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ORIGINAL ARTICLE

Molecular typing and epidemiology of *Clostridium difficile* in respiratory care wards of central Taiwan

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Clostridium difficile;
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Prevalence

Background/purpose: In industrialized countries, *Clostridium difficile* is the major cause of nosocomial diarrhea. This study involved a broad overview of baseline epidemiology for *C. difficile* in Taiwan.
Materials and methods: Point prevalence was estimated from a prospective survey conducted in the respiratory care wards of six hospitals in central Taiwan. Polymerase chain reaction (PCR) ribotyping and multiple-locus variable-number tandem-repeat analysis (MLVA) were performed on all toxigenic *C. difficile* isolates, including asymptomatic and symptomatic strains.
Results: A total of 149 patients were screened for *C. difficile*; the point prevalence for *C. difficile* infection (CDI) and *C. difficile* colonization was 4% and 19%, respectively. CDI cases were significantly related to end-stage renal disease, and *C. difficile* colonization cases were significantly associated with previous admission to an acute-care facility. No hypervirulent PCR

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ribotype 027 strain was found. MLVA detected two clusters of CDI-related and three clusters of asymptomatic *C. difficile* strains circulating in wards.
Conclusion: Our results demonstrate a high prevalence of toxigenic *C. difficile* colonization in hospitals. Infection control personnel should pay attention to the increasing numbers of CDI cases, and molecular typing for *C. difficile* should be performed when necessary.

Introduction

Clostridium difficile is the major cause of nosocomial diarrhea and colitis in industrialized countries.[1,2] The spectrum of *C. difficile* infection (CDI) varies from asymptomatic carriage or mild diarrhea to severe colitis, and it may lead to megacolon, perforation, sepsis, and death.[3] Since 2002, several outbreaks of severe colitis, caused by the hypervirulent *C. difficile* strain NAP1/027, have been recognized in health-care facilities in Canada, the United States, and Europe[4,5]; the disease is highly associated with advanced age, antacid use, antibiotic use, and increased length of hospital stay.[6]

A few epidemiologic studies on *C. difficile* have been conducted in Taiwan. In 2003, a 4-month prospective study of intensive care unit (ICU) and infection wards found that the average incidence of CDI was 1/1000 patient days.[7] During 2007 and 2008, a 14-month retrospective survey showed that the overall incidence in a southern medical center was 4.3/10,000 patient days.[8] Recently, a prospective study showed the average incidence in a northern medical center to be 4.5/10,000 patient days, and indicated a downward trend in CDI incidence.[9] However, these results are limited to single hospitals and may not reflect the overall epidemiology in Taiwan. In addition, no suitable molecular typing method was applied to detect the endemic and/or epidemic strains.

To determine the burden of *C. difficile* on multiple long-term respiratory care wards (RCWs), the baseline prevalence of both colonizing and infectious *C. difficile* strains was estimated. Furthermore, polymerase chain reaction (PCR) ribotyping, a classical method used to represent the virulent strain of *C. difficile*,[10] was performed on all *C. difficile* isolates to detect the possible existence of the hypervirulent *C. difficile* strain NAP1/027. Multiple-locus variable-number tandem-repeat analysis (MLVA) typing, one of the most discriminatory methods used for the molecular analysis of *C. difficile* strains,[11] was used to detect *C. difficile* clusters in wards. In conclusion, we present the epidemiology of *C. difficile* in Taiwan, and provide evidence for the presence of several asymptomatic and CDI-related endemic strains in hospitals.

Materials and methods

Patients, specimens, and facility

This research, including the collection of demographic and clinical patient data and stool specimens, was approved by the Institutional Review Boards at each investigative site. Only those patients who were admitted to wards for more than 48 hours were considered to be hospital-associated patients, as recommended by the *Ad Hoc Clostridium difficile* Surveillance Working Group.[12] Information was collected on patient age, sex, date of birth, date of admission, ward of acquisition, clinical symptoms, underlying disease, and clinical treatment. We gathered this information through consultation with the physician or nurses in charge.

RCWs of six hospitals in central Taiwan were involved in this project. Stool specimens from each RCW were collected over 3 days between April 21, 2009 and October 23, 2009. All stool specimens were transported using anaerobic transport swabs and were delivered within 24 hours to the central-region laboratory at the Centers for Disease Control in Taiwan for isolation of *C. difficile*. Facility data (such as location, type, and number of licensed beds) were obtained from the website of the Bureau of National Insurance, Taiwan Department of Health (http://www.nhi.gov.tw/AmountInfoWeb/).

Definitions

A *C. difficile* strain was confirmed to be toxigenic by a positive toxin test and/or toxigenic type determined by PCR. Diarrhea was defined as $\geq$3 unformed stools per 24-hour period.[12] CDI was defined as the presence of diarrhea, in combination with a positive report for toxigenic *C. difficile* and a negative culture for *Salmonella* species, *Shigella* species, and *Staphylococcus aureus*. *C. difficile* colonization was defined as a positive toxin test for *C. difficile*, regardless of the presence of diarrhea. Previous acute-care admission was defined as hospitalization in an ICU for 2 weeks within 1 year. Antibiotic use was defined as any antibiotic treatment for 1 week within the past 3 months.

DNA preparation

Genomic *C. difficile* DNA was purified using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Genomic DNA isolated from *C. difficile* was then used for PCR amplification of variable-number tandem-repeat sequences and PCR ribotyping.

Isolation and determination of *C. difficile*

All stool specimens were cultured on cycloserine cefoxitin fructose agar (Oxoid Ltd, Hampshire, UK) and were

incubated under anaerobic conditions for 48 hours. All suspected *C. difficile* colonies were analyzed for a species-specific internal fragment of the triose phosphate isomerase (*tpi*) housekeeping gene, and the toxigenic type was characterized by PCR amplification of internal fragments of the toxin A (*tcdA*) and toxin B (*tcdB*) genes, as previously described.[13] Briefly, each candidate colony was dissolved in 1 mL distilled water and boiled for 15 minutes to prepare the DNA. *Tpi*-, *tcdA*-, and *tcdB*-specific primers[13] were used in separate PCR reactions. PCR was performed in 20 μL volumes containing the following components: 50 ng DNA, 10% glycerol, 0.5 μM of each primer, 200 μM dNTPs, and 1 U of Taq DNA polymerase (BioVan, Taichung, Taiwan) in a 1 × amplification buffer solution [10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5 mM $MgCl_2$]. PCR was performed using a PTC-200 Thermal Cycler (MJ Research, Waltham, MA, USA). The PCR cycle conditions were as follows: 95 °C for 3 minutes; followed by 30 cycles of 95°C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds; and a final extension at 72 °C for 3 minutes. PCR products were resolved by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

Enzyme immunoassay of *C. difficile* toxins A and B

All *C. difficile* isolates were analyzed for production of toxins A and B using the ProSpect *C. difficile* Toxin A/B Microplate Assay (Remel, Lenexa, KS, USA), according to the manufacturer's instructions. The kit's sensitivity and specificity are 90.3% and 96.2%, respectively, compared to a tissue culture cytotoxicity assay.

MLVA typing

MLVA was conducted as described previously, using seven loci (A6cd, B7cd, C6cd, E7cd, F3cd, G8cd, and H9cd) from published MLVA schemes.[14] The products were then analyzed on an ABI3130 sequence detection system (Applied Biosystems, Foster City, CA, USA), and the fragment sizes or copy numbers were determined as described.[15] MLVA types with summed tandem-repeat difference of ≤10 were considered a cluster of *C. difficile* isolates.

PCR ribotyping

Genomic DNA from all the *C. difficile* strains was amplified with the primer set designed by Bidet et al,[16] and electrophoresis-based PCR ribotyping was performed using a published method.[15] PCR ribotypes 001, 012, 017, 027, and 106 were set up by comparing the curve files with the five reference strains NCTC11204, NCTC13307, NCTC13366, NCTC13287, and NCTC13404, respectively. All PCR ribotypes were named with an "R" added prior to the serial number.

Point prevalence measurement and statistical analysis

The point prevalence for CDI and *C. difficile* colonization was determined as follows: Point prevalence rate = CDI or *C. difficile* colonization cases

Total RCW residents (149)

To evaluate the influence of different toxin determination methods, characteristics of toxigenic isolates from different groups (A,B, and C in Table S1) were analyzed by Pearson's Chi-square test and Fisher's exact test. To assess the factors associated with CDI and *C. difficile* colonization, Pearson's Chi-square test and Fisher's exact test were used for categorical data. Factors with $p < 0.05$ were confirmed with multivariable logistic regression analysis, by applying the backward stepwise variable selection procedure. All tests were two tailed, and $p < 0.05$ was considered statistically significant. A p value between 0.1 and 0.05 represented a trend. Data were analyzed using the SPSS software, version 14 (SPSS Inc., Chicago, IL, USA) or Excel software (Microsoft, Redmond, WA, USA).

Results

A total of 57 *C. difficile* strains were isolated from 149 RCW residents; among them, 29 toxigenic strains were identified by toxin test and/or toxigenic typing. The results of the toxigenic isolates were as follows (Table S1): one isolate was tested positive for the presence of the toxin but negative for toxigenic type; three isolates were tested negative for the presence of the toxin but positive for toxigenic type; and 25 isolates were tested positive for the presence of both toxin and toxigenic type.

The point prevalence of CDI and *C. difficile* colonization in central Taiwan is shown in Table 1. A total of 149 specimens were collected, and the average point prevalence rates for *C. difficile* colonization and CDI were 19% and 4%, respectively (Table 1). The RCWs of six hospitals in central Taiwan participated in this research, and the total number of beds sampled represented 12.2% (149 in 1221) of the total beds of the RCWs in central Taiwan. The point prevalence of *C. difficile* colonization varied among hospitals and ranged from 4% to 31%. The point prevalence of CDI also varied among different hospitals; the rates in wards (H1, H3, H4, H5, and H6) without CDI clusters ranged from 0% to 4%, whereas that in the ward (H2) with two CDI clusters reached 12%.

Table 1 Point prevalence of *Clostridium difficile* colonization and CDI in RCWs of six hospitals from central Taiwan

Hospital	Hospital type	Sample size	No. of cases (point prevalence rate, %)	
			C. difficile colonization	CDI
H1	Medical center	13	4 (31)	0 (0)
H2	Regional	43	11 (26)	5 (12) [a]
H3	Regional	8	2 (25)	0 (0)
H4	Area	34	8 (24)	0 (0)
H5	Regional	23	1 (4)	1 (4)
H6	Area	28	3 (11)	0 (0)
Total		149	29 (19)	6 (4)

[a] CDI clusters were detected in this ward.
CDI = *C. difficile* infection; RCWs = respiratory care wards.

Table 2 MLVA clusters and PCR ribotypes of *Clostridium difficile* isolates in RCWs of six hospitals from central Taiwan

Hospitals	Strain name	CDI	PCR ribotype	MLVA cluster[a]	MLVA profile[b] A6cd-B7cd-C6cd-E7cd-F3cd-G8cd-H9cd
H1	1	N	R18		30-19-17-5-5-9-8
	2	N	R10	E	32-15-31-5-9-5-7
	3	N	R10	E	32-14-36-5-9-5-7
	4	N	R59		27-17-41-8-5-10-8
H2	5	Y	R10	A	36-16-31-5-9-5-7
	6	Y	R10	A	37-16-31-5-9-5-7
	7	Y	R10	A	37-16-31-5-9-5-7
	8	N	R10	A	36-16-30-5-9-5-7
	9	Y	R106	B	19-15-41-2-6-8-7
	10	N	R106	B	18-14-41-2-6-8-7
	11	Y	R106		24-8-23-2-6-11-7
	12	N	R45	C	32-16-19-4-6-8-11
	13	N	R45	C	33-16-19-4-6-8-11
	14	N	R45	C	25-16-19-4-6-7-11
	15	N	R66		23-17-31-6-8-15-31
H3	16	N	R17		3-12-25-8-6-80-8
	17	N	R106		16-15-19-2-6-10-7
H4	18	N	R106	D	19-16-45-2-6-8-7
	19	N	R106	D	19-15-44-2-6-8-7
	20	N	R106	D	19-15-45-2-6-8-7
	21	N	R106		19-15-27-2-6-8-7
	22	N	R7		16-9-20-4-6-9-7
	23	N	R10		24-16-33-4-9-7-7
	24	ND	R10		43-9-29-5-9-6-7
	25	ND	R10		41-16-32-5-9-5-7
H5	26	Y	R66		18-9-30-6-5-10-8
H6	27	N	ND		41-7-14-8-6-4-7
	28	N	R41		25-20-33-2-6-13-8
	29	N	R52		28-22-17-8-5-20-8

[a] Isolates with an STRD of $\leq$10 are defined as one MLVA cluster.
[b] Identified previously by van den Berg et al.[14]
CDI = *C. difficile* infection; MLVA = multiple-locus variable-number tandem-repeat analysis; N = non-CDI case; ND = no data; PCR = polymerase chain reaction RCW = respiratory care ward; STRD = summed tandem-repeat difference; Y = CDI case.

PCR ribotyping was performed on 29 isolates and 11 PCR ribotypes were identified (Table 2). The hypervirulent 027 strain was not identified and the three most common PCR ribotypes were R10, R106 (UK 106), and R45 (9, 8, and 3, respectively). These PCR ribotypes were detected in multiple hospitals.

Five MLVA clusters were detected in 29 toxigenic *C. difficile* isolates, and one (E), three (A, B, and C), and one (D) clusters were identified in hospitals H1, H2, and H4, respectively (Table 2). All MLVA clusters strains were limited to individual hospitals. When these endemic strains were compared with the corresponding clinical symptoms, 75% (3 of 4) and 50% (1 of 2) of isolates from clusters A and B, respectively, were CDI cases. By contrast, all the isolates in clusters C, D, and E were from asymptomatic *C. difficile* carriers.

Of the 149 patients, 29 (19%) had *C. difficile* colonization and six (4%) were CDI cases. The results for the multivariable analysis of the corresponding data are shown in Table 3. *C. difficile* colonization cases were significantly related to prior admission to an acute-care facility (60.7% vs. 32.7%, $p = 0.009$). CDI patients were more likely to have end-stage renal disease (ESRD) (33% vs. 5%, $p = 0.022$) than non-CDI patients; there was also a trend toward exposure to antacids (67% vs. 29%, $p = 0.072$). No significant differences were found between colonization and non-colonization cases or between CDI and non-CDI patients with respect to sex, age, or previous antibiotic treatment.

Discussion

In the present study, the point prevalence and baseline characteristics for either *C. difficile* colonization or infection of RCW patients were estimated. A high prevalence of toxigenic *C. difficile* colonization in hospitalized patients was discovered, and two CDI-related endemic strains and three asymptomatic endemic strains were detected by MLVA typing.

The CDI prevalence rate (4%) was similar to that obtained in the study by Hung et al[17] in southern Taiwan (3.6%; 7/194) and was much lower than the prevalence rates in countries that have experienced CDI outbreaks (7–14.7%; Table 1).[18–20] All the patients with CDI in this

Table 3 Characteristics for *Clostridium difficile* colonization and CDI cases of RCW patients in central Taiwan[a]

Variable	*C. difficile* colonization			CDI		
	Colonized ($n = 28$)	Not colonized ($n = 113$)	p	Cases ($n = 6$)	Not cases ($n = 135$),	p
Male	14 (50)	69 (61)	—	2 (33)	81 (60)	—
Age (y)						
<65	5 (17.9)	11 (9.7)	—	1 (16.7)	15 (11.1)	—
65–85	13 (46.4)	66 (58.4)	—	3 (50)	76 (56.3)	—
>85	10 (35.7)	36 (31.9)	—	2 (33.3)	44 (32.6)	—
Past history						
Acute care admission	17 (60.7)	37 (32.7)	0.009	2 (33.3)	52 (38.5)	
Fecal incontinence	7 (25)	15 (13)	—	1 (17)	18 (13)	—
Antacid	13 (46)	30 (27)		4 (67)	39 (29)	0.072
Antibiotic used	19 (68)	61 (54)		3 (33)	78 (57)	
Fluoroquinolone	0 (0)	6 (5.3)	—	0 (0)	6 (4.4)	—
Vancomycin	3 (10.7)	9 (8)	—	1 (16.7)	11 (8.1)	—
Clindamycin	0 (0)	1 (0.9)	—	0 (0)	1 (0.7)	—
Penicillin	13 (46.4)	30 (26.5)	—	2 (33.3)	41 (30.3)	—
Cephalosporin	2 (7.1)	22 (19.5)	—	0 (0)	24 (17.8)	—
Imipenem	2 (7)	10 (8.8)	—	1 (16.7)	11 (8.1)	—
Aminoglycoside	3 (10.7)	10 (8.8)	—	1 (16.7)	12 (8.9)	—
Any underlying disease	18 (64.3)	64 (56.6)	—	5 (83.3)	77 (57)	—
Diabetes	10 (36)	39 (35)	—	3 (50)	46 (34)	—
ESRD	3 (11)	6 (5)	—	2 (33)	7 (5)	0.022
Malignancy	2 (7)	7 (6)	—	(0)	(0)	—
Stroke	9 (32)	35 (31)	—	1 (17)	43 (32)	—

[a] Stepwise backward logistic regression, final model retained variables with a $p < 0.1$.
Data are presented as *n* (%).
CDI = *C. difficile* infection; CI = confidence interval; ESRD = end-stage renal disease; NG tube = naso gastric tube; OR = odds ratio; RCW = respiratory care ward; —= not significant.

study experienced only mild symptoms. Moreover, PCR ribotyping of *C. difficile* isolates did not identify any hypervirulent PCR ribotype 027 strain in the wards studied, which was also true of two medical centers in northern and southern Taiwan.[17,21] Both the predominant PCR ribotypes and their proportions in Taiwan differed significantly from those found in England and North America. In England, the three most common PCR ribotypes are 027, 106, and 001, which accounted for 55%, 13%, and 9%, respectively, of all CDI cases during the epidemic period of CDI between 2007 and 2009, whereas the rate decreased to 21%, 7%, and 7%, respectively, in the endemic period.[22] In Canada during the epidemic period of CDI, the PCR ribotype 027 accounted for 80% isolates from all CDI cases.[23] In Taiwan, the three most common PCR ribotypes were reported to be R10 (31%), R106 (UK106; 27.5%), and R45 (10%); the proportions of dominant strains were much less than those the other countries experienced during the epidemic of CDI. The lack of highly epidemic strains such as PCR ribotype 027 may explain the low prevalence of CDI cases.

In this study, a strain that was positive for either toxigenic type or presence of toxin was defined as toxigenic; therefore, our method was supposed to be more sensitive than studies that have used only one method for toxin detection. Riggs et al[18] and the Canadian Nosocomial Infection Surveillance Program (CNISP)[19] used only the enzyme immunoassay toxin test, whereas Hung et al's[17] study used only the real-time PCR method. We defined CDI as diarrhea that occurred three or more times in 1 day, with a positive laboratory test for *C. difficile*. These criteria were weaker than those of CNISP, which defined CDI as diarrhea for more than 2 days with a positive laboratory test.[19] Therefore, the low CDI prevalence observed in our study was not attributable to either the study method or our definition of CDI.

Substantial colonization with *C. difficile* strains was found in several hospitals (31% for H1, 26% for H2, 25% for H3, and 24% for H4). These rates are similar to those in hospitals with outbreaks that were reported in previous studies (approximately 20%; Table 1),[18,24] but only patients in H2 progressed to diarrhea. Although colonization is commonly believed to be a transmission source for *C. difficile* and was used to predict the burden of CDI in wards,[25] our result indicates that the prevalence of CDI was not confirmatively related to the number of toxigenic *C. difficile* colonizing isolates.

MLVA typing differentiated CDI-related endemic strains (A and B) from asymptomatic endemic strains (C, D, and E). This observation may be a result of two factors. First, MLVA cluster strains A and B may be more virulent than the other MLVA cluster strains (C, D, and E; Table 2), and may be responsible for the subsequent progression to diarrhea. Second, the RCW residents may have had protective immunity against MLVA cluster strains C, D, and E; thus, these strains could retain asymptomatic colonization, as was observed by Samore et al.[24] Therefore, while evaluating the

burden of CDI, the MLVA types of toxigenic *C. difficile* strains should be considered in addition to recording the total number of colonizing isolates. This deduction has some limitations: the asymptomatic carriers were not traced for CDI development; and the results could partially be a result of errors that occurred during the observation period. These MLVA types of strains should be monitored to confirm our conclusions.

By contrast, single PCR ribotypes (R10 and R106) included the CDI-related MLVA cluster (A and B) and the carriage-related MLVA cluster (E and D; Table 2); this shows that PCR ribotyping was less sensitive for detecting invasive strains than MLVA typing, although the former has been the traditional method for detecting virulent strains.[10]

Patients with ESRD, or those who had been treated with antacids, had higher rates of CDI in our study, and the data were consistent with another study (Table 3).[26] However, the other conventional risk factors for CDI (advanced age and antibiotic use)[27] were not statistically significant (Table 3). The most probable reason for this result is the small number of CDI cases (6 cases). Another possibility is that the results indicate an endemic situation, because no large outbreak was detected in any of the study wards; this situation was also observed by Hensgens et al.[28]

Previous admission to an acute-care facility was a risk factor for *C. difficile* colonization, which is consistent with other studies[24,29] (Table 3). This may reflect increased severity of the underlying disease or be explained by the treatment in acute-care facilities. However, this factor is not found to be significant in Riggs et al's research.[18] The difference may be due to the fact that RCW patients are usually transferred directly from an ICU; therefore, our CDI cases might have had a shorter interval between prior ICU exposure and the admission day than Riggs et al's patients and were more likely to have been influenced by treatment in the ICU. In addition, different acute-care wards may use different medical care strategies. Thus, the risk factors for ICU patients in developing CDI should be studied further.

In other studies, antibiotic exposure was found to be a major risk factor for *C. difficile* colonization.[17,18] In our study, previous antibiotic exposure was found in more colonized patients than in noncolonized patients (68% vs. 54%), but was not statistically significant. Penicillin exposure was significantly related to *C. difficile* colonization by Chi-square test analysis ($p = 0.037$), but not an independent risk factor for *C. difficile* colonization when analyzed by multivariable logistic regression. The results showed that antibiotic exposure would increase the tendency of developing *C. difficile* colonization. The low significance of our statistics may be due to confounding factors such as sample population, sample size, duration of antibiotic treatment, and exposure to multiple antibiotics.[29]

Our survey has some limitations. First, different durations of exposure to antacids were defined as a single factor, which may produce less significant statistics with respect to risk factors. Second, the prevalence study was carried out between June and August in different hospitals, and there was no control for seasonal influences, which might have resulted in measurement bias.

In summary, the baseline characteristics and prevalence for *C. difficile* in multiple RCWs in Taiwan were determined. The results of this study revealed a high prevalence of toxigenic *C. difficile* colonization in hospitals in Taiwan. Several asymptomatic and CDI-related endemic strains that belonged to different MLVA types were circulating in the wards of several hospitals. Infection control personnel should be alert to the increasing number of CDI cases.

Conflicts of interest

All contributing authors declare that they have 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.2013.03.014

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