Title

Molecular epidemiology of *Clostridioides difficile* and risk factors for the detection of toxin gene-positive strains

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Abstract

In this study, we investigated all *Clostridioides difficile* strains isolated from stool samples in Nagasaki University Hospital between January 2012 and December 2014. Toxin genes (*tcdA*, *tcdB* and *cdtA*/*cdtB*) were analyzed for multiplex PCR in a total of 213 strains. In the toxin gene-positive strain, PCR ribotyping was conducted using capillary gel electrophoresis-based PCR and the Webribo database. Patients' backgrounds were analyzed by departments, disorders, antimicrobials, and clinical dates. The positive rates of *tcdA*, *tcdB*, and *cdtA*/*cdtB* genes were 62.9%, 63.4%, and 2.8%, respectively. The most frequent PCR ribotype was 047 (14.1%), followed by 014/0 (11.1%) and 002/0 (8.2%). In univariate analysis, the risk factors for the detection of toxin gene-positive strains in patients were older age ($p = 0.0036$), over ≥ 65 years old ($p = 0.0175$), the patients hospitalized at Department of Digestive Surgery (P = 0.0059), higher CRP level (P = 0.0395), and lower albumin level ($p = 0.0014$). In the multivariate analysis, the risk factor for detection of toxin gene-positive strains was the patients hospitalized at Department of Digestive Surgery (OR; 4.62, 95% CI; 1.18-18.0, $p = 0.0274$). In this study, the percentage of toxin gene-positive and *cdtA*/*cdtB* gene-positive strains was almost the same as that reported in previous studies, but the ribotype was different. In addition, we revealed that the risk factor associated with

the detection of toxin gene-positive strains was the patients hospitalized at Department of digestive surgery.

Keywords: *Clostridioides difficile*; molecular epidemiology; risk factor; toxin gene

Introduction

Clostridioides difficile is a gram-positive anaerobic bacterium and a causative pathogen of antibiotic-associated diarrhea (AAC) [1]. In *C. difficile* infections (CDIs), toxin A and B play important roles [2,3]. Both toxins have cytotoxic activities and induce the release of various immunomodulatory mediators from epithelial cells, phagocytes, and mast cells [2]. Recently, the spread of binary toxin (CDT)-producing strains has raised a major concern in the United States and Europe. CDT-producing strains are highly resistant to fluoroquinolones and increase mortality [4–6]. In European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guideline for the diagnosis of CDI, glutamate dehydrogenase (GDH) screening test, toxin A and toxin B EIA testing, and nucleic acid amplification tests (NAATs) for toxin genes are recommended[7].

Although the sensitivities of NAATs were higher than that of the EIA test, it was difficult to conduct NAATs on all specimens in routine examinations, because of the high cost and labor involved. The first European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guideline recommend a two-step algorithm: first, samples should be analyzed by EIA testing; if the result is positive for GDH and negative for toxin A/B, samples are then analyzed by NAATs[7]. Recently, the fully

automated molecular test (FAMT) has been developed, making it easier to perform NAATs [8,9] and allowing for the detection of CDT. Many hospitals are expected to implement the NAATs including FAMT, but there have been few molecular epidemiological studies of *C. difficile* and CDT in Japan[10–12].

In this study, we investigated the molecular epidemiology of *C. difficile*. Additionally, to clarify the patients who were recommended for performance of the NAATs, we investigated and compared the patients' backgrounds between toxin-positive and -negative *C. difficile* to clarify the risk factors for CDI.

Materials and Methods

Study design and sample collection

This study was conducted between January 2012 and December 2014. This study was approved by the ethics committee of Nagasaki University Hospital (13062425). We collected *C. difficile* isolated from stool samples between January 2012 and December 2014 in Nagasaki University Hospital. To avoid duplicates, only the first isolate from each patient was collected.

The patient background information, such as age, gender, length of hospitalization, underlying diseases, history of surgery within 90 days before submission of stool sample, length of hospitalization before submission of stool sample, oral and intravenous administration of antimicrobial agents, use of proton pump inhibitors (PPIs), and use of histamine-2 receptor antagonists (H2 blockers) within 60 days before submission of stool sample, CDI symptoms associated with CDI, and mortality rate were analyzed. We defined administration of metronidazole and oral vancomycin after submission of stool sample as CDI treatment. Because we did not evaluate the Bristol scale in the laboratory during the study period, we investigated the development of diarrhea, based on medical record. We also investigated the laboratory data for white blood cell count ($\times 10^3/\mu l$), red blood cell count ($\times 10^6/\mu l$),

C-reactive protein (CRP) (mg/dl), blood urea nitrogen (BUN) (mg/dl), serum creatinine (mg/dl), uric acid (UA) (mg/dl), total protein (TP) (g/dl), albumin (g/dl), and lactate dehydrogenase (LD) (U/l) at one day before and after submission of the stool sample.

Genetic analysis

In this study, we used the ATCC9689 strain as a positive control. We inoculated all *C. difficile* strains with cycloserine-cefoxitin mannitol agar (CCMA; Nissui Pharmaceutical Co., Ltd., Japan). The plates were incubated at 37°C for 48–72 h under anaerobic conditions. Bacterial DNA was extracted by using Achromopeptidase solution (Wako Chemical Co., Ltd., Osaka, Japan), 10% Chelex-100 (Bio-Rad Laboratories, Hercules, CA, USA), and the boiling method [13]. We confirmed the genes for toxins (*tcdA* and *tcdB*), CDT (*cdtA* and *cdtB*), and 16S rRNA by PCR as previously described [14]. The 16S rRNA primers were as follows: forward 8UA, 5'-AGAGTTTGATCMTGGCTCAG-3'; and reverse 1458B, 5'-TACGGTTACCTTGTTACGAC-3' [15].

PCR ribotyping was evaluated by capillary gel electrophoresis-based PCR, which was performed according to the protocol of a previous study [16]. In this method, we

111 used the Applied Biosystems 3130 genetic analyzer with POP7 polymer (Applied
112 Biosystems, Foster City, CA, USA) and GeneScan-1200Liz size standard (Applied
113 Biosystems). The size of each peak was determined using Peak Scanner software v1.0
114 (Life Technologies, Carlsbad, CA), and PCR ribotypes were determined by the
115 Webribo database of the Austrian Agency for Health and Food Safety
116 (https://webribo.ages.at).

117 We defined a following situation as outbreak, based on the notification from
118 Regional Medical Care Planning Devision, Healthy Policy Bureau, Ministry of Health,
119 Labor and Welfare, Japan (December 19, 2014): same PCR ribotype strain was
120 isolated from three or more patients hospitalized in same ward within 4 weeks.

121

122 *Statistical analysis*

123 The statistical software used in this study was JMP Pro 10.0.2 (SAS Institute Inc,
124 Cary NC, USA). Continuous variables were compared using the Student's *t*-test. The
125 chi-square was used to compare categorical variables. Variables with a *P* value less
126 than 0.20 according to the univariate analysis were considered for inclusion in the
127 forward stepwise multivariate logistic regression analysis.

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Results

Genetic analysis

C. difficile strains were isolated from a total of 213 (122 men and 91 women) patients during the study period (Fig. 1). The positive rates of *tcdA*, *tcdB*, and CDT genes were 62.9% (n = 134), 63.4% (n = 135), and 2.8% (n = 6), respectively. We defined strains harboring the *tcdA* and/or *tcdB* genes as toxin gene-positive strains. Among the toxin gene-positive strains, the positive rate of CDT gene was 4.4%.

PCR ribotyping was performed on toxin gene-positive strains (Table 1). The most frequent PCR ribotype was the 047 strain (14.1%), followed by 014/0 (11.1%), 002/0 (8.2%), 020 (6.7%), and 018 (4.4%). The PCR ribotypes of CDT-positive strains were 016 (two strains), 131 (three strains), and 413 (one strain). There was no outbreak of each PCR ribotype in this study.

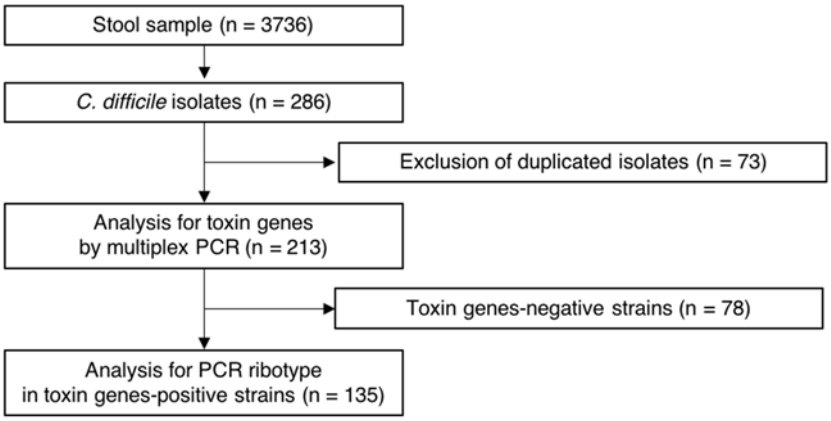

Fig 1. Analysis of *C. difficile* isolates
C. difficile isolated from stool sample of 213 patients were analyzed for toxin genes by multiplex PCR. Toxin genes-positive strains were analyzed for PCR ribotype (n = 135).

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Table1 PCR ribotype of toxin genes-positive strains

Ribotype	(N)	(%)
047	19	14.1
014/ 0	15	11.1
002/ 0	11	8.1
020	9	6.7
018	6	4.4
043	5	3.7
449	5	3.7
076	4	3.0
209	4	3.0
001	3	2.2
012	3	2.2
056	3	2.2
153	3	2.2
638	3	2.2
015	2	1.5
046	2	1.5
131	2	1.5
404	2	1.5
496	2	1.5
541	2	1.5
660	2	1.5
014/ 5	2	1.5

Other	26	19.3

Other: less than 1%

Comparison of toxin gens-positive and -negative strains

The patient backgrounds with toxin gene-positive and -negative strains are shown in Table 2. The average age of the patients was significantly higher in patients with toxin gene-positive strains (54.2 years) than in those with toxin gene-negative strains (42.4) (p = 0.0034). There were significant differences between the two groups in terms of the inpatient department. The percentage of patients with toxin gene-positive strains who were hospitalized at the Department of Pediatrics was significantly lower in patients with toxin-positive gene (25 patients, 15.6%) than in those with toxin gene-negative strains (25 patients, 32.1%) (p = 0.0055). In contrast, the percentage of patients with toxin gene-positive strains who were hospitalized at the Department of Digestive Surgery was significantly higher than in patients with toxin-positive gene strains (27 patients, 20.0%) than in those with toxin gene-negative strains (four patients, 5.2%) (p = 0.0015). When analyzing the laboratory data, the serum albumin levels in patients with toxin gene-positive strains was significantly lower (3.0 ± 0.7 g/dl) than in those with toxin gene-negative strains (3.3 ± 0.7 g/dl) (p = 0.0017).

Administration of antimicrobials, PPIs, and H2 blockers and antimicrobial agents is

160 summarized in Table 3. The PPIs, H_2 blockers, and antimicrobial agents were
161 administered to 84 patients (39.4%), 15 patients (7.0%), and 157 patients (70.5%),
162 respectively. The percentage of those administered with macrolides was significantly
163 higher in the patients with toxin gene-positive strains (seven patients, 5.2%) than in
164 those with gene-negative strains (0 patients, 0.0%) ($p = 0.0107$).

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Table2 Comparison of the patient background between the patients toxin genes-positive and negative strains

Background	Total (n = 213)	Patients with toxin genes-positive strain (n = 135)	Patients with toxin genes-negative strain (n = 78)	*P* value
Age	49.9 ± 28.4	54.2 ± 26.5	42.4 ± 29.9	0.0034
< 15 years old	49 (23.0 %)	23 (17.0 %)	26 (33.3 %)	0.0072
≥ 65 years old	91 (42.7 %)	66 (48.9 %)	25 (32.0 %)	0.0159
Sex (male %)	122 (57.3 %)	80 (59.3 %)	42 (53.8 %)	0.4422
length of hospitalization (day)	32.3 ± 55.8	33.2 ± 60.2	30.6 ± 47.2	0.9466
diarrhea (%)	128 (60.1 %)	83 (61.5 %)	45 (57.7 %)	0.5869
abdominal tenderness	59 (27.7 %)	39 (28.9 %)	20 (25.6 %)	0.6086
temperature (≥ 38.5 ° C)	49 (23.0 %)	29 (21.5 %)	20 (25.6 %)	0.4893
previous surgery within 90 days	36 (16.9 %)	25 (18.6 %)	11 (14.1 %)	0.4023
mortality within 90 days	24 (11.3 %)	15 (11.1 %)	9 (11.5 %)	0.9244
Treatment with CDI (vancomycin or metronidazole)	44 (20.7 %)	33 (24.4 %)	11 (14.1%)	0.0663
Department				
Hematology	47 (22.1 %)	30 (22.2 %)	17 (21.8 %)	0.9422
Pediatrics	46 (21.6 %)	21 (15.6 %)	25 (32.1 %)	0.0055
Gastroenterology	44 (20.7 %)	28 (20.7 %)	16 (20.5 %)	0.9684
Digestive Surgery	31 (14.6 %)	27 (20.0 %)	4 (5.2 %)	0.0015

Respiratory medicine	11 (5.2 %)	9 (6.7 %)	2 (2.6 %)	0.1700
Anesthesiology	7 (3.3 %)	4 (3.0 %)	3 (3.9 %)	0.7302
Disorder				
Hematology	54 (25.4 %)	33 (24.4 %)	21 (26.9 %)	0.6895
Liver	41 (19.2 %)	26 (19.2 %)	15 (19.2 %)	0.9959
Kidney	12 (5.6 %)	7 (5.2 %)	5 (6.4 %)	0.7110
Intestinal diseases	27 (12.7 %)	21 (15.7 %)	6 (7.7 %)	0.0858
cardiac	12 (5.6 %)	9 (6.7 %)	3 (3.9 %)	0.3767
Laboratory data				
White Blood Cell ($\times 10^3$/ μl)	7.5 ± 6.6	7.4 ± 6.2	7.7 ± 7.4	0.9546
Red Blood Cell ($\times 10^6$/μl)	3.4 ± 0.8	3.4 ± 0.7	3.5 ± 0.8	0.3977
C-reactive protein (mg/dl)	5.1 ± 6.1	5.8 ± 6.5	3.9 ± 5.1	0.0565
Blood urea nitrogen (mg/dl)	19.1 ± 15.0	19.8 ± 16.4	17.8 ± 11.9	0.5033
Serum creatinine (mg/dl)	0.9 ± 1.1	1.0 ± 1.0	0.9 ± 1.3	0.0659
Uric acid (mg/dl)	4.5 ± 2.3	4.8 ± 2.6	4.2 ± 1.7	0.6126
Total protein (g/dl)	6.2 ± 1.0	6.2 ± 1.0	6.4 ± 0.9	0.2012
Albumin (g/dl)	3.1 ± 0.7	3.0 ± 0.7	3.3 ± 0.7	0.0017
Lactate dehydrogenase (U/l)	246.9 ± 160.6	244.0 ± 179.9	252.5 ± 116.2	0.1717

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Table3 Comparison of administration of PPI, H2 blocker and antimicrobial agents between the patients with toxin genes-positive and negative strains

Variable	Total (n = 213)	Patients with toxin genes-positive strain (n = 135)	Patients with toxin genes-negative strain (n = 78)	*P* value
PPIs	84 (39.4 %)	54 (40.0 %)	30 (38.5 %)	0.8247
H2 blocker	15 (7.0 %)	9 (6.7 %)	6 (7.7 %)	0.7793
Administration of antimicrobial agents within 60 days	157 (73.7 %)	102 (75.6 %)	55 (70.5 %)	0.4288
Penicillin	49 (23.0 %)	31 (23.0 %)	18 (23.1 %)	0.9848
Cephalosporin	81 (38.0 %)	51 (37.8 %)	30 (38.5 %)	0.9211
1st generation Cephalosporin	17 (8.0 %)	12 (8.9 %)	5 (6.4 %)	0.5139
2nd generation Cephalosporin	7 (3.3 %)	3 (2.2 %)	4 (5.1 %)	0.2625
3rd generation Cephalosporin	15 (7.0 %)	12 (8.9 %)	3 (3.8 %)	0.1478
4th generation Cephalosporin	48 (22.5 %)	28 (20.7 %)	20 (25.6 %)	0.4124

Carbapenems	64 (30.0 %)	40 (29.6 %)	24 (30.8 %)	0.8614
Aminoglycoside	7 (3.3 %)	4 (3.0 %)	3 (3.8 %)	0.7302
Fluoroquinolones	30 (14.1 %)	20 (14.8 %)	10 (12.8 %)	0.6852
Macrolides	7 (3.3 %)	7 (5.2 %)	0 (0.0 %)	0.0107

PPI, proton pump inhibitors

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168 *Risk factor for detection of toxin gene-positive strains*

169 We compared the background of the patients with toxin gene-positive and -negative
170 strains by univariate and multivariate analyses to clarify the risk factors for the
171 detection of toxin gene-positive strains (Table 4). In the univariate analysis, the risk
172 factors for the detection of toxin genes positive-strain were older age (OR, 0.99; 95%
173 CI, 0.98–1.00; p = 0.0036), over ≥ 65 years old (OR, 2.03; 95% CI, 1.13–3.63; p =
174 0.0175), patients hospitalized at the Department of Digestive Surgery (OR, 4.63; 95%
175 CI, 1.55–13.8; p = 0.0059), higher CRP level (OR, 0.94; 95% CI, 0.89–1.00; p =
176 0.0395), and lower albumin level (OR, 2.02; 95% CI, 1.29–3.14; P = 0.0014). In the
177 multivariate analysis, the risk factors for the detection of toxin gene-positive strains
178 were patients hospitalized at Department of Digestive Surgery (OR, 4.62; 95% CI,
179 1.18–18.0; p = 0.0274).

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Table4 Univariate and multivariate analysis of the risk factor for the detection of toxin genes-positive strains

Risk factor	Univariate analysis			Multivariate analysis		
	Odds ratio	95 % CI	*P* value	Odds ratio	95 % CI	*P* value
Age	0.99	0.98-1.00	0.0036	1.01	0.96-1.06	0.7574
< 15 years old	0.41	0.21-0.79	0.0073	-	-	-
≥ 65 years old	2.03	1.13-3.63	0.0175	1.67	0.44-6.37	0.4495
Department of Pediatrics	0.39	0.20-0.76	0.0056	-	-	-
Department of Digestive Surgery	4.63	1.55-13.8	0.0059	4.62	1.18-18.0	0.0274
Intestinal diseases	2.21	0.85-5.74	0.1032	1.28	0.38-4.28	0.6915
C-reaction protein (mg/dl)	0.94	0.89-1.00	0.0395	0.97	0.91-1.04	0.3974
Albumin (g/dl)	2.02	1.29-3.14	0.0014	1.51	0.80-2.84	0.2010
Administration of 3rd generation Cephalosporin within 60 days	2.44	0.67-8.93	0.1478	7.22	0.85-61.0	0.0694

Multivariate analysis; Predictors with a *P* value < 0.20 in the univariate analysis were included in forward stepwise multivariate logistic regression analysis.

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183 Characteristics of the patients with CDT gene-positive strains

184 CDT gene-positive strains were cultured from 6 patients (3 men and 3 women). The
185 age range was 5–84 years, and the patients under 18 years included 2 patients. All
186 patients had diarrhea, 3 had temperatures of over 38.5°C, and the white blood cell
187 count was high only in one case (29,700/μl). There were no severe cases, such as
188 pseudomembranous enteritis, in the 6 patients. Treatment with CDI (vancomycin or
189 metronidazole) was administered only in 3 cases. All cases were cured from CDI.

Discussion

This study revealed the molecular epidemiology of *C. difficile* and risk factors for the detection of toxin gene-positive strains. In a previous Japanese study, the percentage of toxin gene-positive strains and CDT gene-positive strains was 73% and 2%, respectively [10]. In other Japanese study, CDT-gene positive strains was 5% [17]. In comparison with the previous studies, the percentage of toxin gene-positive strains that was lower than that of CDT gene-positive strains was nearly the same in this study. The lower percentage of toxin gene-positive strain was caused by a difference in collection of samples. The previous report investigated suspected CDI, but in this study, we investigated all strains cultured from stool samples. Therefore, our study involved the patients with colonization of *C. difficile*. The percentage of CDT gene-positive strain in Japan seemed to be lower than that in other countries: the percentage of CDT gene-positive strain in Colombia was 17.5% [18]; in United Kingdom and Finland, the percentage of CDT gene-positive strains was 16.9% and 37%, respectively. [19,20]. Additionally, in this study, we did not detect NAP1/027 and 078 strains, which were reported as the causative pathogens of serious and epidemic CDI in Europe and United States [21]. The PCR ribotype of CDT gene-positive strains in this study was 016, 131, and 413.

In this study, we clarified the PCR ribotype in all toxin gene-positive strains. The most frequent ribotypes as revealed by PCR for toxin gene-positive strains were 047, 014/0, and 020 in this study. In a previous Japanese study, the ribotypes present at a high frequency were strains 018, 002, 052, and 369 [22]. In other Japanese study, the ribotypes present at a high frequency was strains 449 [17]. The frequent PCR ribotypes varied by the studies in Japan [17, 22]. In the United States, the most frequent PCR ribotypes were strains 002, 014-020, 027, and 053-163 [23], but the most frequent PCR ribotypes were different according to area. Other reports from the United States revealed that the most frequent PCR ribotypes in the northeast, southern, midwestern, and western United States were 027, 014/020, 106, 001, 053, respectively [24]. We hypothesized that ribotypes differed from area to area, and that it is important to conduct surveillance in each area. Additionally, 42% of the PCR ribotype strain 047 was detected from the patients at Department of Digestive Surgery in this study, but we did not observe outbreak of the strain. This result was indicating that epidemic ribotypes differed from ward to ward.

We investigated the risk factors for the detection of toxin-positive strains by comparing the characteristics of patients with toxin gene-positive and negative strains. In the univariate analysis, there was no significant difference in the administration

226 history of the antimicrobial drugs or the type of antibiotics between the patients with toxin
227 gene-positive and -negative strains. Since the antimicrobial drug administration history
228 was observed at almost the same ratio in both groups, administration of antimicrobial
229 drugs may lead to the colonization of *C. difficile*. In the analysis of laboratory data, the
230 patients with toxin gene-positive strains showed higher CRP and lower albumin levels.
231 However, there were no significant differences in the multivariate analysis. It seems to be
232 difficult to discriminate between the presence or absence of toxin gene-positive strains by
233 using laboratory data. In the multivariate analysis, the risk factors for the detection of
234 toxin gene-positive strains included hospitalized patients at the Department of
235 Digestive Surgery. On the other hand, if *C. difficile* was detected in children, the
236 possibility that they were also positive for toxin genes was relatively low in this study.
237 A previous report revealed that CDI was more frequently caused in solid-organ
238 transplant recipients than in other hospitalized patients, because solid-organ transplant
239 recipients had a several CDI risk factors, such as PPIs, H_2 blockers, age greater than 65
240 years old, severe underlying disease, uremia, gastrointestinal surgery, presence of
241 nasogastric or endotracheal tube, and prolonged hospitalization [25]. The Department
242 of Digestive Surgery in our hospital conducts solid-organ transplants, and it seemed
243 to have had an influence on the higher percentage of toxin gene-positive strains in the

Department of Digestive Surgery.

There were some limitations in this study. First, we studied only *C. difficile* strains cultured from stool samples, and CDIs diagnosed by only enzyme immunoassays were not included in this study. Second, we could not investigate the Bristol scale. If we could investigate the Bristol scale, we would evaluate the association between diarrhea condition and harboring toxin genes including Bristol scale. Third, we did not determine the PCR ribotype of toxin gene-negative strains. The PCR ribotype of the toxin gene-negative strains remained unknown. It is necessary to understand the tendency to colonized *C. difficile* with toxin negative strains. Therefore, further investigations are needed.

Conclusion

In this study, the percentages of toxin gene-positive and CDT gene-positive strains were almost the same as those reported in previous studies, but the ribotypes differed. In addition, we revealed that the risk factors associated with the detection of toxin gene-positive strains included patients hospitalized at the Department of Digestive Surgery.

Conflict of interest

The authors declare no conflicts of interest.

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Figure legend

Fig 1. Analysis of *C. difficile* isolates

C. difficile isolated from stool sample of 213 patients were analyzed for toxin genes by multiplex PCR.

Toxin genes-positive strains were analyzed for PCR ribotype (n = 135).