. perfringens* infection and required a much longer duration of antibiotic treatment compared to those with SD (data not shown).

Molecular Subtyping and Phylogenetic Analysis

Genotyping analysis by MLST revealed that out of 153 *C. perfringens* isolates, 143 exhibited a wide variety of sequence types (STs), including many new ST types. Ten isolates could not be characterized due to double peaks in the sequences of PCR products which could not be resolved despite repeated experiments. The sequences of new ST types, corresponding to new isolates of *C. perfringens* had been submitted to *C. perfringens* MLST database (Table S1). A total of 83 isolates were chosen and analyzed genetically (Table 5), which included all type F and type C isolates, and 2 type A isolates (*cpb2*-positive isolates). The profiles of STs and toxin genes in the remaining type A isolates were shown in supplementary results (Table S2). MLST phylogenetic analysis indicated that type F^C isolates predominantly belonged to ST77 (6/15 isolates), which is more closely related to ST382, and the remaining 8 isolates grouped into a separate cluster. Six ST77 isolates were cultured from different wards and at different times, therefore ruling out the origination from a single source ward. In contrast, most of type F^P isolates exhibited considerable diversity, and only 5 out of 48 isolates belonged to ST41. Type F^P isolates were genetically divergent from type F^C isolates. Similarly, type C and type A carrying *cpb2* gene isolates were assigned into different ST types respectively, and had high genetic diversity between ST types (Figure 1).

Discussion

To our knowledge, this is the first in-depth investigation of fecal carriage of *C. perfringens* in clinical diarrheal patients in central China. We have reported the clinical features associated with infection by various subtypes of *C. perfringens* and molecular characterization and genetic diversity of various isolates. In this study, we found a high prevalence of *C. perfringens* type F^C infections in patients with foodborne GI diseases and *C. perfringens* type F^P were mostly associated with non-foodborne GI diseases. This observation is similar to recent reported studies.[4,19] Most *C. perfringens* type F isolates carrying *cpe* gene on the chromosome (F^C) caused food poisoning, some of them also caused AAD and SD. Likewise, most type F isolates carrying *cpe* gene on a plasmid (F^P) caused AAD, but a significant number of these isolates also caused food poisoning GI diseases and SD. Thus, *C. perfringens* type F isolates were a significant cause of AAD, food poisoning GI disease and SD diseases. Interestingly, we also observed that patients with AAD had longer hospital stays and duration of antibiotic treatments and more serious of *C. perfringens* infections. This finding supports a significantly pathogenic role of *C. perfringens* in AAD.

Beta toxin (encoded by *cpb* gene) is a pore-forming cytopathic toxin that can cause vascular necrosis, intestinal necrosis and systemic enterotoxaemia in humans and animals.[7,20] A recent case report described how *C. perfringens* type C triggered a life-threatening acute hemorrhagic necrotizing enteritis (AHNE) with high mortality.[21] In the present study, we found that in spite of the small number of *C. perfringens* type C isolates cultured from these patients, infection with these isolates was characterized by vomiting, abdominal cramps, bloody diarrhea and even necrosis of intestinal mucosa. Some *C. perfringens* type C isolates (C-1) carried three toxin genes (*cpa, cpb*. and *cpe*). The increased virulence of type C isolates might be due to the synergistic effect of several toxins such as beta toxin with CPE. In this study, the clinical features and laboratory findings confirmed that type C isolates caused more serious clinical symptoms than type A isolates.

Beta2 toxin encoded by plasmid-borne *cpb2* gene was associated with food poisoning and AAD. Five isolates carrying *cpb2* gene were detected in diarrheal patients in this study. In a similar study performed in Japan, *cpb2* gene prevalence was only present at a low rate.[13] However, the *C. perfringens* subtypes containing *cpb2* gene have been found to play an important role in preterm necrotizing enterocolitis (NEC).[22,23] Additionally, *cpb2* gene-positive *C. perfringens* isolates have also been

Table 5 Sequence Types (ST) and Toxin Gene Profiles of *C. Perfringens* Isolates

ST (No. of Isolates)	Allelic Profile	Toxin Types	Toxin Genes				Location of *cpe*
			cpa	*cpb*	*cpe*	*cpb2*	
ST 77 (6)	4-3-4-1-6-4-4-3-1	F^C	+	-	+	-	Chromosome
ST382 (1)	4–34-1-6-4-4-3-93	F^C	+	-	+	-	Chromosome
ST348 (1)	3-5-1-3-56-2-39-72	F^C	+	-	+	-	Chromosome
ST396 (1)	112–34-1-130-4-4-3-1	F^C	+	-	+	-	Chromosome
ST318 (1)	23–12-24-23-16-17-14-20	F^C	+	-	+	-	Chromosome
ST321 (1)	4-5-1-5-4-4-3-3	F^C	+	-	+	-	Chromosome
ST335 (1)	66-3-1-50-1-41-4-1	F^C	+	-	+	-	Chromosome
ST149 (1)	23–12-29-20-16-17-14-20	F^C	+	-	+	-	Chromosome
ST377 (1)	66–58-1-5-1-41-4-1	F^C	+	-	+	-	Chromosome
ST378 (1)	12–14-1-9-3-5-4-52	F^C	+	-	+	-	Chromosome
ST 41 (5)	19-5-1-5-5-2-3-1	F^P	+	-	+	-	Plasmid
ST171 (3)	19–34-1-5-5-2-2-3	F^P	+	-	+	-	Plasmid
ST241 (2)	20-1-14–19-5-2-2-72	F^P	+	-	+	-	Plasmid
ST370 (2)	4–19-1-4-3-71-20-1	F^P	+	-	+	-	Plasmid
ST372 (2)	66–58-1-3-1-41-4-4-1	F^P	+	-	+	-	Plasmid
ST 29 (1)	20-1-14-19-5-2-2-1	F^P	+	-	+	-	Plasmid
ST108 (1)	3-1-3-1-3-5-1-1-1	F^P	+	-	+	-	Plasmid
ST130 (1)	66–58-1-50-1-41-4-1	F^P	+	-	+	-	Plasmid
ST143 (1)	19-5-1-1-5-2-3-1	F^P	+	-	+	-	Plasmid
ST179 (1)	4-19-1-4-3-2-1-1	F^P	+	-	+	-	Plasmid
ST252 (1)	97–95-89-103–25-12-4-105	F^P	+	-	+	+	Plasmid
ST310 (1)	66–84-1-50-1-41-4-1	F^P	+	-	+	-	Plasmid
ST314 (1)	3–85-3-10-3-5-20-1	F^P	+	-	+	-	Plasmid
ST320 (1)	14–58–92-92-25-62-48-106	F^P	+	-	+	-	Plasmid
ST322 (1)	3-5-1-5-4-2-3-1	F^P	+	-	+	-	Plasmid
ST323 (1)	4-1-5-1-3-2-1-13	F^P	+	-	+	-	Plasmid
ST324 (1)	3–29-3-10-3-5-20-1	F^P	+	-	+	-	Plasmid
ST328 (1)	26–41-109-116-25-2-50-1	F^P	+	-	+	-	Plasmid
ST331 (1)	71–63-1-94-45-45-7-61	F^P	+	-	+	-	Plasmid
ST332 (1)	20-1-1-19-5-2-2-72	F^P	+	-	+	-	Plasmid
ST334 (1)	19-5-111-5-1-2-4-1	F^P	+	-	+	-	Plasmid
ST338 (1)	4-5-104-5-4-4-3-1	F^P	+	-	+	-	Plasmid
ST343 (1)	14–24-9-3-38-8-8-11	F^P	+	-	+	-	Plasmid
ST346 (1)	77–56-6-11-25-4-11-13	F^P	+	-	+	-	Plasmid
ST348 (1)	3-5-1-3-56-2-39-72	F^P	+	-	+	-	Plasmid
ST352 (1)	3-5-1-3-5-3-3-1	F^P	+	-	+	-	Plasmid
ST354 (1)	3-29-1-43-5-5-4-1	F^P	+	-	+	-	Plasmid
ST364 (1)	4-3-5-5-1-3-4-1	F^P	+	-	+	-	Plasmid
ST365 (1)	18–104–107-114-67-67-8-99	F^P	+	-	+	-	Plasmid
ST367 (1)	3–49-1-70-3-5-4-4	F^P	+	-	+	+	Plasmid
ST368 (1)	125–105-108-113-3-2-8-101	F^P	+	-	+	-	Plasmid
ST371 (1)	23-5-1-5-1-2-3-1	F^P	+	-	+	-	Plasmid
ST373 (1)	4-106-4-43-70-2-3-1	F^P	+	-	+	-	Plasmid
ST374 (1)	14–58-92-92-25-68-48-106	F^P	+	-	+	-	Plasmid
ST375 (1)	26-4-1-5-5-2-3-1	F^P	+	-	+	-	Plasmid
ST376 (1)	71-3-1-109-25-7-4-1	F^P	+	-	+	-	Plasmid
ST379 (1)	89–93-89-105-25-4-4-89	F^P	+	-	+	-	Plasmid
ST383 (1)	3–19-1-10-3-5-20-1	F^P	+	-	+	-	Plasmid
ST385 (1))	8-94-81-9-2-18-1-1-	F^P	+	-	+	-	Plasmid

(*Continued*)

Table 5 (Continued).

ST (No. of Isolates)	Allelic Profile	Toxin Types	Toxin Genes				Location of *cpe*
			cpa	*cpb*	*cpe*	*cpb2*	
ST387 (1)	19–85-3-10-3-5-20-1	F^P	+	-	+	-	Plasmid
ST388 (1)	71–63-1-109-25-45-48-61	F^P	+	-	+	-	Plasmid
ST390 (1)	3-3-5-4-3-3-4-1	F^P	+	-	+	-	Plasmid
ST392 (1)	109–72-23-34–38-61-41-105	F^P	+	-	+	-	Plasmid
ST393 (1)	4-34-1-3-5-4-3-1	F^P	+	-	+	-	Plasmid
ST397 (1)	14–14-9-131-38-8-8-52	F^P	+	-	+	-	Plasmid
ST399 (1)	4-3-3-133-3-2-1-1	F^P	+	-	+	-	Plasmid
ST400 (1)	113-3-114-124-56-2-1-72	F^P	+	-	+	-	Plasmid
ST401 (1)	20–66-113-117-25-12-4-108	F^P	+	-	+	-	Plasmid
ST5 (1)	4-1-3-4-3-2-1-4	C-2	+	+	+	-	Plasmid
ST392 (2)	109–72-23-34-38-61-41-105	C-2	+	+	+	-	Plasmid
ST311 (1)	20–12-29-23-18-17-16-29	C-1	+	+	-	-	-
ST313 (1)	119–19-23-54-65-5-4-94	C-1	+	+	-		-
ST 29 (1)	20-1-14-19-5-2-2-1	C-2	+	+	+	-	Plasmid
ST330 (1)	85–19-100-104-38-3-8-105	C-1	+	+	-	-	-
ST336 (1)	116–93-89-117-25-4-4-97	C-1	+	+	-	-	-
ST341 (1)	119–19-1-106-38-63-8-11	C-2	+	+	+	-	Plasmid
ST379 (1)	85–93-89-105-25-4-4-89	C-2	+	+	+	-	Plasmid
ST351 (1)	109–72-23-108-38-61-41-92	C-2	+	+	+	-	Plasmid
ST360 (1)	122-7-1-112-25-66-53-13	C-1	+	+	-	-	-
ST333 (1)	66-3-1-50-1-7-4-1	H2	+	-	-	+	-
ST362 (1)	123–12-106-20-16-17-14-29	H2	+	-	-	+	-

Abbreviations: F^C, type F isolates with *cpe* gene on a chromosome; F^P, type F isolates with *cpe* gene on a plasmid; C-1, type C isolates carry *cpa, cpb*, and *cpe* genes; C-2, type C isolates carry *cpa* and *cpb* genes.

found at higher rates in feces of autism spectrum disorder (ASD) children and infants aged <6 months.[24–26] Another study demonstrated that beta 2 toxin contributed to necrotizing soft tissue infections in hospitalized patients.[27] Further studies are required to investigate the pathogenic significance of beta 2 toxin produced by *C. perfringens* isolates in humans.

Patients older than 50 years-of-age were more likely to have *C. perfringens* infection. Similar results were reported in a study on *C. perfringens* infection in AAD patients from Iran.[4] However, another study on *C. perfringens* infection in a community in England found an older average age for increased susceptibility.[28] The possible reasons that patients older than 50-years were prone to *C. perfringens* infection include three different aspects: (1) the majority of these patients have more comorbid diseases and weakened immune system function which cause them to be more susceptible to *C. perfringens* infection; (2) the diversity of intestinal microbiota in these patients decreases and loss of colonization resistance may result in an increased risk for developing *C. perfringens* infection after the use of antimicrobial therapy;[29] and (3) other therapeutic factors in these patients may impair colonization resistance, including surgery, cancer chemotherapy and invasive procedures that can lead to *C. perfringens* infection. The ratios of male to female and the length of hospital stay in the three groups of patients were similar to those reported in previous studies.[4,30] Typically, *C. perfringens* food poisoning is a self-limiting disease lasting 12–24 h; mortality is uncommon.[31] However, we found that patients in our study positive for *C. perfringens* food poisoning experienced diarrhea lasting for more than 24 h, and had to be given antibiotic therapy during hospitalization. The most common underlying diseases in patients infected with both *C. perfringens* type F and type A, in decreasing order of prevalence, were cancers, especially GI tumors requiring chemotherapy and radiation therapy, hepatobiliary diseases, cerebral infarction and cardiovascular diseases. This finding is in agreement with the results of a previous study.[4] Interestingly, we observed that patients with diseases of the hepatobiliary system such as

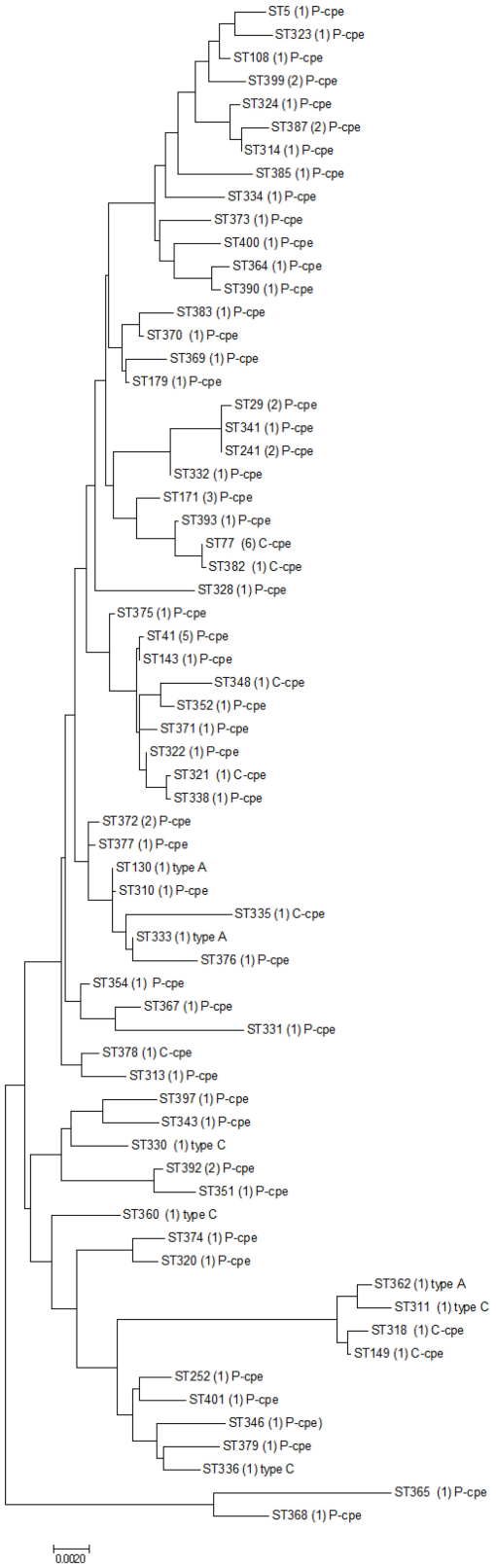

Figure 1 Phylogenetic analysis of several toxin types (F^c, F^p, C and A carrying *cpb2* gene) of *C. perfringens* isolates created by the maximum likelihood method based on composite sequences of eight housekeeping gene fragments using MEGA 7.0 software.

decompensated liver cirrhosis and cholecystitis, were at greater risk of developing *C. perfringens* type F and type A infections. The main reasons are perhaps an imbalance in the enteric microbiota, alterations of the intestinal barrier, probably due to portal hypertension, and reticuloendothelial system dysfunction in these patients.[32,33] We found that a subset of patients infected with *C. perfringens* type F and A suffered from UC, confirmed by colonoscopy. These data are in disagreement with research by Aleksandra et al where the numbers of Crohn's disease patients were higher than those of UC patients, but this may be attributed to the difference in the average age of the patients in these two studies.[34] The average age of patients in our study was much higher than the average age of 11.7 years in their study. It is important to emphasize the impact of *C. perfringens* type A isolates on patients with decompensated liver cirrhosis and UC patients with dysfunctional intestinal epithelial barriers. *C. perfringens* type A isolates can easily penetrate the destroyed intestinal epithelial barriers, enter the vasculature and cause bacteremia.

Based on the MLST scheme previously reported by Xiao et al,[12] we observed a considerable genetic diversity in the *C. perfringens* isolates. The phylogenetic analysis indicated that most *C. perfringens* type F^C isolates belonged to a single distinct cluster that evolved independently from *C. perfringens* type F^P isolates. Moreover, *C. perfringens* type F^P isolates do not have a common genetic background, which supports the idea of horizontal transfer of *cpe* gene among *C. perfringens* type F^P strains via conjugation (*cpe*-positive to *cpe*-negative transfer).[35] These results are similar to earlier studies that demonstrated a wide genetic diversity of *C. perfringens* type F^P isolates.[12,13] Our findings indicate that MLST assays can be used as tools to investigate the reservoirs and transmission among *cpe*-positive clinical isolates of *C. perfringens*.

The limitations of this study should be considered. Because there was not enough volume of stool specimens, we were only able to identify the toxigenic types of *C. perfringens* isolates using multiplex PCR, which is one of the diagnostic methods for ascertaining *C. perfringens* infection.[17,36] Indeed, several studies reported that *C. perfringens* infection was diagnosed by detection of CPE toxin using ELISA, reversed passive latex agglutination (RPLA) and enzyme immunoassay (EIA) tests.[28,37,38]

Further experiments should be performed to detect the CPE toxin in stool specimens of patients.

Conclusions

We believe that testing for the *cpe* gene or CPE toxin should be included in the routine diagnosis of vulnerable patients over 50 years of age, or in patients with underlying diseases such as cancer, liver cirrhosis or ulcerative colitis. In addition, for patients younger than 50 years, if they are suspected of having food poisoning due to contaminated food, we also suggest screening them for *cpe* gene or CPE toxin. Lastly, our data add to the evidence base that despite its relatively low prevalence, *C. perfringens* type C can cause severe clinical symptoms in patients. Further studies are needed to carefully evaluate the potential role of *C. perfringens* type A in the etiology of diseases that involve violation of the intestinal barrier such as UC and spontaneous bacterial peritonitis in liver cirrhosis.

Data Sharing Statement

The datasets generated for this study are available from the corresponding author Pro Yi Li on request.

Ethics Approval and Informed Consent

This study was approved by the Ethics Committee of Zhengzhou University (20190211). Adult patients wrote the informed consent and a parent or legal guardian of patients under 18 years of age provided informed consent prior to the molecular analysis described below. This study was conducted in accordance with the Declaration of Helsinki.

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Disclosure

The authors declare no conflicts of interest in this work.

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