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ORIGINAL ARTICLE

Prevalence and characterization of enterotoxigenic *Bacteroides fragilis* and toxigenic *Clostridium difficile* in a Taipei emergency department

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toxin genotypes

Background/Purpose: Enterotoxigenic *Bacteroides fragilis* (ETBF) and toxin-encoding *Clostridium difficile* (TXCD) are associated with gastroenteritis. Routine anaerobic blood culture for recovery of these anaerobic pathogens is not used for the detection of their toxins, especially for toxin-variant TXCD. The aim of this study was to investigate the prevalence and risk factors of the genotypes of these anaerobes in patients with acute diarrheal illnesses.
Methods: The data and samples of 513 patients with gastroenteritis were collected in a Taipei emergency department from March 1, 2006 to December 31, 2009. Nonenterotoxigenic *B. fragilis* (NTBF) and ETBF and the toxin genotypes of TXCD were detected by molecular methods.
Results: The prevalence rates of NTBF, ETBF, and TXCD infections were 33.14%, 1.56%, and 2.34%, respectively. ETBF infections often occurred in the elderly (average age = 67.13

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years) and during the cold, dry winters. TXCD infections were widely distributed in age and often occurred in the warm, wet springs and summers. The symptoms of ETBF-infected patients were significantly more severe than those of NTBF-infected patients.
Conclusion: This study identified and analyzed the prevalence, risk factors, and clinical presentations of these anaerobic infections. Future epidemiologic and clinical studies are needed to understand the role of ETBF and TXCD in human gastroenteritis.

Introduction

Acute gastroenteritis caused by infectious aerobic and/or anaerobic etiological agents is often observed in the emergency department (ED). The common symptoms include vomiting, diarrhea, and dyspepsia. In Taiwan, viruses are the leading cause of gastroenteritis, followed by bacteria, such as diarrheagenic *Escherichia coli*, *Salmonella* spp. and *Vibrio parahaemolyticus*.[1] The major microfloral populations of the human gut are not only *E. coli* and other fecal facultative microbes but also obligate anaerobes.[2] However, due to the time-consuming culture technique and the difficulties of bacterial identification, the routine testing of these pathogenic anaerobes is difficult, and approximately 70—80% are unculturable from symptomatic patients.[3] Many anaerobic bacterial infections have reemerged as important clinical problems; among these bacteria, enterotoxigenic *Bacteroides fragilis* (ETBF) and toxin-encoding *Clostridium difficile* (TXCD) are the most frequent and important anaerobic infectious agents of diarrheal illness in humans.[4—10]

Although nonenterotoxigenic *B. fragilis* (NTBF) is a common component of the colonic, ETBF strains can cause gastrointestinal tract infections, acute inflammatory diarrhea, and other severe infections.[4,6,11,12] ETBF secretes the only known virulence factor, a 20-kDa zinc metalloprotease toxin—*B. fragilis* enterotoxin, also termed fragilysin.[13,14] Because the diagnoses of the *B. fragilis* strains are technically difficult, no studies have reported the prevalence of *B. fragilis* or the toxin-encoding rate in Taiwan.

C. difficile is one of the most common enteric anaerobic pathogens. It was not considered a human pathogen until it was implicated as the cause of antibiotic-associated pseudomembranous colitis.[15,16] TXCD strains cause human infections ranging from mild diarrhea to potentially life-threatening pseudomembranous colitis through the production of three toxins: toxins A and B and a less well-known enzyme component, *C. difficile* binary toxin (CDT). Recent studies suggest that toxins A and B are similar enterotoxins and that toxin B is even more potent than toxin A in humans.[17] Toxins A and B are encoded by two genes, *tcdA* and *tcdB*,[18] and have been reported in several countries with varying prevalence rates.[8,9,19—21] In addition to toxins A and B, CDT has been found in one strain (CD196) of *C. difficile*.[22] Stubbs et al[23] showed that CDT is an ADP-ribosyltransferase consisting of the enzymatic component and the binding component. Approximately 6% of the *C. difficile* isolates isolated from patients in the USA and Europe, and from veterinary and environmental sources contain the genes for CDT: *cdtA* (the enzyme) and *cdtB* (the binding component).[23] Because the virulence potential of CDT is still unclear and commercial toxin detection kits for *C. difficile* do not cover CDT, the prevalence of the genotypes of the TXCD strains in Taiwan remains largely unknown.

The specific aims of this study were to investigate the prevalence and to identify the molecular characteristics of NTBF, ETBF, and TXCD infections. We describe the clinical presentations and potential risk factors of these acute gastroenteritis cases in order to recall the medical personnel to the importance of these neglected pathogens.

Methods

Patients

The study site was a Taipei ED. The data of 513 patients were collected by a triage nurse from March 1, 2006 to December 31, 2009. The primary case definitions were as follows: (1) at least three loose stools or three instances of vomiting; or (2) either diarrhea and/or vomiting plus two or more additional symptoms, including abdominal pain, fever, nausea, blood in the stool, or stool mucus.[1] Patients were excluded from the study if they were younger than 15 years, exhibited coughing, a sore throat, or runny nose, or were bedridden. Patients aged >65 years were excluded from the control group. Stool specimens were tested for recognized enteropathogens, including viruses (norovirus, rotavirus, and astrovirus), parasites (*Giardia lamblia* and *Entamoeba histolytica*), and bacteria (suspected diarrheagenic *E. coli*, *Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Campylobacter* spp., *Aeromonas* spp., and *Staphylococcus aureus* with related enterotoxins).[1]

DNA extraction

A total of 0.5 g of each stool sample or 500 μL mixtures of each rectal sample were mixed into 2.0 mL of 5.3M guanidine thiocyanate. The mixture was heated at 95°C while shaking for 30 minutes and cooled to room temperature prior to centrifuging for 5 minutes at 20,000 × *g*. MagNA Pure systems (Roche Diagnostics, Mannheim, Germany) were used to isolate a total of 100 μL of DNA from 250 μL of the supernatant according to the manufacturer's instructions. Polymerase chain reaction (PCR) was

performed on the stool specimens after DNA extraction. The DNA was tested for the presence of the small subunit rRNA gene.

gyrB-based real-time PCR for detecting B. fragilis

The *B. fragilis gyrB*-based real-time PCR system was performed according to the protocol established by Lee and Lee.[24] A CFX96 real-time PCR System (Bio-Rad, Hercules, CA, USA) was used with a total of 25 μL of mixture, which consisted of 5 μL of template DNA, 12.5 μL of a KAPA Probe Fast qPCR kit (KAPA Biosystems, Wilmington, MA, USA), 500nM of each *gyrB*-based primer (Bf904f/Bf958R), and 250nM of the *gyrB*-based TaqMan probe (Bf923MGB) labeled with 6-carboxyfluorescein (FAM; Table 1).[24,25,26] Water, instead of template DNA, served as an additional negative control for each PCR reaction. The reactions were incubated in a 96-well plate at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute.

Detection of the B. fragilis toxin gene by PCR

The *B. fragilis* enterotoxin gene, *bft*, was detected by PCR to yield a 1-kb DNA band as described with some modifications.[7,25] In brief, 5 μL of template DNA, 10 μL of EconoTaq Plus Green 2 × master mix (Lucigen, Middleton, WI, USA), 500nM of each *bft*-based primer (*bft* F/*bft* R; Table 1) were mixed and brought to a final volume of 20 μL. Water, instead of template DNA, served as an additional negative control for each PCR reaction. The reactions were incubated at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 65°C for 1 minute, extension at 72°C for 1 minute and a final extension at 72°C for 3 minutes. PCR products were verified by 1% agarose gel electrophoresis with ethidium bromide staining.

Multiplex real-time PCR validation for C. difficile toxin genes

Multiplex real-time PCR, which included two duplex reactions, was used to characterize the *C. difficile* toxin genes.[26] One reaction was used to detect *tcdA* (FAM-labeled probe) and *tcdB* [hexachloro-6-carboxy-fluorescein (HEX)-labeled probe], and the other was used for *cdtA* (FAM-labeled probe) and *cdtB* (HEX-labeled probe; Table 1). A CFX96 real-time PCR System (Bio-Rad) was used with a total of 25 μL of mixture, which consisted of 5 μL of template DNA, 12.5 μL of a KAPA probe fast qPCR kit (KAPA Biosystems), two sets of primers at 50nM each (*tcdA*F/*tcdA*R, *tcdB*F/*tcdB*R, *cdtA*F/*cdtA*R, and *cdtB*F/*cdtB*R), and the two respective probe sets at 100nM in duplex reactions. Water, instead of template DNA, served as an additional negative control for each PCR reaction. The reactions were incubated at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 1 minute, annealing at 54°C for 30 seconds, and extension at 72°C for 1 minute.

Results

A total of 513 acute diarrheal fecal samples were collected from the hospital ED in Taipei and screened for *B. fragilis* by the *gyrB*-based real-time PCR system (Table 1). The ETBF strains were further identified from *B. fragilis*-positive samples by the PCR detection of the virulence factor gene *bft*. The expected 1-kb product was amplified in ETBF infections.[7,25] Overall, Table 2 shows that there were 178 *B. fragilis* infections, and eight isolates carried the *bft* gene. The total prevalence rates of *B. fragilis* and ETBFs were 34.70% and 1.56%, respectively. The rate of *B. fragilis* enterotoxin gene (*bft*) detection in the BF infections was 4.49%.

The annual *B. fragilis*- and ETBF-positive rates were 39.44% and 2.11% in 2006, 30.77% and 2.31% in 2007,

Table 1 Nucleotide sequences and targets of primers and probes used for real-time polymerase chain reaction and polymerase chain reaction assay in this study

Name	Sequence (5′—3′)	Target	Gene	Refs
*bft*F	CGCGGCATTATTAGCTGCATGTTCTAATG	*Bacteroides fragilis*	*B. f* toxin	25
*bft*R	GATACATCAGCTGGGTTGTAGACATCCCA			
Bf904F	GGCGGTCTTCCGGGTAAA	*B. fragilis*	gyrB	24
Bf923MGB	FAM-TGGCCGACTGCTC-MGBNFQ			
Bf958R	CACACTTCTGCGGGTCTTTGT			
*tcdA*F	TTCAAGCAGAAATAGAGCACTC	*Clostridium difficile*	*tcd*A	26
*tcdA*R	TATCAGCCCATTGTTTTATGTATTC			
tcdA probe	FAM-TCACTGACTTCTCCACCTATCCATACAA-BHQ			
*tcdB*F	GGTATTACCTAATGCTCCAAATAG		*tcd*B	
*tcdB*R	TTTGTGCCATCATTTTCTAAGC			
tcdB probe	HEX-ACCTGGTGTCCATCCTGTTTCCCA-BHQ			
*cdtA*F	GGGTAAAGCAAATTATAATGATTGG		*cdt*A	
*cdtA*R	CTATATACAGTTAAATTAGTTGGAATAGG			
cdtA probe	FAM-AATTAACACCTAATGAACTTGCTGATGT-BHQ			
*cdtB*F	TGGTGTGTCTGTTAATGTAGG		*cdt*B	
*cdtB*R	CTTTGTTTATACTTAATCCAGTATTCC			
cdtB probe	HEX-CTCCATTACTATCTTGAACAGCAGTTGA-BHQ			

Table 2 Prevalence of *Bacteroides fragilis* and toxigenic *Clostridium difficile* in fecal samples

	Samples	BFs	BF positive rate (%)	ETBFs	ETBF rate (%)	ETBFs/BFs (%)	TXCDs	TXCD rate (%)
2006	142	56	39.44	3	2.11	5.36	4	2.82
2007	130	40	30.77	3	2.31	7.50	3	2.31
2008	134	51	38.06	2	1.49	3.92	4	2.99
2009	107	31	28.97	0	0	0.00	1	0.93
Total	513	178	34.70	8	1.56	4.49	12	2.34

BF = *Bacteroides fragilis*; ETBF = enterotoxigenic BF; TXCD = toxin-encoding *Clostridium difficile*.

38.06% and 1.49% in 2008, and 28.97% and 0% in 2009, respectively. There were no significant variances in the annual prevalence rates of *B. fragilis* and ETBF infections, except for ETBFs in 2009 (no positive cases). Interestingly, half of the ETBF infections occurred in winter months (December–February) during 2006–2009. Fig. 1A shows that the prevalence rate of ETBF infections in winter months (December–February; 2.61%) was significantly higher than in the springs (March–May; 0.74%), summers (June–August; 0.86%), and autumns (September–November; 1.83%) included in the study. However, the prevalence rate of NTBF infections was 35.29% in the winter months and did not significantly differ from other seasons (Fig. 1B). These data show that the prevalence rate of ETBF infections was significantly higher during the cold, dry winter months than during others; this was not true for NTBF infections.

Of the 513 patients from whom acute diarrheal fecal samples were collected in the ED, 493 (96.1%) returned completed and usable questionnaires. According to the triage records, no differences were noted between participants and nonparticipants in terms of age, gender, or the distribution of diarrhea symptoms, but nonparticipants exhibited more cases of vomiting and abdominal pain. We used case–case comparisons to study the risk factors associated with the infections.[27] Fig. 2 shows the distribution of the age of ETBF-infected participants. The mean ± standard deviation age of patients with NTBF and ETBF infections was 42.86 ± 18.66 years and 67.13 years and 12.62 years, respectively. The analyses demonstrate that ETBF infections are more common among elderly than younger individuals (aged > 15 years).

We analyzed the symptoms reported by the participants and linked to the other pathogens. The frequency of diarrhea within 24 hours was significantly different between ETBF and NTBF infections (Table S1). The median diarrhea frequency within 24 hours in ETBF infections (10.0) was also significantly higher than for NTBF infections (6.0).

The multiplex real-time PCR assay for TXCD was used to screen and characterize the toxin genotypes of TXCD isolates from the 513 ED samples from 2006 to 2009 (Table 1). The toxin genes were detected in twelve samples, and the total prevalence rate was 2.34%. There were no significant differences in the annual prevalence rate from 2006 to 2008 (Table 2). Similar to the low incidence of ETBF in the same year, only one TXCD-positive case was identified in 107 samples during 2009. However, unlike ETBF infections, which often occur in dry, cold winters in Taiwan, the prevalence rate of TXCD infections peaked in wet, warm springs (4.44%) and summers (2.59%; Fig. 1A).

We also studied the risk factors associated with TXCD infection and compared them to the other infections. Unlike ETBF infections, the mean ± standard deviation of patients with TXCD (46.42 ± 21.61 years) were similar to

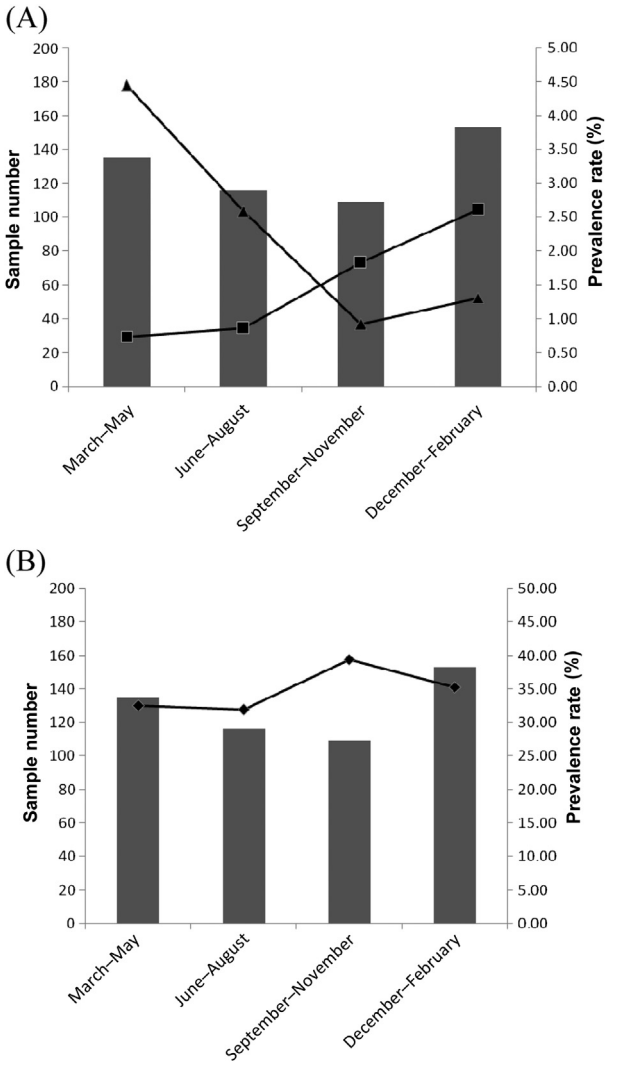

Figure 1. The prevalence rate of enterotoxigenic *Bacteroides fragilis*, toxigenic *Clostridium difficile* (A) and nonenterotoxigenic *B. fragilis* (B) in seasons during 2006–2009. Gray bars, sample number in each seasons; (A) (■, prevalence rate (%) of enterotoxigenic *B. fragilis* in each season; ▲, prevalence rate (%) of toxigenic *C. difficile* in each season; (B) ◆, prevalence rate (%) of nonenterotoxigenic *B. fragilis* in each season.

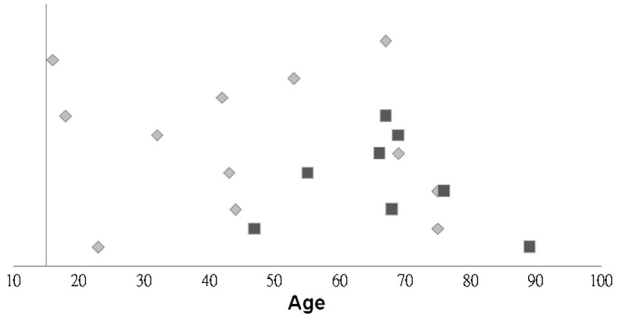

Figure 2. The distribution of the age (years) of enterotoxigenic *Bacteroides fragilis* (■) and toxigenic *Clostridium difficile* (◆) patients.

NTBFs, and the distribution of the age of TXCD-infected participants had a nearly random distribution (Fig. 2).

Twelve TXCDs were detected by multiplex real-time PCR for the toxin-encoding genes of TXCD: *tcdA*, *tcdB*, and CDT (*cdtA*, *cdtB*). These 12 TXCD-positive samples were categorized into three different TXCD toxin genotypes (Table 3). Nine samples were $tcdA^{-}tcdB^{+}CDT^{-}$ in 2006–2008, two were $tcdA^{+}tcdB^{+}CDT^{-}$ in 2006 and 2009, and one was $tcdA^{-}tcdB^{+}CDT^{+}$ in 2006. There was no significant correlation between the symptom severity and the toxin genotypes of TXCD and the patients with $tcdA^{+}tcdB^{+}CDT^{-}$ TXCD infection did not show a more serious symptom.

The specimens were also analyzed for viruses (norovirus, rotavirus, and astrovirus), parasites (*G. lamblia* and *E. histolytica*), and bacteria (suspected diarrheagenical *E. coli*, *Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Campylobacter* spp., *Aeromonas* spp., and *S. aureus* with related enterotoxins). Interestingly, there was no significant correlation between ETBF, TXCD, and other pathogens with the exception of *G. lamblia*. Table 4 shows that the rates of *G. lamblia* coexistence with ETBF (37.50%) and TXCD (33.33%) were significantly higher than coexistence with NTBF (22%; Table S2) and also higher than the prevalence of *G. lamblia* in all ED specimens (15.59%).

Discussion

This is the first ED-based epidemiological study of ETBF and TXCD infections in Taiwan. We identified and characterized NTBF and ETBF infections and the toxin genotypes of TXCD isolates from acute gastroenteritis patient diarrhea stool samples using PCR and real-time PCR. This study demonstrates the prevalence rates, epidemiological data, and clinical presentations of NTBF, ETBF, and TXCD in ED patients suffering from acute gastroenteritis.

Table 3 The toxin types of toxigenic *Clostridium difficile* (TXCD) in fecal samples

Toxin genes			% of TXCD-positive samples
tcd A	tcd B	CDT	
−	+	−	75.00 (9/12)
+	+	−	16.67 (2/12)
−	+	+	8.33 (1/12)

CDT = *Clostridium difficile* toxin.

Table 4 Prevalence of *Bacteroides fragilis* and toxigenic *Clostridium difficile* coinfected with *Giardia* in fecal samples

	Numbers	*Giardia* coinfections (%)
ETBF	8	3 (37.50)
TXCD	12	4 (33.33)
NTBF	170	22 (12.94)
Total fecal samples	513	80 (15.59)

ETBF = enterotoxigenic *Bacteroides fragilis*; NTBF = nonenterotoxigenic *B. fragilis*; TXCD = toxin-encoding *Clostridium difficile*.

From our observations, the annual prevalence rates of NTBF, ETBF, and TXCD infections were stable during 2006–2008. However, in 2009, there were no ETBF infections and only one TXCD infection in the collected samples, which may have been the result of the 2009 influenza H1N1 pandemic. A laboratory-based hand hygiene campaign in Egypt demonstrated a 30% reduction in absenteeism caused by diarrhea during the H1N1 pandemic.[28] Although the mechanism of this phenomenon was not clear and may be complicated, hand hygiene may be one of the most important intervention methods.

Interestingly, the seasonal rates of NTBF infections were stable, but the rates of ETBF infections were significantly higher in the winter months than other months during 2006–2009 (Fig. 1). The observed seasonal variance of NTBF and ETBF infections differs from a previous study in Dhaka, Bangladesh.[7] In Bangladesh, the infection rates of NTBF and ETBF during the hot, dry spring months (March–May) were significantly higher than during other months, suggesting that the variation was due to ambient humidity in addition to the season. Humidity level may affect ETBF pathogenicity. Both of the ETBF infection peaks occurred in the dry season in Taiwan and Bangladesh.

The toxin types of the TXCD strains found in this study are in agreement with the findings of the presence of the $tcdA^{-}tcdB^{+}$ strains in clinical material, which have been reported with prevalence rates varying from 0.2% to 56%.[19–21,29] Our results show that the prevalence rate of $tcdA^{-}tcdB^{+}$ *C. difficile* was 1.75% in Taiwan (9/513), which is similar to its prevalence in the USA and also from recent epidemiological data here in Taiwan.[21,29,30] A previous study suggested that $tcdA^{-}tcdB^{+}$ *C. difficile* strains had spread throughout Far East Asia prior to 2000.[9] In Korea, the mean prevalence of $tcdA^{-}tcdB^{+}$ *C. difficile* strains was <7% until 2002, but it began to increase in 2003. By contrast, the mean prevalence of $tcdA^{+}tcdB^{-}$ *C. difficile* strains was >93% until 2002 but decreased to 35.2% in 2004.[9] In the present study, we demonstrated that the $tcd^{-}tcdB^{+}$ strains were more prevalent than $tcdA^{+}tcdB^{+}$ strains, which is in agreement with the findings of the previous studies.[30] The primary limitation of this study was the small number of positive TXCD cases, which makes the statistical findings less conclusive. In addition, this retrospective study was performed without first isolating the *C. difficile* strains.

However, previous studies has shown that in Northern Taiwan *tcdA*$^{-}$*tcdB*$^{+}$strains predominate in patients with confirmed *C. difficile* infections, which correlated with our own study.

In conclusion, this ED-based epidemiological study shows the prevalence rates and seasonal distribution of NTBF and ETBF infections and the toxin genotypes of TXCD isolates in Taipei during 2006—2009. This study analyzed the risk factors and clinical presentations of these anaerobic infections. Putting this result with other results from related studies together, we propose that ETBF and TXCD have strong association with acute gastroenteritis.[7,31—34] This study demonstrates that ED staff must also pay attention to anaerobic bacterial infections.

Conflicts of interest

All authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jmii.2014.12.005.