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ARTICLE

Clostridium difficile isolates with increased sporulation: emergence of PCR ribotype 002 in Hong Kong

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Abstract We identified a predominant clone of *Clostridium difficile* PCR ribotype 002, which was associated with an increased sporulation frequency. In 2009, 3,528 stool samples from 2,440 patients were tested for toxigenic *C. difficile* in a healthcare region in Hong Kong. A total of 345 toxigenic strains from 307 (13.3%) patients were found. Ribotype 002 was the predominant ribotype, which constituted 35 samples from 29 (9.4%) patients. The mean sporulation frequency of ribotype 002 was 20.2%, which was significantly higher than that of the 56 randomly selected ribotypes other than 002 as concurrent controls (3.7%, $p<0.001$). Patients carrying toxigenic ribotype 002 were more frequently admitted from an elderly home ($p=0.01$) and received more β-lactam antibiotics in the preceding 3 months compared with the controls ($p=0.04$) . The identification of toxigenic ribotype 002 in 2009 was temporally related to a significant increase in both the incidence of toxigenic *C. difficile* from 0.53 to 0.95 per 1,000 admissions ($p<0.001$) and the rate of positive detection from 4.17% to 6.28% ($p<0.001$) between period 1 (2004–2008) and period 2 (2009). This finding should alert both the physician and the infection control team to the establishment of and possible outbreaks by ribotype 002 in our hospitals, as in the case of ribotype 027.

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Introduction

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacillus which causes gastrointestinal diseases ranging from antibiotic-associated diarrhea to pseudomembranous colitis. Though community-acquired infection can occur, most cases are found in the hospital and long-term care facilities [1]. The fecal colonization rate in ambulatory individuals is up to 2% [2, 3] and this increased to 15–30% among hospitalized patients due to the acquisition from healthcare workers and the hospital environment [4]. About 15–60% of the colonized patients develop symptomatic diarrhea during hospitalization [5–7], which may increase the nosocomial transmission of *C. difficile* with hospital outbreaks. Recently, a virulent strain identified as PCR ribotype 027, toxinotype III, or North American pulse-field type 1 has emerged to cause severe colitis, leading to a high mortality rate [8–10]. The mechanism of increased virulence is still under investigation, but it may be related to the 18-bp deletion and single-base-pair deletion at position 117 in the toxin regulator gene (*tcdC*), leading to the hyperproduction of toxins A and B [11, 12]. In addition, increased sporulation frequency of certain epidemic strains of *C. difficile* PCR ribotype 027 may also contribute to its better survival and nosocomial spread [13, 14]. Sporulation occurs when the ability to maintain vegetative growth has failed and facilitates the transmission of *C. difficile* in the healthcare setting, as the spores remain infective and persist in the environment for many months [15].

A sporadic case of *C. difficile* PCR ribotype 027 was reported in Hong Kong in 2008 [16]. In response to this

emerging infectious agent, further investigation was performed on all *C. difficile* isolates in a healthcare region in Hong Kong. We also conducted a retrospective review of the clinical and epidemiological data related to the different ribotypes of toxigenic *C. difficile* isolated in 2009. The findings and their potential implications in disease transmission and infection control practice are discussed.

Materials and methods

Setting

A surveillance program was conducted in a regional microbiology laboratory in Hong Kong. The laboratory provided service to a healthcare network of five hospitals, including one acute care university teaching hospital with 1,400 beds and four chronic care hospitals with 110 to 524 beds. The hospital network provided clinical service to a population of approximately 0.53 million people. Between 1 January 2009 and 31 December 2009, stool specimens from hospitalized patients sent for culture and cytotoxin assay of *C. difficile* were performed by our routine service protocol as usual, but the toxigenic strains were further characterized by molecular tests. The incidence of toxigenic *C. difficile* per 1,000 admissions and the rate of detection identified between period 1 (2004 and 2008) and period 2 (2009) were analyzed using databases of the laboratory information and hospital record system.

Bacterial culture for *C. difficile*

Liquid or semisolid stool samples were tested within 24 h of receipt. The samples were inoculated onto cycloserine–cefoxitin–fructose agar (CCFA) containing 4% proteose peptone, 0.5% Na_2HPO_4, 0.1% KH_2PO_4, 0.01% $MgSO_4$, 0.2% NaCl, 0.6% fructose, 1.5% agar at pH 7.4 (#CM0601, Oxoid, UK), with added selective supplement containing 250 mg/L D-cycloserine, 8 mg/mL cefoxitin (#SR0096, Oxoid, UK), and 7% horse blood. Culture plates were incubated anaerobically at 35°C to 37°C for 48 h. Obligatory anaerobic, large Gram-positive bacilli that were isolated from the CCFA and were susceptible to 5 μg of vancomycin were presumptively identified as *C. difficile*. The identification was confirmed by the Vitek® Anaerobe Identification Card (ANI) (bioMérieux, Inc., USA). Antimicrobial susceptibility testing against metronidazole, vancomycin, and ciprofloxacin were performed for selected strains using Etest strips according to the manufacturer's instructions (AB Biodisk, Sweden).

Cell culture cytotoxicity neutralization assay

Approximately 3 to 5 g of stool was suspended in 5 ml of phosphate-buffered saline (PBS) in a 1:2 dilution at a pH level of 7. The sample was centrifuged at 3,000 rpm for 55 min to produce a supernatant, which was subsequently transferred into eppendorf tubes and centrifuged at 13,000 rpm for 15 min at 4°C to clarify the supernatant. The supernatant was passed through a 0.22-μm-pore-size membrane filter. A toxin-producing *C. difficile* strain (UKEQAS QC 6109) was used as the positive control for the cell culture cytotoxicity neutralization assay (CCCNA), which was performed in sterile 96-well plates coated with the HeLa cell line as previously described [16]. In addition to the direct detection of cytotoxin from stool filtrates, CCCNA was also performed on the stationary-phase culture supernatant of each *C. difficile* isolate. *C. difficile* isolates were subcultured to brain heart infusion broth and incubated anaerobically for 96 h. Culture supernatant was subjected to CCCNA as for stool filtrate with the same interpretation criteria as previously described [16].

PCR ribotyping

DNA was extracted from *C. difficile* colonies using alkaline lysis as described previously [17]. Polymerase chain reaction (PCR) ribotyping was performed according to the method described by Bidet et al. [18]. After the electrophoresis of PCR products, the phylogenetic tree was constructed using BioNumerics v6.0 software (Applied Maths, Belgium).

slpA typing

slpA typing was performed for selected strains belonging to the same ribotype according to Joost et al. [19]. The purified PCR product was subjected to cycle sequencing by ABI BigDye terminator v1.1 (Applied Biosystems, Foster City, USA) and the resulting *slpA* sequence was compared to those in the NCBI database by using Nucleotide BLAST.

Aerotolerance sporulation experiment

The Miles and Misra method was adopted to study all PCR ribotype 002 isolates [20]. *C. difficile* was subcultured to *C. difficile* agar (Mast Group Ltd., Merseyside, UK) and incubated at 37°C anaerobically for 72 h. Bacterial suspension was prepared in saline to a turbidity of McFarland standard 0.5, followed by serial dilutions in saline to 10^1–10^4. Twenty microliters of each bacterial suspension with dilutions from 10^2 to 10^4 were transferred to three sectors of 12 blood agar plates from a height of 2.5 cm. The inoculum was spread over an area of 1.5–2.0 cm diameter. One set of agar plates (six blood agar plates) were incubated

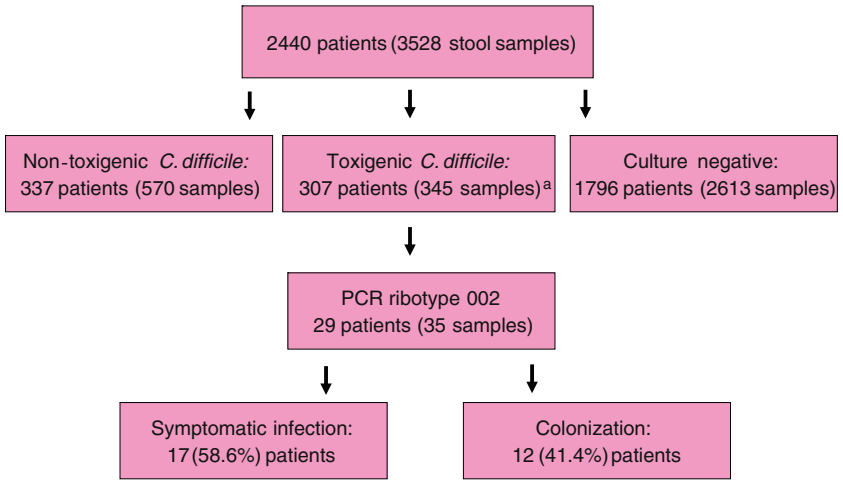


Fig. 1 Workup for *Clostridium difficile* PCR ribotype 002 in a healthcare region in Hong Kong (1 January 2009 to 31 December 2009). [a]169 strains from 145 patients were found to have cytotoxin production by cell culture cytotoxicity neutralization assay (CCCNA) from both stool filtrates and *C. difficile* isolates. Another 176 strains from 162 patients had negative cytotoxin production in stool filtrates but positive toxin production in the culture supernatant of *C. difficile* isolates

for 72 h at 37°C anaerobically and the other set of agar plates (six blood agar plates) were incubated for 48 h at 37°C aerobically, followed by a further incubation of 72 h at 37°C anaerobically. The sporulation frequency (the spore/total cell ratio), expressed as a percentage, was determined by dividing the average cell count of the second set over the first set. To rule out cross-contamination by other *Clostridium* species in the experiment [21], PCR for *C. perfringens* and *C. difficile* was performed on bacterial suspensions and colonies according to previously described methods [22, 23].

ELISA for the detection of *C. difficile* toxins A and B

Toxins A and B were measured by the *C. difficile* TOX A/B II™ kit (TechLab, the Netherlands), according to the manufacturer's instructions. All strains belonging to the PCR ribotype 002 were subcultured into brain heart infusion broth and incubated for 72 h anaerobically. Bacterial suspension was centrifuged at 3,000×g for 10 min and 50 μl of supernatant was mixed with 200 μl of diluent supplied in the kit. One drop (50 μl) of conjugate was added to the well of a microassay plate with immobilized affinity-purified polyclonal goat antibody against toxins A and B. The 100 μl diluted sample was transferred and mixed with the conjugate in the wells. The plate was incubated at 37°C for 50 min, washed four times with 1X wash solution, followed by the addition of 100 μl substrate solution. Stop solution was added to terminate the reaction after 5 min of incubation at room temperature. The resulting yellow color change was quantified by measuring the optical density 450 nm/620 nm. An A450/620 value of 1.0 corresponded to 1 U of the total amount of toxins (A and B).

Epidemiology of PCR ribotype 002 in our healthcare region

Patients with toxin-producing *C. difficile* were assessed by infection control nurses for symptomatology. Case records were reviewed if the patients had been discharged. Patients with diarrhea at the time of specimen collection were classified as having symptomatic infection, while those without diarrhea were classified as having asymptomatic colonization. Patients with symptomatic infection were further classified as having healthcare-associated infection, of which the development of symptoms started more than 48 h after admission to the hospital or within 4 weeks after discharge from the hospital, or community-associated infection, of which the development of symptoms started within 48 h after admission to the hospital or more than 12 weeks after discharge from the hospital, as previously described [24, 25]. When symptoms developed 4–12 weeks after hospital discharge, the association was indeterminate. The disease severity was stratified according to a scoring system described previously [26]. Briefly, two points were given to

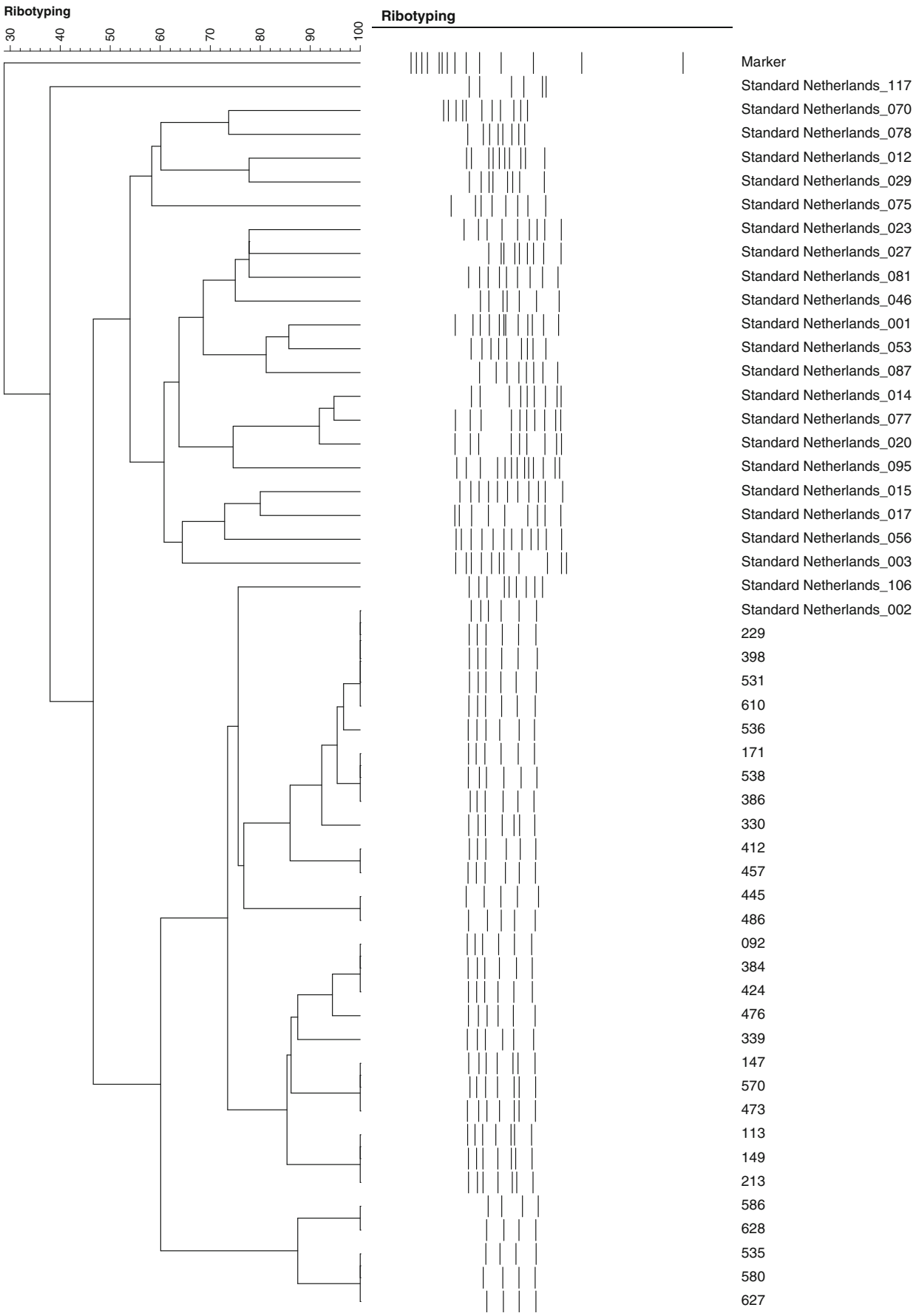
Table 1 Ribotype distribution of toxin-producing strains of *Clostridium difficile* among 307 patients in Hong Kong

	Total number of strains (%)	Total number of patients (%)
PCR ribotype 002[a]	35 (10.1%)	29 (9.4%)
PCR ribotype og39[b]	13 (3.8%)	11 (3.6%)
PCR ribotype 012	8 (2.3%)	7 (2.3%)
PCR ribotype 014	4 (1.2%)	4 (1.3%)
PCR ribotype 017	2 (0.6%)	2 (0.7%)
PCR ribotype 001	1 (0.3%)	1 (0.3%)
PCR ribotype 027	0	0
Other pattern	242 (70%)	221 (72%)[c]
Non-typable	40 (11.6%)	32 (10.4%)
Total	345	307

[a] PCR ribotype 002 constituted 55.6% (35/63) of strains and 53.7% (29/54) of patients with known ribotyping results

[b] This cluster of strains was identified by *slpA* typing

[c] Using 80% similarity in the dendrogram as the cutoff value, there were 106 distinct patterns with no more than eight isolates in each pattern


Ribotyping
30
40
50
60
70
80
90
100
Ribotyping
Marker
Standard Netherlands_117
Standard Netherlands_070
Standard Netherlands_078
Standard Netherlands_012
Standard Netherlands_029
Standard Netherlands_075
Standard Netherlands_023
Standard Netherlands_027
Standard Netherlands_081
Standard Netherlands_046
Standard Netherlands_001
Standard Netherlands_053
Standard Netherlands_087
Standard Netherlands_014
Standard Netherlands_077
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330
412
457
445
486
092
384
424
476
339
147
570
473
113
149
213
586
628
535
580
627

◀ **Fig. 2** Dendrogram of *Clostridium difficile* PCR ribotype 002 among 29 patients. Note: the strain number among 29 patients' isolates and the standard strains of different PCR ribotypes from the Netherlands are listed

patients with endoscopic confirmation of pseudomembranous colitis or those requiring intensive care support. One point each was given for age >60 years, temperature >38.3°C, albumin level <2.5 mg/dL, and peripheral WBC count >15,000 cells/mm^3 within 48 h of symptom onset. Patients with two points or more were considered as having "severe *C. difficile*-associated diarrhea (CDAD)". Otherwise, the patient was stratified as having "*C. difficile* infection". The demographics of the patients with *C. difficile* ribotype 002 and 56 randomly selected patients with PCR ribotypes other than 002 were compared.

Sporulation frequency and toxin level of *C. difficile* PCR ribotype 002

The toxin level of all strains of *C. difficile* PCR ribotype 002 were determined by ELISA. The sporulation frequency of *C. difficile* PCR ribotype 002 was measured by the quantitative method described above. The correlation between the sporulation frequency and toxin level was assessed. The sporulation frequencies and toxin levels of the strains isolated from patients with symptomatic infection were compared to those from asymptomatic patients. The sporulation frequency was tested in 56 randomly selected strains of PCR ribotypes other than 002 (one strain of 001, six strains of 012, three strains of 014, two strains of 017, eight strains of og39, and 36 strains of other patterns) as the control.

Statistical analysis

Fisher's exact test and Student's *t*-test were used in the analysis, where appropriate. Changes in the incidence of toxigenic *C. difficile* per 1,000 admissions between period 1 (2004–2008) and period 2 (2009) was analyzed by Poisson regression. Correlation between sporulation frequency and toxin level was assessed by Spearman's rank correlation coefficient. All reported *p*-values were two-sided. A *p*-value of <0.05 was considered to be statistically significant. Computation was performed using the Statistical Package for the Social Sciences (SPSS) version 15.0 for Windows.

Results

Between 1 January 2009 and 31 December 2009, a total of 3,528 stool samples from 2,440 patients were tested for *C. difficile* culture and cytotoxin assay. A total of 169 samples from 145 (5.9%) patients were found to have cytotoxin production by CCCNA from both stool filtrates and *C. difficile* isolates. Another 176 samples from 162 (6.6%) patients had negative cytotoxin production in stool filtrates but positive toxin production in the culture supernatant of *C. difficile* isolates (Fig. 1). A total of 345 toxigenic *C. difficile* strains from 307 (12.5%) patients were further tested for ribotyping and the results are listed in Table 1. All PCR ribotypes concurred with *slpA* typing results. PCR ribotype 002 was the most predominant ribotype, constituting 35 strains from 29 (9.4%) patients with toxin-producing *C. difficile*. The dendrogram of *C. difficile* PCR ribotype 002 is shown in Fig. 2. All 35 strains of PCR ribotype 002 were resistant to ciprofloxacin (minimum inhibitory concentration [MIC]>32 μg/ml). The MIC_{50} of metronidazole and vancomycin were 0.5 μg/ml and 0.75 μg/ml, respectively, whereas the MIC_{90} were 0.75 μg/ml and 1.5 μg/ml, respectively. The change in incidence of toxigenic *C. difficile* per 1,000 admissions and the rate of positive detection in our healthcare region from 2004 to 2009 are shown in Fig. 3. There was a significant increase in both the incidence of toxigenic *C. difficile* from 0.53 to 0.95 per 1,000 admissions ($p<0.001$) and the rate of positive detection from 4.17% to 6.28% ($p<0.001$) between period 1 (2004–2008) and period 2 (2009).

Epidemiology of PCR ribotype 002 in our healthcare region

During the study period, a total of 29 patients shed *C. difficile* ribotype 002 in the stool. There were 20 males and 9 females, with a median age of 76 years (range 14–97 years). Eleven (37.9%) patients were old age persons home residents. Symptomatic infection was diagnosed in 17 (58.6%) patients, while ten of them had severe CDAD according to the scoring system (Table 2). All 17 patients with symptomatic infection were defined as healthcare-associated infection. Colonization of *C. difficile* was found in 12 (41.4%) patients. Most of the patients (24, 82.8%) were managed in the medical ward, while three patients were managed in the surgical ward, one patient in the emergency department, and one patient in the pediatric and adolescent unit. These patients were located in 20 different wards from three different hospitals (one acute care and two chronic care hospitals) without any epidemiological linkage. There were five patients with repeated isolation of *C. difficile* during the same episode of hospitalization, among whom four patients had two episodes and one patient had three episodes. Among these five patients, three had symptomatic infection and one had asymptomatic colonization. The other progressed from asymptomatic colonization to symptomatic infection during hospitalization. The demographic characteristics of patients with *C. difficile* PCR ribotype 002 and ribotypes other than 002 are illustrated in Table 3. Patients carrying toxigenic *C. difficile*

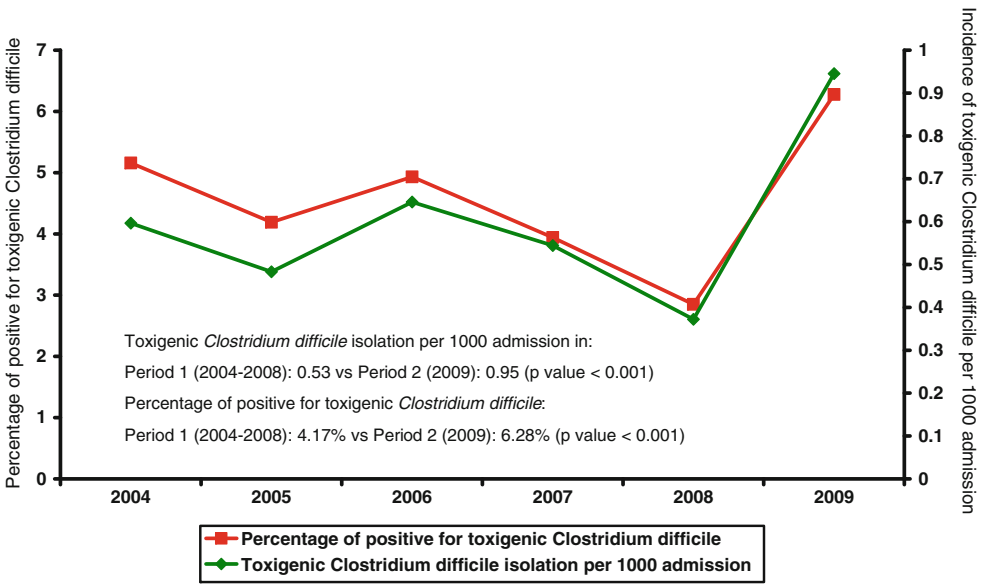

Fig. 3 Trend of isolation of toxigenic *Clostridium difficile* in a healthcare region in Hong Kong. Note: toxigenic *Clostridium difficile* isolation per 1,000 admissions in: period 1 (2004–2008): 0.53 vs. period 2 (2009): 0.95 (p-value<0.001); Percentage of patients positive for toxigenic *Clostridium difficile*: period 1 (2004–2008): 4.17% vs. period 2 (2009): 6.28% (p-value<0.001)

PCR ribotype 002 were more frequently admitted from an elderly home (p=0.01) and received more β-lactam antibiotics in the preceding 3 months (p=0.04).

Sporulation frequency and toxin level of *C. difficile* PCR ribotype 002

There is no statistically significant correlation between the sporulation frequency and the measured toxin levels of the *C. difficile* isolates (Spearman's rho 0.0311, p=0.858). The mean sporulation frequency of 35 strains of *C. difficile* PCR ribotype 002 was 20.2%, which was significantly higher than that of the 56 randomly selected PCR ribotypes other than 002 (3.7%, p<0.001). Among the 23 strains of *C. difficile* PCR ribotype 002 isolated from patients with symptomatic infection, the mean sporulation frequency was significantly higher than 12 strains of PCR ribotype 002 cultured from patients with asymptomatic colonization (28.9% vs. 3.5%, p=0.02). The mean levels of toxin production by ELISA among strains collected from patients with asymptomatic colonization and symptomatic infection were 96.13 U/ml and 123.85 U/ml, respectively (p=0.29). Subgroup analysis for the *C. difficile* isolated from symptomatic patients with or without severe CDAD showed no statistically significant difference in terms of sporulation frequency and toxin production.

Discussion

C. difficile has become a re-emerging pathogen since the outbreak of a hypertoxigenic strain of PCR ribotype 027 in North America and Europe since 2003 [8, 10, 11]. This was followed by its global dissemination to the other continents in 2008 [27]. On the other hand, *C. difficile* PCR ribotypes other than 027 have also been increasingly reported recently. PCR ribotype 078 has been implicated in community-associated *C. difficile* infection in the Netherlands [24], while PCR ribotypes 001 and 018 were the most commonly identified ribotypes in the healthcare facilities in Germany, the UK, and Italy [28–30]. Although *C. difficile* ribotype 027 has also been reported in Hong Kong in 2008 [16], our present study showed that PCR ribotype 002 was the most predominant strain and constituted 56% (35 out of 63 typed strains with well-characterized PCR ribotyping) of the *C. difficile* strains isolated in 2009. This finding might explain the significant increase in the positive detection rate of toxigenic *C. difficile* and the incidence of toxigenic *C. difficile* per 1,000 admissions observed in 2009 as compared to the baseline data between 2004 and 2008.

C. difficile PCR ribotype 002 was commonly found in patients with both symptomatic infection and asymptomatic colonization. The mean sporulation frequency (20%) of our strains was significantly higher than the other PCR ribotypes in our locality, but was comparable to that of the epidemic strains of *C. difficile* PCR ribotype 027 [13]. Since the epidemic strains of *C. difficile* were reported to have an inherently increased sporulation frequency [13, 31], and the transmission of *C. difficile* in the healthcare facilities was mediated primarily by spores found in the environment or carried by healthcare workers [32, 33], *C. difficile* PCR ribotype 002 might, thus, become an epidemic strain and contribute to hospital outbreaks. In fact, all 17 symptomatic patients carrying PCR ribotype 002 had healthcare-associated *C. difficile* infection. Furthermore, as the proportion of patients residing in elderly homes was significantly higher among those with PCR ribotype 002 than those with other ribotypes, further

Table 2 Demographic characteristics of 29 patients with culture isolation of *Clostridium difficile* ribotype 002 in Hong Kong

Case and (strain) no.	Sex/age	Disease status (disease score)[a]	Underlying disease(s)	Antibiotic therapy 1 week before culture of *C. difficile*	Days between the identification of *C. difficile* and admission	Days between the identification of *C. difficile* and death
1 (171)	M/89	Symptomatic (1)	IHD, MDS	Nil	1[b]	330
2 (196)	F/81	Symptomatic (2)	CRF, HT	Cefuroxime, levofloxacin	61	302
3 (213)	M/38	Symptomatic (3)	Refractory ALL	Meropenem	7	27
4 (229)	M/14	Colonization	ALL	Cefoperazone/sulbactam, amikacin	1	NA
5 (330)	M/54	Symptomatic (2)	NPC	Nil	1[b]	NA
6 (339)	M/55	Symptomatic (4)	Liver Tx recipient	Nil	25	131
7 (341)	M/46	Symptomatic (1)	AML	Imipenem/cilastatin	13	NA
8 (386)	F/48	Symptomatic (0)	CA of the urethra	Levofloxacin	4	148
9 (398)	F/91	Symptomatic (1)	CA of the lung	Amoxicillin/clavulanate	45	14
10 (408)	M/25	Colonization	Lymphoma	Meropenem, vancomycin	27	NA
11 (412)	M/33	Colonization	HT, renal Tx recipient	Amoxicillin/clavulanate	30	NA
12 (445)	M/93	Symptomatic (3)	Sick sinus syndrome	Cefuroxime	7	87
13 (457)	M/59	Colonization	CRF, DM, HT	Piperacillin/tazobactam, vancomycin	31	142
14 (570)	M/95	Symptomatic (1)	CRF, DM, HT	Ciprofloxacin, co-trimoxazole	61	246
15 (428)	M/86	Colonization	IHD, parkinsonism	Amoxicillin/clavulanate	88	297
16 (486)	M/73	Symptomatic (1)	CA of the lung, IHD	Nil	13	NA
17 (476)	F/42	Colonization	Lymphoma	Nil	79	24
18 (536)	M/86	Symptomatic (1)	IHD	Nil	1[b]	NA
19 (526)	F/96	Colonization	CA of the colon, CHF	Amoxicillin/clavulanate	21	114
20 (531)	M/76	Colonization	CRF, HT	Levofloxacin	7	291
21 (538)	F/21	Colonization	AML	Piperacillin/tazobactam,vancomycin	4	191
22 (535)	F/96	Symptomatic (2)	CHF	Nil	1[b]	110
23 (580)	M/97	Symptomatic (2)	CHF, dementia	Amoxicillin/clavulanate	14	113
24 (586)	M/75	Colonization	Alzheimer's disease, DM,HT	Ceftibuten	10	130
25 (610)	M/89	Symptomatic (1)	Dementia	Amoxicillin/clavulanate	49	8
26 (627)	M/62	Colonization	IHD	Cefuroxime	8	NA
27 (92)	F/90	Symptomatic (1)	HT	Amoxicillin/clavulanate	18	309
28 (113)	M/85	Colonization	Dementia	Nil	17	47
29 (129)	F/80	Symptomatic (1)	CRF, DM, HT	Amoxicillin/clavulanate	376	NA

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CA, carcinoma; CHF, congestive heart failure; CRF, chronic renal failure; DM, diabetes mellitus; HT, hypertension; IHD, ischemic heart disease; MDS, myelodysplastic syndrome; NA, not applicable; NPC, nasopharyngeal carcinoma; Tx, transplant

[a] The disease score is defined as previously described [26]. Briefly, two points were given to patients with endoscopic confirmation of pseudomembranous colitis or those requiring intensive care support. One point each was given for age >60 years, temperature >38.3°C, albumin level <2.5 mg/dL, or peripheral WBC count>15,000 cells/mm^3 within 48 h of symptom onset. Patients with two points were considered to have severe *C. difficile*-associated diarrhea (CDAD). Otherwise, the patient was stratified as infection

[b] Case was defined as healthcare-associated infection, as the symptoms onset was within 4 weeks after discharge from the hospital

Table 3 Demographic characteristics of patients with culture isolation of *Clostridium difficile* ribotype 002 and non-ribotype 002 in Hong Kong

	Clostridium difficile ribotype 002 (n=29)	*Clostridium difficile* ribotype other than 002[b] (n=56)	p-value
Age (mean±SD)	68.1±25.5	58.3±26.2	0.97
Sex (M/F)	20/9	33/23	0.37
Residence in elderly home	11 (37.9%)	7 (12.5%)	0.01[a]
Patients with			
Malignancy	12 (41.4%)	20 (35.7%)	0.61
Organ transplant	2 (6.9%)	5 (8.9%)	1.0
Cardiopulmonary condition	9 (31.0%)	10 (17.9%)	0.18
Renal failure	5 (17.2%)	7 (12.5%)	0.55
Cerebrovascular accident	5 (17.2%)	6 (10.7%)	0.40
Diabetes mellitus	4 (13.8%)	9 (16.1%)	0.78
Patients with			
Asymptomatic colonization	12 (41.4%)	26 (46.4%)	0.66
Severe CDAD[c]	7/17 (41.2%)	13/30 (43.3%)	0.90
Number of hospitalizations in the past year, median (interquartile range)	6 (4–12)	5.5 (2–12.75)	0.52
Number of patients with isolation of toxigenic *C. difficile* in the past year	3 (10.3%)	8 (14.3%)	0.74
Antibiotic therapy in the week preceding the culture of *C. difficile*	21 (72.4%)	34 (60.7%)	0. 29
Days of antibiotics[d] received by patients in the past 3 months (mean±SD)			
β-lactams	23.7±17.9	16.2±14.6	0.04
Fluoroquinolones	2.6±5.1	5.6±10.0	0.14
Clindamycin	0	0.2±1.2	0.35
Number of patients using proton pump inhibitors			
Within 90 days	14 (48.3%)	19 (33.9%)	0.24
91–180 days	8 (27.6%)	12 (21.4%)	0.59
181–365 days	5 (17.2%)	10 (17.9%)	1
Mean (range) days between the identification of *C. difficile* and admission	35.2 (1–376)	31.4 (0–416)	0.99
30-day survival after the identification of *C.difficile* and admission	3 (10.3%)	6 (10.7%)	0.96

[a] Odds ratio 1.89; 95% confidence interval 1.04–3.42

[b] Patients with *Clostridium difficile* ribotype 001 (n=1), 012 (n=6), 014 (n=3), 017 (n=2), og39 (n=8), and unrecognized ribotype (n=36) were randomly selected as the control

[c] Severe CDAD (*C. difficile*-associated diarrhea) is defined according to the disease score, as previously described [26]

[d] Penicillin group included ampicillin, amoxicillin–clavulanate, ampicillin–sulbactam, ticarcillin–clavulanate, piperacillin, and piperacillin–tazobactam; cephalosporin group included cefazolin, cefuroxime, cefotaxime, ceftriaxone, ceftazidime, cefoperazone, cefoperazone–sulbactam, cefepime; carbapenem group included imipenem–cilastatin, meropenem, and ertapenem; fluoroquinolones included ciprofloxacin, levofloxacin, and moxifloxacin

investigations should be conducted in order to determine whether elderly homes might serve as an epidemic center amplifying this particular ribotype in our locality.

Interestingly, *C. difficile* PCR ribotype 002 isolated from patients with symptomatic infection had a significantly higher rate of sporulation, but not to the level of toxin production in vitro. Furthermore, there was no correlation between toxin production and sporulation. Our finding was in concordance with a recent study which suggested toxin production and sporulation to be opposite survival strategies for *C. difficile* in response to nutrient limitation by demonstrating an inverse correlation between toxin level and spore count in stationary-phase cultures [34]. However, a more recent analysis of a hypervirulent strain of *C. difficile* exhibited an increased sporulation as well as toxin production [14]. Since sporulation and toxin production is a complex process [35], and the disease severity of *C. difficile* is also determined by host factors such as the age,

underlying diseases, and levels of toxin-neutralizing antibodies [36, 37], the relationship between sporulation frequency and disease severity remains to be elucidated.

The emergence of *C. difficile* with an inherently increased sporulation such as PCR ribotypes 027, 001, and 002 poses a great challenge to the infection control team. There is increasing evidence that the use of fluoroquinolones is a major risk factor for CDAD in the hospital and long-term care facilities [38–42]. Similar to the other emerging PCR ribotypes of *C. difficile*, all of our ribotype 002 strains were resistant to fluoroquinolones with an MIC>32 μg/ml against ciprofloxacin. Since the use of fluoroquinolones is increasing globally and locally [43, 44], our antimicrobial stewardship program should no longer target broad-spectrum antimicrobial agents only and should extend to include fluoroquinolones as well [45, 46]. Of note, patients carrying *C. difficile* PCR ribotype 002 were more frequently found to have used β-lactam antibiotics, including penicillin, cephalosporin, and carbapenem, in the preceding 3 months to our analysis (Table 3). As most of the β-lactams have a relatively lower MIC against *C. difficile* [47–49], strains with inherently increased sporulation, such as PCR ribotype 002, may survive better than poorly sporulating ribotypes, despite β-lactam antibiotics therapy. The waterless alcohol-based hand rub, which has been highly advocated in the healthcare setting for hand hygiene, was not effective against the spores of *C. difficile* in the hands [50, 51], even with directly observed hand hygiene practice [52]. Therefore, a timely laboratory diagnosis to recognize this high-risk strain would facilitate strategic infection control interventions, which should include the early identification of symptomatic cases for contact precautions with single-room isolation and thorough environmental cleaning with chlorine-based germicides in order to inactivate *C. difficile* spores. In fact, the use of non-chlorine-based cleaning agents has been associated with a paradoxical increase in sporulation, which may further enhance the nosocomial transmission of *C. difficile* [31, 53]. A close collaboration between the microbiology laboratory and the infection control team is essential for the successful prevention of nosocomial outbreaks of *C. difficile* PCR ribotype 002 in our healthcare region.

There were several limitations to this study. Firstly, as there was no standardized guideline on when to order stool testing for *C. difficile* in our hospital, the population of our samples may have been biased. This might contribute to the apparently higher proportion of asymptomatic colonization of ribotype 002 among our population than that previously reported for *C. difficile* in general [2–4]. However, the exact asymptomatic colonization rate of ribotype 002 has not been clearly documented elsewhere. Secondly, we only had 23 (20%) out of 116 control strains available for the full panel of PCR ribotyping [54]. There might be a possibility of missing some important PCR ribotypes in our series. However, among 221 patients with unrecognized PCR ribotypes, there were 106 distinct patterns and no major cluster. Thirdly, we could not retrieve the archived strain from the early period and, thus, the origin of this *C. difficile* PCR ribotype 002 could not be ascertained. From the epidemiological perspective, the incidence of toxigenic *C. difficile* remained static (0.53 per 1,000 admissions) between 2004 and 2008, but it suddenly increased to 0.95 per 1,000 admissions in 2009. Although the incidence was lower than some centers in Europe and was still within an acceptable limit of less than 5 cases per 1,000 admissions as recommended by some experts [55, 56], an 80% upsurge should alert both the physician and the infection control team to possible outbreaks of ribotype 002 before it could become established in our hospitals, as in the case of *C. difficile* PCR ribotype 027.

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