MICROBIOLOGY, MOLECULAR AND CLINICAL CHARACTERISTICS OF *CLOSTRIDIOIDES DIFFICILE* INFECTION AND IDENTIFICATION OF RISK FACTORS IN A CHINESE HOSPITAL

Wei Chen, Yan-ming Li and Wen-en Liu*

Department of Clinical Laboratory, Xiangya Hospital of Central South University, Changsha, People's Republic of China

Abstract. *Clostridioides difficile* infection (CDI) is not widely prevalent in China but there are regional differences in epidemiology and microbiology of CDI. Incidence of CDI, molecular characteristics of virulence and clinical characteristics of *C. difficile* from clinical isolates in China were investigated to identify scale of the problem and formulate appropriate infection control actions and interventions to avoid CDI outbreaks. Diarrhea fecal specimens (n = 400) from Xiangya Hospital of Central South University, Changsha were selectively cultured for *C. difficile* and identified by API20A, a test kit for the identification of anaerobes. PCR was employed to detect *tcdA*, *tcdB*, *cdtA*, *cdtB*, and 16S-23S internal spacer region. Rate of CDI occurrence was 23.25%, 23.66% of which were toxin A-negative and toxin B-positive strains (no binary toxins strains) and 64.52% obtained from healthcare-associated CDI. Twenty-nine different ribotypes were identified: 8.60% CD001, 12.90% CD012,15.05% CD046, and 21.51% CD017. Independent risk factors associated with CDI were being >55 years of age [odds ratio (OR) = 2.34, 95% confidence interval (95% CI): 1.25-4.37)], installation of a catheter (OR = 2.31, 95% CI: 1.28-4.19), surgical procedure within previous two months (OR = 3.28, 95% CI: 1.30-8.27), and fluoroquinolones use (OR = 2.84, 95% CI: 1.33-6.03). The findings provide evidence CDI was highly prevalence in hospitalized diarrheal patients, and efforts must be undertaken to diagnose CDI allowing appropriate therapy and to raise awareness of this hitherto under-recognized disease.

Keywords: *Clostridioides difficile*, diarrhea, ribotype, risk factor

INTRODUCTION

Clostridium difficile is a Gram-positive spore-forming rod shape bacterium, first detected in the mid-1930s (Hall and O'Toole, 1935) and identified as a primary cause of pseudo-membranous colitis (PMC) in 1978 (George *et al*, 1978). Patients with CDI have clinical manifestations ranging from asymptomatic to severe life-threatening PMC and can result in death. *C. difficile* is the main cause of nosocomial diarrhea contributing to high morbidity and mortality among traditionally recognized at-risk groups, *eg* elderly, prolonged hospital stay, antibiotic treated, immuno-suppressed, and with

Correspondence: Wen-en Liu, Department of Clinical Laboratory, Xiangya Hospital of Central South University, Changsha 410008, People's Republic of China
Tel: +86 731 84327437; Fax: +86 731 84327332
E-mail: wenenliu@163.com

indwelling devices (Chen *et al*, 2015; Piepenbrock *et al*, 2019).

Prevalence of CDI in North America is generally higher than other regions of the world (Lessa *et al*, 2015; Balsells *et al*, 2019), especially during the rise in prevalence and severity in the early 2000s due to an epidemic of a hyper-virulent *C. difficile* strain BI/NAP1/027 (restriction endonuclease analysis group BI, North American pulsed-field type 1, PCR ribotype 027). In USA, *C. difficile* causes an estimated 29,000 deaths in 2012, more than 80% of these deaths occurring in those 65 years of age and above (Lessa *et al*, 2015). Challenges remain in controlling CDI prevalence in other regions, such as Asia (Collins *et al*, 2013), Europe (Bauer *et al*, 2011; Davies *et al*, 2014; Lessa *et al*, 2015) and South Africa (Legenza *et al*, 2018). The overall rate of hospital-acquired (HA)-CDI is 2.24/1,000 admission/year and 3.54/10,000 patient-day/year globally (Balsells *et al*, 2019).

CDI is not widely detected in China due to low sample submission rate and insufficient laboratory diagnostic capacity (Cheng *et al*, 2016b). In recent years, *C. difficile* in various Chinese hospitals were reported demonstrating increased awareness of CDI in the country (Huang *et al*, 2009; Cheng *et al*, 2011; Han *et al*, 2013; Hawkey *et al*, 2013; Chen *et al*, 2014; Fang *et al*, 2014; Huang *et al*, 2014; Wang *et al*, 2014; Chen *et al*, 2015;Wei *et al*, 2015a; Wei *et al*, 2015b; Cheng *et al*, 2016a; Jin *et al*, 2017; Li *et al*, 2017; Li *et al*, 2018a; Li *et al*, 2018b); however, the studies focused on a relatively small number of local strains. Regional differences play a key role in epidemiology and microbiology of CDI. Hence, the prevalence of CDI, molecular characteristics of virulence and clinical characteristics of *C. difficile* of clinical isolates from Xiangya Hospital of Central South University, Hunan were investigated to identify the scale of the problem and to formulate appropriate infection control measures and interventions.

MATERIALS AND METHODS

Enrollment of patients

The study was conducted at Xiangya Hospital of Central South University, a 3,500-bed tertiary care facility, located in Hunan, China. Fecal specimens were collected from July to October 2012 from in-patients with diarrhea, defined as loose, watery stool passages >3 times/day on three consecutive days or 6 times within 36 hours, assessed for fecal shape/texture and examined by microscopy for presence of white blood cells. CDI was diagnosed when *C. difficile* was cultured on selective agar and identified by API20A (bioMérieux, Marcy l'Étoile, France). *C. difficile* isolates must also carry toxin genes *tcdA* and *tcdB* (see below). A CDI patient who developed diarrhea at least 72 hours after hospitalization or within two months following hospital discharge is considered as HA-CDI, while a CDI patient who developed diarrhea prior to or within 72 hours after admission with a negative history of hospitalization in the previous two months is considered as community-associated (CA)-CDI (Gravel *et al*, 2009). A non-CDI patient is defined one who was culture negative for *C. difficile* or culture positive for *C. difficile* that did not carry *tcdA* or *tcdB*. Information for the previous 2-month history of a case was obtained by interview and review of hospital records. Data collected for each case were basic demographics (age, sex, length of hospital stay before collection of fecal sample, hospitalization history during the previous two months and underlying diseases, such as diabetes

mellitus, liver cirrhosis, class IV heart failure, malignancy, and hematological, neurological and renal diseases), clinical findings (abdominal cramp, diarrhea frequency, duration of diarrhea before sampling, and fever), laboratory findings (WBC counts, albumin and C-reactive protein (CRP) levels in blood, stool characteristics, and presence of WBC in feces), use of antibiotics (carbapenems, clindamycins, extended-spectrum cephalosporins, fluoroquinolone, glycopeptides, β-lactam/β-lactamase inhibitors) and other medications/medical procedures (chemotherapies, H2 blockers, installation of catheter, probiotics, proton pump inhibitors, steroids, and surgical procedures).

Research protocol was approved by Xiangya University Hospital Institutional Review Board. Written informed consent was obtained from all participating patients.

Fecal culture

Fecal sample was stored in alcohol prior to inoculation on selective *C. difficile* moxalactam–norfloxacin–taurocholate agar (CDMN-TA) (Oxoid Ltd, Cambridge, UK). Suspected *C. difficile* colonies were identified based on morphology on agar plate, Gram staining and characteristic odor, biochemical properties identified by API20A test (bioMérieux, Marcy l'Étoile, France) (Moukhaiber *et al*, 2015). Presence of toxin genes *cdtA*, *cdtB tcdA*, and *tcdB* were determined by multiplex-PCR as previously described (Persson *et al*, 2008). In brief, DNA was extracted from colony using by a DNeasy kit (Qiagen, Hilden, Germany). Gene-specific primers and amplicon sizes are listed in Table 1. The amplification reactions were performed in 25 μl final volume containing 12.5 μl HotStar Taq Master Mix (Qiagen, Hilden, Germany), 10 pmol of each primer and 2 μl DNA. Thermocycling was conducted in a GeneAmp PCR system 9700 cycler (Applied Biosystems, Gouda, The Netherlands) as follows: 94°C for 10 minutes; 35 cycles of 94°C for 50 seconds, 54°C for 40 seconds and 72°C for 50 seconds; with a final step of 72°C for 3 minutes. Amplicons were separated by 1.0% agarose gel-electrophoresis and visualized by staining with ethidium bromide. *C. difficile* strains were stored at -80°C until used.

PCR-based ribotyping

PCR-based ribotyping was performed as previously described (Janezic *et al*, 2014). Gene-specific primers and amplicon sizes are listed in Table 1. In short, reaction mixture (50 μl) contained 25 μl HotStar Taq Master Mix (Qiagen, Hilden, Germany), 50 pmol of each primer and 1.5 μl of DNA. Thermocycling was conducted in a GeneAmp PCR system 9700 cycler (Applied Biosystems, Gouda, The Netherlands) as follows: 95°C for 15 minutes; 35 cycles of 94°C for 1 minute, 57°C for 1 minute and 72°C for 1 minute; with a final step of 72°C for 7 minutes. Amplicons were separated by 1.5% agarose gel-electrophoresis and visualized by staining with ethidium bromide. PCR ribotypes for which reference strains were available are designated using standard Cardiff nomenclature (CD001, CD002 and so on), while other undefined ribotypes are designated (in-house) $XYn_1n_2n_3$.

Statistical analysis

SPSS version 18.0 for Windows was used for statistical analysis. Results are presented as mean ± standard deviation for continuous variables, calculated using the independent *t*-test or Mann-Whitney U-test, and percentage for categorical variables, calculated using Pearson's chi-square test or Fisher's exact

Table 1
Primers used in the study.

PCR product	Primer	Sequence (5′-3′)	Fragment length(bp)
tcdA	tcdA-F3345	5- GCATGATAAGGCAACTTCAGTGGTA -3	629
	tcdA-R3969	5- AGTTCCTCCTGCTCCATCAAATG-3	
tcdB	tcdB-F5670	5- CCAAARTGGAGTGTTACAAACAGGTG-3	410
	tcdB-R6079A	5- GCATTTCTCCATTCTCAGCAAAGTA-3	
	tcdB-R6079B	5-GCATTTCTCCGTTTTCAGCAAAGTA-3	
cdtA	cdtA-F739A	5- GGGAAGCACTATATTAAAGCAGAAGC-3	221
	cdtA-F739B	5- GGGAAACATTATATTAAAGCAGAAGC-3	
	cdtA-R958	5- CTGGGTTAGGATTATTTACTGGACCA-3	
cdtB	ctdB-F617	5- TTGACCCAAAGTTGATGTCTGATTG -3	262
	cdtB-R878	5- CGGATCTCTTGCTTCAGTCTTTATAG -3	
internal spacer region	PRB	5-GTGCGGCTGGATCACCTCCT-3	variable
	PRBas	5-CCCTGCACCCTTAATAACTTGACC-3	

test. Multivariate logistic regression was employed to identify independent risk factors, with a 95% confidence interval (CI) to quantify relationship of risk factors. A p-value <0.05 in a two-tailed test of significance is considered statistically significant.

RESULTS

During the study period, diarrheal fecal specimens ($n = 400$) were examined, 93 (23.25%) of which met CDI case definition. Median age of the 93 CDI patients was 60 years and 57 (61.29%) were male. Risk factors for contracting CDI were assessed by a blinded review of the medical record of each patient. Clinical characteristics were compared among the 93 CDI with 307 non-CDI patient. Univariate analysis revealed age, length of hospital stay prior to fecal collection, hospitalization history during the prior two months, underlying diseases, duration of diarrhea before fecal collection, blood albumin level, blood CRP level, use of antibiotics (carbapenems, clindamycins, fluoroquinolones, and extended-spectrum cephalosporins), installation of catheter, and surgical procedure during the prior two months are significantly associated with CDI (Table 2). However, multivariate logistic regression analysis revealed age >55 years [odds ratio (OR) = 2.34, 95% CI: 1.25-4.37)], installation of a catheter (OR =2.31, 95% CI: 1.28-4.19), surgical procedure during the prior two months (OR = 3.28, 95% CI: 1.30-8.27), and use of fluoroquinolones (OR = 2.84, 95% CI: 1.33-6.03) were independent risk factors for CDI. There is a significant association of sex, length of hospital stays prior to fecal collection, underlying diseases, duration of diarrhea prior to fecal collection, watery stool, and use of carbapenems, extended-spectrum cephalosporins, glycopeptides, and H2 blockers with HA-CDI (Table 2). However, multivariate logistic regression analysis revealed only two parameters,

Table 2
Demographic data, clinical and laboratory characteristics of patients enrolled at Xiangya Hospital of Central South University, Hunan, China (July - October 2012).

Demographic data, clinical and laboratory characteristics	Unit	CDI *vs* non-CDI			HA-CDI *vs* CA-CDI		
		CDI (n = 93)	Non-CDI (n = 307)	p-value*	HA-CDI (n = 60)	CA-CDI (n = 33)	p-value*
Sex (male)	Number (%)	57 (61.29)	199 (64.82)	0.308	41 (68.33)	16 (48.48)	0.049
Age (years)	Mean ± SD	60.61±16.54	47.31±22.48	0.020	62.77±15.64	56.70±17.63	0.204
Length of hospital stay prior to fecal collection (days)	Mean ± SD	22.10±11.68	14.92±9.21	0.000	23.97±11.62	18.70±11.18	0.032
Hospitalization history in the prior two months	Number (%)	24 (25.81)	173 (56.35)	0.000	19 (31.67)	5 (15.15)	0.065
Underlying diseases	Number (%)	64 (68.82)	179 (58.31)	0.044	51 (85.00)	13 (39.39)	0.000
Clinical findings							
Abdominal cramp	Number (%)	35 (37.63)	98 (31.92)	0.184	25 (41.67)	10 (30.30)	0.196
Fever (>38.3°C)	Number (%)	48 (51.61)	131 (42.67)	0.081	32 (53.33)	16 (48.48)	0.409
Diarrhea frequency (times/day)	Mean ± SD	4.35±3.78	4.28±3.53	0.493	4.78±4.15	3.58±2.88	0.052
Duration of diarrhea prior to fecal collection (days)	Mean ± SD	11.85±9.60	5.46±4.10	0.000	11.73±9.88	6.61±6.39	0.000
Laboratory findings							
WBC count in blood (10^9/l)	Mean ± SD	10.89±5.55	9.66±5.90	0.566	10.95±5.93	10.78±4.88	0.103
Albumin level in blood (g/dl)	Mean ± SD	3.27±0.84	3.25±0.69	0.035	3.22±0.82	3.36±0.88	0.586
CRP level in blood (mg/l)	Mean ± SD	69.19±122.97	50.04±90.32	0.006	80.07±129.52	49.42±109.18	0.033
Watery stool	Number (%)	23 (24.73)	63 (20.52)	0.213	20 (33.33)	3 (9.09)	0.007
Presence of WBC in fecal sample	Number (%)	31 (33.33)	93 (30.29)	0.332	20 (33.33)	11 (33.33)	0.588
Antibiotic use							
Fluoroquinolones	Number (%)	30 (32.26)	58 (18.89)	0.006	23 (38.33)	7 (21.21)	0.071

Table 2 (Continued)

Demographic data, clinical and laboratory characteristics	Unit	CDI *vs* non-CDI			HA-CDI *vs* CA-CDI		
		CDI ($n = 93$)	Non-CDI ($n = 307$)	p-value*	HA-CDI ($n = 60$)	CA-CDI ($n = 33$)	p-value*
Clindamycins	Number (%)	17 (18.28)	33 (10.75)	0.044	12 (20.00)	5 (15.15)	0.389
Extended-spectrum cephalosporins	Number (%)	54 (58.06)	137 (44.63)	0.016	41 (68.33)	13 (39.39)	0.006
Beta-lactam/beta-lactamase inhibitors	Number (%)	26 (27.96)	75 (24.43)	0.288	18 (30.00)	8 (24.24)	0.367
Carbapenems	Number (%)	23 (24.73)	49 (15.96)	0.041	20 (33.33)	3 (9.09)	0.007
Glycopeptides	Number (%)	12 (12.90)	42 (13.68)	0.502	11 (18.33)	1 (3.03)	0.031
Use of other medicine/medical devices							
Probiotics	Number (%)	47 (50.54)	128 (41.69)	0.083	34 (56.67)	13 (39.39)	0.084
Proton pump inhibitors	Number (%)	73 (78.49)	224 (72.96)	0.176	48 (80.00)	25 (75.76)	0.410
H2 blockers	Number (%)	18 (19.35)	65 (21.17)	0.414	16 (26.67)	2 (6.06)	0.013
Steroids	Number (%)	29 (31.18)	86 (28.01)	0.320	20 (33.33)	9 (27.27)	0.359
Chemotherapy	Number (%)	26 (27.96)	77 (25.08)	0.334	17 (28.33)	9 (27.27)	0.557
Installation of catheter	Number (%)	48 (51.61)	109 (35.50)	0.004	33 (55.00)	15 (45.45)	0.253
Surgical procedure in the prior two months	Number (%)	20 (21.51)	32 (10.42)	0.006	10 (16.67)	10 (30.30)	0.104

*p-value from univariate analysis: category variables calculated using Pearson's chi-square test or Fisher's exact test, and continuous variables using independent t-test or Mann-Whitney U-test; CRP: C-reactive protein; WBC: white blood cells.

namely, watery stool (OR = 38.28, 95% CI: 2.49-588.02) and use of extended-spectrum cephalosporins (OR = 10.96, 95% CI: 1.14-104.81) were independent risk factors for HA-CDI.

Seventy-one (76.34%) *C. difficile* isolates were *tcdA*- and *tcdB*-positive (A^+B^+) strains, 22 (23.66%) were A^-B^+ strains and none harbored a binary toxin. Sixty (64.52%) *C. difficile* isolates were obtained from HA-CDI and 33 (35.48%) were from CA-CDI. Twenty-nine ribotypes were identified: CD001 (*n* = 8), CD002 (*n* = 6), CD009 (*n* = 1), CD012 (*n* = 12), CD014 (*n* = 2), CD017 (*n* = 20), CD020 (*n* = 1), CD046 (*n* = 14), XY001(*n* = 3), XY002 (*n* = 2), XY003 (*n* = 4), XY004 (*n* = 2), XY005 (*n* = 2), and XY006-XY021 (*n* =1 each) (data not shown).

DISCUSSION

Anaerobic culture of stools samples from diarrheal patients at a hospital in China allowed isolation of *C. difficile* that was subsequently shown by PCR to carry *tcdA* and *tcdB*. The prevalence of CDI was within the range (4-34.6%) observed previously in Hong Kong (Cheng *et al*, 2011) and subsequently in other hospitals in the country, namely, Beijing (Han *et al*, 2013), Hunan (Hawkey *et al*, 2013; Chen *et al*, 2015; Li *et al*, 2018b), Shanghai (Huang *et al*, 2014), Sichuan (Wang *et al*, 2014), Taiwan (Wei *et al*, 2015b), and Zhejiang (Chen *et al*, 2014; Fang *et al*, 2014; Gu *et al*, 2015). The broad range of CDI prevalence reflect differences in number of patients, sample selection methodologies and *C. difficile* detection strategies.

PCR-ribotyping, widely employed for molecular epidemiological investigations of *C. difficile* infections, is based on size variation in 16S-23S rRNA intergenic spacer region (Janezic *et al*, 2014). The possibility of an CDI outbreak should be suspected if the same ribotype is found clustered in a particular hospital setting. Although geographical variations within China result in different locations having different ribotypes (Cheng *et al*, 2011; Collins *et al*, 2013; Lee *et al*, 2014; Jin *et al*, 2017), it is striking ribotypes CD017 and CD012 are always among the most predominant types; this was also the case in the present study. Ribotypes CD027 and CD078 associated with hypervirulent disease (Li *et al*, 2018a) were not detected. The predominant ribotypes showed similarity to a previous study, which reported CD012, CD017 and CD046 prevalence of 14.00%, 48.00% and14.00%, respectively (Hawkey *et al*, 2013). *C. difficile* A^-B^+ ribotype CD017 is well recognized as the most common strain associated with pseudomembranous colitis (PMC) (Shin *et al*, 2008; Cairns *et al*, 2012) and the altered virulence properties of the typically toxin A-negative CD017 ribotype are believed to be responsible for A^-B^+ ribotype CD017 predominance and ability to cause CDI outbreaks (Drudy *et al*, 2007). Ribotypes CD001 and CD002 have not been previously reported in our hospital indicating changes to the local *C. difficile* population.

Subsequent to the present study, CDI has received much attention in recent years as the incidence and severity of the disease appear to be increasing, particularly in severe cases of CA-CDI (Balsells *et al*, 2019). Only a limited number of investigations on risk factors for CDI has been conducted in China (Gu *et al*, 2015; Li *et al*, 2017). The present study identified independent risk factors for CDI were elderly patients (mean age of 60 years), consistent with other reports (Huang *et al*, 2014; Jin *et al*, 2017; Li *et al*, 2017), probably owing to greater

use of antimicrobials and decrease in immunity (Balsells *et al*, 2019); and use of fluoroquinolones, although use of other classes of antibiotics should not be discounted (Faleck *et al*, 2016). Long hospitalization stays (≥14 days) was identified as an independent risk factor for CDI (Huang *et al*, 2014), possibly due to an increase in opportunity of exposure to *C. difficile* spores (Wang *et al*, 2014). This parameter was not observed in the present study, possibly due to the limited number of patients in this category.

Over the last two decades, there has been a significant reduction in HA-CDI and a rise in CA-CDI (Dingle *et al*, 2017). Undoubtedly the reduction in antibiotics use and control of infection risk behaviors have played a key role (Hawkey *et al*, 2013). Percent CA-CDI (35.48%) was lower than that of Northern Ireland (49.0%) (Maisa *et al*, 2019) but both studies identified young females were more at risk, but other risk parameters are not significantly different between CA- and HA-CDIs in the present study.

In conclusion, the study is one of the earliest investigations to provide evidence of CDI in a hospital in China (Xiangya Hospital of Central South University, Hunan). Efforts must be undertaken to make testing of *C. difficile* a routine practice to allow for targeted therapy and to provide a better understanding of this underdiagnosed but virulent disease, particularly regarding recognized common risk factors. Detailed analysis of *C. difficile* isolates, *eg* presence of toxins and ribotypes, should assist in evaluating virulence status and identifying epicenters of HA-CDI outbreaks.

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