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ORIGINAL ARTICLE

A retrospective study of the epidemiology of *Clostridium difficile* infection at a University Hospital in Japan: genotypic features of the isolates and clinical characteristics of the patients

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Abstract *Clostridium difficile* is a major cause of antibiotic-associated diarrhea and frequently results in healthcare-associated infections. The epidemiology of *C. difficile* infection (CDI), including the prevalent polymerase chain reaction (PCR) ribotypes and the clinical characteristics of the patients, is not well known in Japan, compared to the situation in the United States and Europe. We performed PCR ribotyping of *C. difficile* isolates from 71 consecutive patients with CDI at a University Hospital over a 3-year period and investigated the clinical features of those patients. CDI was diagnosed when a patient with diarrhea or colitis was found to have toxin B-positive *C. difficile* with no other enteropathogenic microorganisms. Toxin A-positive, toxin B-positive, binary toxin-positive ($A^+B^+CDT^+$) strains; toxin A-positive, toxin B-positive, binary toxin-negative ($A^+B^+CDT^-$) strains; and toxin A-negative, toxin B-positive, binary toxin-negative ($A^-B^+CDT^-$) strains were isolated from 4, 58, and 9 patients, respectively, indicating that infections with binary toxin-positive strains were uncommon (5.6%). PCR ribotyping of the isolates demonstrated that among the 71 strains, 20 different PCR ribotypes were identified and that types smz, yok, and hr were predominant (19, 14, and 13 isolates, respectively), all of which were $A^+B^+CDT^-$. No specific time periods or wards were found to be associated with the three types; PCR ribotyping analysis clearly showed that the three types spread almost evenly in all wards for the 3 years studied. Comparative analysis of the clinical characteristics of patients harboring the three *C. difficile* types indicated that the duration of CDI was longer in the yok group than in the hr group. PCR ribotyping, which is easy to perform, appears to give us useful information to trace CDI cases in clinical settings. Further, the analysis of a large number of CDI cases may allow evaluation of the possible relationship between specific *C. difficile* types and the clinical features of patients.

Keywords *Clostridium difficile* · PCR ribotyping · Binary toxin

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Introduction

Clostridium difficile is a major cause of antibiotic-associated diarrhea (AAD) and colitis, and the clinical characteristics of *C. difficile* infection (CDI) range from mild diarrhea to severe diseases including pseudomembranous colitis and toxic megacolon. *C. difficile* is involved in 15–25% of AAD and 100% of pseudomembranous colitis cases [1, 2]. Typing technology has been employed to investigate the prevalence of particular types of *C. difficile* and the relationship between types and enteropathogenicity. Binary toxin-producing strains including the PCR ribotype 027 (BI/NAP1/027) and 078 strains have been reported to cause outbreaks and severe CDI [3–7]. In

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Japan, the PCR ribotype smz has been documented to cause healthcare associated infections in several hospitals [8, 9]. Furthermore, the emergence of CDI caused by A^-B^+ strains has been reported [10–12]. Because the epidemiology of CDI is known to vary from region to region, it is crucial to appreciate the incidence of CDI in an individual region or healthcare institute. The aims of this study were to investigate the prevalent PCR ribotype(s) in our hospital and to examine the clinical characteristics of patients infected with each type.

Subjects and methods

The subjects were patients whose stools were found to be positive for *C. difficile* culture, between April 2005 and March 2008, at Nagoya City University Hospital, which is a teaching hospital with 800 beds spread across 24 wards. CDI was diagnosed if a patient showing the symptoms of diarrhea or colitis was found to have toxin B-positive *C. difficile* with no other enteropathogenic microorganisms. The clinical characteristics, outcomes, and clinical laboratory data of the patients, and the details of the antimicrobial agents administered were retrospectively examined.

Stool specimens were treated with alcohol for spore selection, before being cultured anaerobically on cycloserine–cefoxitin–mannitol agar (Nissui Pharmaceutical, Tokyo, Japan) for 48 h. The identification of *C. difficile* was carried out as described previously [13]. The toxin producibility of *C. difficile* isolates was determined by a PCR technique as follows: if the repeating sequences of the toxin A gene were 1,266 bp in size, it was determined that toxin A was produced [13, 14]; if the nonrepeating sequences of the toxin B gene with the expected size (204 bp) were detected, it was determined that toxin B was generated [13, 14]; and if PCR yielded part of the gene encoding component B (510 bp), it was determined that binary toxin was produced [15]. PCR ribotyping of the isolates was performed according to the method of Stubbs et al. [16].

Recurrence of CDI was defined as the patient suffering from CDI again within 2 months after recovery from the previous CDI episode.

The χ^2 test and Fisher's exact test were used for comparison of categorical data. Differences in continuous variables were tested using the Kruskal–Wallis test and Mann–Whitney *U*-test with Bonferroni correction. A *P* value of <0.05 was considered statistically significant.

Results

During the 3 years of the study, the stool specimens of 610 patients were submitted to a *C. difficile* culture test, and *C. difficile* was isolated from 106 patients. Of these 106 patients, 35 were excluded from further studies because 21 were determined to be asymptomatic carriers of the organism, based on their clinical characteristics, and 14 had only nontoxigenic strains. Thus, 71 patients were subjected to further evaluation; 70 were inpatients and 1 was an outpatient. Although 9 of the 71 patients developed recurrence of CDI, their clinical characteristics in the first episode were adopted for the analysis (Table 1).

As a result of the toxigenic analysis of 71 isolates from the 71 patients, toxin A-positive, toxin B-positive, binary toxin-positive ($A^+B^+CDT^+$) isolates; toxin A-positive, toxin B-positive, binary toxin-negative ($A^+B^+CDT^-$) isolates; and toxin A-negative, toxin B-positive, binary toxin-negative ($A^-B^+CDT^-$) isolates were recovered from 4, 58, and 9 patients, respectively. None of the four patients with the $A^+B^+CDT^+$ strains had severe CDI.

The results of PCR ribotyping of the 71 isolates were as follows: of the 4 $A^+B^+CDT^+$ isolates, 2, 1, and 1 were PCR ribotypes j52, nc07109, and km0403, respectively; of the 58 $A^+B^+CDT^-$ isolates, 19, 14, and 13 were PCR ribotypes smz, yok, and hr, respectively, and each of the remaining 12 $A^+B^+CDT^-$ isolates were identified as different PCR ribotypes; and of the 9 $A^-B^+CDT^-$ isolates, 6, 2, and 1 were PCR ribotypes trf, fr, and sgf, respectively. An epidemiological study of the 46 patients harboring the 3 predominant PCR ribotypes (smz, yok, and hr) isolates with $A^+B^+CDT^-$ showed that patients with *C. difficile* isolates of these 3 PCR ribotypes were hospitalized in 18 wards. Only one PCR ribotype was found in eight wards, while two PCR ribotypes and three PCR ribotypes were detected in eight and two wards, respectively. There was more than one patient infected with the same PCR ribotype isolate in a few wards (smz isolates in 4 wards, yok isolates in 2 wards, and hr isolates in 2 wards). There were two wards where two patients were infected with the smz isolate at the same time. Similarly, two patients were infected with the yok isolate in two wards at the same time. Regarding the hr isolate, two patients were infected in one ward at the same time.

The prevalence of all PCR ribotype isolates detected between April 2005 and March 2008 in our hospital is illustrated in Fig. 1. In the 3 years examined, the number of CDI cases was less than five per month, and none of wards or departments were associated with a particular incidence of CDI. Thus, all CDIs were considered to be sporadic. Further, none of the PCR ribotypes were predominant with a significant number occurring at a particular time period.

Table 1 presents the clinical characteristics of all 71 patients with CDI and the 58 patients infected with $A^+B^+CDT^-$ CDI strains. There were no significant differences in the number of antimicrobials administered, the duration of administration before the onset of CDI, clinical

Table 1 Demographics and clinical characteristics of all patients with *Clostridium difficile* infection and patients infected with *C. difficile* $A^+B^+CDT^-$ strains

Characteristic	All patients	Patients infected with $A^+B^+CDT^-$ strains of			
		PCR ribotype smz	PCR ribotype yok	PCR ribotype hr	Others
No. of patients	71	19	14	13	12
Age in years (mean ± SD)	67.4 ± 16.6	67.4 ± 19.2	77.4 ± 6.26	64.2 ± 18.6	60.1 ± 17.4
Sex (male: female)	36: 35	8: 11	5: 9	8: 5	8: 4
Antimicrobials before the onset of CDI					
No. of patients who received antimicrobials	69	19	13	13	11
Median number of antimicrobials administered	2 (1–7)	2 (1–6)	2 (1–7)	2 (1–6)	2 (1–5)
Median days of administration	11 (1–35)	11 (1–32)	12 (3–32)	10 (1–35)	7 (1–31)
Median days from the start of antimicrobials to the onset of CDI	16 (1–63)	13 (5–53)	29 (3–57)	21 (1–63)	11 (1–60)
Risk factors for the onset of CDI					
No. of patients who received H_2RA or PPI	52	12	12	11	9
No. of patients who received anticancer drugs	8	0	2	5	0
No. of patients who received steroids	11	1	3	2	3
No. of patients who received tube feeding	11	3	1	2	1
No. of patients in whom oral intake was suspended	14	5	2	3	3
Vancomycin					
No. of patients who received vancomycin	47	18	10	6[a]	5
Days administered	7 (1–28)	7 (1–19)	7 (3–14)	7.5 (5–16)	7 (3–14)
Total dose (g)	9.5 (2–56)	8.75 (2–38)	6.0 (3.5–24)	12.0 (4–24)	10.0 (6–19)
Clinical findings of patients with CDI					
Duration of intestinal symptoms (days)	7 (2–25)	7 (2–19)	11 (3–25)	6 (2–19)[b]	8.5 (2–15)
Maximum frequency of diarrhea (no. of episodes per day)	6 (1–16)	6 (1–14)	6 (4–16)	6.5 (1–13)	7 (1–11)
Peak WBC count (/μL)	8,600 (1,800–29,700)	10,400 (4,400–16,000)	8,050 (2,800–17,500)	6,350 (1,800–29,700)	8,250 (6,400–18,600)
Peak CRP (mg/dl)	5.63 (0.05–37.39)	4.88 (0.22–22.07)	7.28 (0.42–37.39)	4.36 (1.11–23.97)	7.02 (0.09–26.59)
Maximum body temperature (°C)	38.0 (36.4–40.6)	38.1 (36.8–39.0)	37.9 (36.9–40.6)	38.0 (36.8–38.8)	38.2 (36.4–39.8)
No. of patients who suffered CDI recurrence	9	5	2	1	0
No. of patients who received antimicrobials before CDI recurrence	3	1	1	1	0
CDI-related mortality (no. of cases)	2	1	0	0	1

Figures in parentheses are ranges

CDI, *Clostridium difficile* infection; *CRP*, C-reactive protein; *H_2RA*, H_2 receptor antagonist; *PPI*, proton pump inhibitor, *WBC*, white blood cell

[a] hr versus smz $p < 0.05$

[b] yok versus hr $p < 0.05$

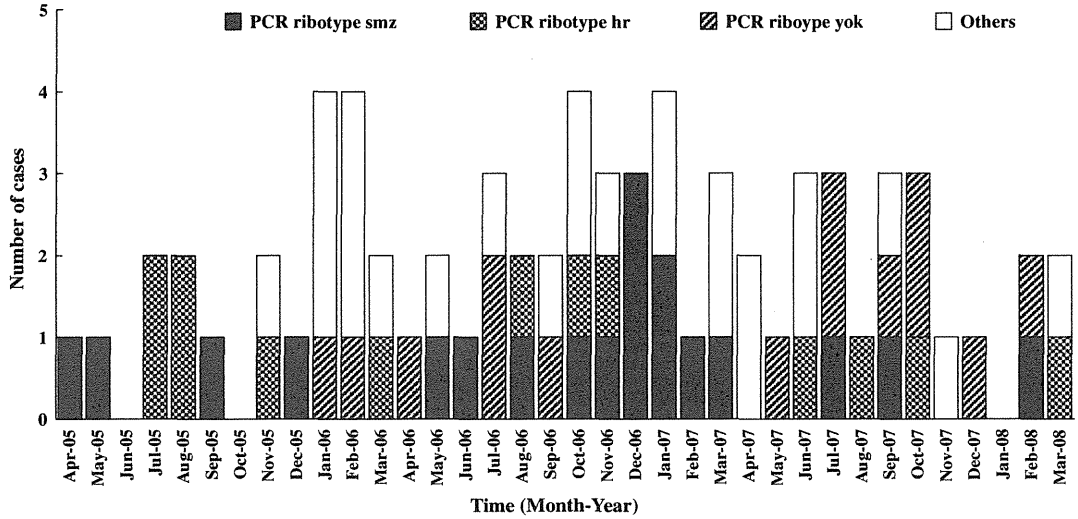

Fig. 1 Numbers of patients with *Clostridium difficile* infection and the distribution of polymerase chain reaction (PCR) ribotypes, with numbers of cases shown in *parentheses*: PCR ribotypes *smz* (19), *yok* (14), *hr* (13), and *others; others* includes PCR ribotype fr (2), gc0578 (1), j52 (2), km0403 (1), nc0803 (2), nc0910 (1), nc0915 (1), nc0923 (1), nc0930 (1), nc0934 (1), nc0938 (1), nc07109 (1), nc0 08162 (1), nc08176 (1), og39 (1), sgf (1), and trf (6)

laboratory data, or clinical symptoms between the three PCR ribotype groups (smz, yok, and hr groups). However, the duration of CDI was longer in the yok group than in the hr group ($p < 0.05$). The number of patients in the smz group treated with vancomycin was higher than that in the hr group ($p < 0.05$). Five patients in the smz group developed CDI recurrence; four of these five patients developed CDI in spite of the absence of antimicrobial readministration after the first episode of CDI. In the yok group, two patients developed CDI recurrence; one did so after reexposure to an antimicrobial, and the other did so during the administration of an anticancer drug. In the hr group, only one patient suffered CDI recurrence, after the readministration of an antimicrobial.

Discussion

Toxigenic *C. difficile* strains produce toxin A, toxin B, and/or binary toxin. Toxin A^-B^+ strains can cause gastrointestinal infection, leading to outbreaks of severe CDI, such as those caused by toxin A^+B^+ strains [10–12]. It was reported that there were no significant differences in the clinical characteristics of patients infected with A^+B^+ *C. difficile* and those infected with A^-B^+ *C. difficile* [10]. That study also demonstrated that there were no significant differences in clinical symptoms or laboratory data between patients with $A^+B^+CDT^-$ CDI and those with $A^-B^+CDT^-$ CDI [10]. In the present study, A^-B^+ strains were isolated from 12.7% (9 cases) of patients with CDI. However, nosocomial spread can easily change the incidence rates of A^-B^+ strains. In fact, nosocomial spread of these strains cannot be ruled out in the present study also, because six strains were the same PCR ribotype. A similar situation seemed to exist in the previous study [10].

Binary toxin-positive strains have been isolated from 6 to 11% of patients with CDI [15, 17–19]. In Europe and North America, where PCR ribotype 027 (BI/NAP1/027) is endemic, the incidence of CDI with binary toxin-positive *C. difficile* appears to be increasing [6], whereas the incidence of CDI with a binary toxin-positive strain has not been so high in Japan [17]. The incidence of CDIs with binary toxin-positive strains was 5.6% in the present study. In the present investigation, four $A^+B^+CDT^+$ *C. difficile* strains were isolated from patients with nonsevere CDI. This result may have been due to the fact that PCR ribotypes 027 and 078, which are hypervirulent strains, were not isolated from patients in this study. Further studies are necessary to investigate the pathogenicity of binary toxin-positive *C. difficile* including ribotypes 027 and 078.

Molecular biological typing techniques have been utilized for epidemiological studies as well as for investigations of the relationship between the molecular type of specific strains and their pathogenicity.

The PCR ribotyping analysis of the 71 *C. difficile* strains in the present study revealed that no specific PCR ribotype was spreading, but that three dominant types, smz, yok, and hr, were almost constantly predominant in the wards of our hospital over the 3-year period. The changing predominance of specific *C. difficile* types over time has been

reported in hospitals [9, 20]. Thus, it is interesting that there was no persistent changing of predominant types with time over the 3-year period in our hospital.

C. difficile strains of PCR ribotype smz have been reported to be highly prevalent in hospitals in Japan, with occasional outbreaks [8, 9]. However, the incidence of this type, smz, in countries other than Japan remains unknown.

PCR ribotype hr is equivalent to PCR ribotype 014 reported by Stubbs et al. [16], which is the dominant PCR ribotype in France, Hungary, The Netherlands, Switzerland, and the United Kingdom [21]. PCR ribotype yok is equivalent to PCR ribotype 002 reported by Stubbs et al. [16] and is highly prevalent in France, Italy, and Switzerland [21]. The incidence of PCR ribotypes hr and yok in our hospital was similar to that in those countries. Because there have been no reports regarding the specific clinical characteristics associated with an individual PCR ribotype, we attempted to elucidate this issue. We found that PCR ribotypes smz, yok, and hr did not show any clinical characteristics specific to each type. However, the durations of CDI varied between the PCR ribotype yok and the PCR ribotype hr. This result suggests that if a large scale study is conducted, differences in clinical characteristics may be found between individual PCR ribotypes. Future study of this issue will surely be worth conducting.

Furthermore, the present study proved that PCR ribotyping analysis was useful for evaluating the spread of particular *C. difficile* strains among wards and for widely implementing infection control in the whole hospital.

So far, few epidemiological studies of CDI have been reported in Japan. In North America and Europe, the dominant PCR ribotype has changed to type 027, and a similar change could occur in Japan in the future. PCR ribotyping is an easy-to-use analytical tool. Thus, we believe that the monitoring of predominant *C. difficile* strains using PCR ribotyping is valuable for conducting appropriate CDI control in hospitals.

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Laboratory Practice

微生物

Clostridium difficile 国内外の優勢株・流行株について

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Clostridium difficile 感染症とタイピング法について

C. difficile 感染症（*C. difficile* infections，CDI）は，医療関連感染の一つとしてよく知られているが，最近は，市中感染としても注目されている．さらに，ウシやブタにおける *C. difficile* 感染[1]や，食品における汚染などにも大きな関心が寄せられている[2]．CDI は医療関連感染として重要であるため，感染源や感染経路の調査目的にさまざまなタイピング法が開発・応用されてきた．タイピング解析を，医療施設内での菌株間の比較だけでなく，施設や地域を超えて分離された菌株の比較検討に応用すると，特定の菌株が複数の医療施設において流行株や優勢株となっていたり，菌株間で病原性の差異が認められていたりすることが，明らかになってきた．

C. difficile のタイピング法としては，さまざまな方法が開発・評価されており，表現型別では血清型別[3]，遺伝子型別では restriction endonuclease analysis（REA），pulsed field gel electrophoresis（PFGE）解析，PCR ribotyping，multilocus sequence typing（MLST），multilocus variable-number tandem-repeat analysis（MLVA），amplified fragment length polymorphism（AFLP）解析，および surface layer protein A gene sequence typing（*slpA* sequence typing）などがある[4]．多くの研究室で採用されているタイピング法は，PCR ribotyping と PFGE 解析で，Stubbs らにより確立された PCR ribotyping[5]による解析は英国を中心としたヨーロッパで，PFGE による解析は主に米国やカナダで行われている．一方，*C. difficile* の産生する毒素には，toxin A，toxin B，および binary toxin がある．toxin A 遺伝子と toxin B 遺伝子が位置している pathogenicity locus（PaLoc）に認められる多様性は toxinotype として分類される[6]．

国内外で優勢株・流行株として報告されている菌株について（表）

1．PCR ribotype 001 株

Stubbs ら[5]に PCR ribotype 001 と命名されたタイプの菌株は，toxin A 陽性 toxin B 陽性 binary toxin 陰性株で，toxinotype 0 に属す．本タイプに属す菌株は，REA type J[7]，*slpA* sequence type gr[8,9]であり，PFGE 解析では泳動時にチオウレアを使用するなどの工夫をしないと DNA degradation によりタイピングができない菌株である[10]．1990 年代には，特に英国，米国で最優勢であった菌株で[5,7]，北米では，現在も後述の PCR ribotype 027 株の次に優勢であり，ヨーロッパでは地域によっては現在も最優勢であるとの報告がある[11]．本株は，芽胞形成能が高いという報告があり[12]，医療関連感染を引き起こしやすい要因のひとつと考えられる．わが国の医療施設でも散発例より分離される．

2．PCR ribotype smz 株

PCR ribotype smz は，わが国の医療施設で頻繁に分離されるタイプで，Kato らにより命名された[8,9]．本株は toxin A 陽性 toxin B 陽性 binary toxin 陰性で，toxinotype 0 に属し，PCR ribotype 001 株と同様に，DNA degradation により PFGE 解析が難しい菌株である[8]．わが国では，

表　国内外で優勢株・流行株として報告されている菌株

PCR ribotype	*slpA* sequence type	toxin type	toxinotype	臨床分離背景
001	gr	$A^+B^+CDT^-$	0	2000年以前には米国や英国で多くの医療施設で最優勢株であった．現在も欧米では優勢株のひとつである．
smz	smz	$A^+B^+CDT^-$	0	わが国の医療施設で優勢となっている．
017/trf	fr	$A^-B^+CDT^-$	VIII	カナダ，オランダ，日本でアウトブレイク事例の報告がある．ポーランド，アイルランド，日本，韓国では，優勢株のひとつと報告されている．
027	gc8	$A^+B^+CDT^+$	III	2000年以降に，CDI症例の増加とともに，北米やヨーロッパのいくつかの国で最優勢となった．
078	078	$A^+B^+CDT^+$	V	従来，ウシやブタなどの動物より分離される菌株として知られていたが，近年，ヒトからの分離株として注目されている．

$A^+B^+CDT^-$：toxin A陽性，toxin B陽性，binary toxin陰性．
$A^-B^+CDT^-$：toxin A陰性，toxin B陰性，binary toxin陰性．
$A^+B^+CDT^+$：toxin A陽性，toxin B陽性，binary toxin陽性．

本株が最優勢になっていた施設の複数事例[9]，他のタイプから本タイプへ優勢株がシフトした事例[13]，本タイプ株を含めた3タイプ菌株が同時に施設内に拡がっていた事例[14]などが報告されている．海外では，ドイツの医療施設における分離株において，*slpA* sequence typingで解析したところ，本タイプが分離株の3%に認められたと報告されたが[11]，他の地域での本株の分布については不明である．

3．PCR ribotype 017/trf株

PCR ribotype 017はStubbsら[5]による命名で，PCR ribotype trfはKatoら[9]による命名である．2タイプはPCR ribotype patternにおいて多くのバンドを共有する．両タイプ菌株ともtoxin A陰性toxin B陽性binary toxin陰性で，toxinotype VIIIに属し，同一の*slpA* sequence major typeに分類される[9]．PCR ribotype 017株によるオランダのアウトブレイク事例[15]や，PCR ribotype trf株によるわが国のアウトブレイク事例[16,17]が報告されている．toxin A陰性toxin B陽性株は，わが国の医療施設で頻繁に分離されるが，同様にポーランド，アイルランド，韓国でも優勢株であると報告されている[18,19]．

4．PCR ribotype 027株

Stubbsら[5]にPCR ribotype 027と命名されたタイプの菌株は，REAではBI，PFGE解析ではNorth America PFGE type 1(NAP1)と命名され，BI/NAP1/027株と呼ばれる[20,21]．toxin A陽性toxin B陽性株であり，*in vitro*で対照とする菌株と比較して両毒素産生性が高いと報告された[21]．toxinotype IIIに属すPaLoc変異株で，toxin Aおよびtoxin Bの産生に負の調節遺伝子である*tcdC*の変異[21,22]がtoxin Aとtoxin B産生性が高い原因の一つといわれているが，すべてを説明しているわけではない．toxin Aおよびtoxin Bのほかにbinary toxinを産生する．

2000年ごろから認められたCDI症例数急増と並行して[23]，本タイプ菌株の分離が増加し，本株の高い病原性が示唆された．PCR ribotype 027株の分離とCDIの重症化との関連について報告があり[18,24]，英国における多数の死亡例を認めた施設内アウトブレイク事例は非常に注目された(http://www.cqc.org.uk/_db/_documents/Stoke_Mandeville.pdf)．PCR ribotype 027株は，1990年代およびそれ以前にも分離されているが(historic isolate)，散発例からの分離で流行株とはなっていない．2000年以降に分離されたepidemic isolateは，historic isolateがガチフロキサシンやモキシフロキサシンなどのフルオロキノロン系抗菌薬に感性であるのに比較して，ガチフロキサシンやモキシフロキサシンに耐性であることから，これらのフルオロキノロン系抗菌薬の

使用が選択圧となり流行の原因の一つとなったとも考えられた[20]．一方，PCR ribotype 027株のhistoric isolateの遺伝子とepidemic isolateの遺伝子を比較解析した検討では，epidemic isolateには，historic isolateにはない約20－kbのG＋C contentの高いphage islandが認められたと報告され，本株が新しく遺伝子を獲得することにより高い病原性をもつに至っていることが示唆された[25]．

わが国においては，PCR ribotype 027株による散発症例は認められているが，現在のところ，本菌株が優勢になっている医療施設や，本菌株が流行株となっているアウトブレイク事例は認められていない[13,26]．

5．PCR ribotype 078株

PCR ribotype 078株[5]は，toxin A陽性toxin B陽性binary toxin陽性株で，toxinotype Vに属す．従来，ウシやブタなどの動物からの分離[1]が問題となっていたが，米国では小売りの食肉製品からも認められることが報告された[2]．最近では，本タイプ菌株はヒト感染症例からも分離され，オランダからはPCR ribotype 027株と同様に重症例から分離されるとの報告があり[27]，病原性の点からも，感染経路の点からも注目されている．

おわりに

わが国においては，調査解析を行った医療施設が限られているため，どのような菌株が流行し優勢となっているのか，実態は不明である．個々の医療施設で適切な細菌学的検査を行うと同時に，全国的なCDIのサーベイランス・システムの構築整備が必要である．一方で，CDIは，抗菌薬の使用を含めた宿主側因子がその発症や重症化に大きく影響するため，上記のように国内外で優勢株・流行株として問題となっている菌株以外の菌株によっても，重篤な経過や死の転帰をとる感染を引き起こしたり，複数の症例に伝播したりすることがありうることにも留意すべきである．

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Use of the loop-mediated isothermal amplification method for identification of PCR ribotype 027 *Clostridium difficile*

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The loop-mediated isothermal amplification (LAMP) assay detecting the *slpA* gene of *slpA* sequence type gc8 (*slpA-gc8*) was established for the identification of a hypervirulent *Clostridium difficile* strain, PCR ribotype 027. Of 107 isolates examined, 27 belonging to PCR ribotype 027 were all positive for the LAMP assay. The remaining 80 isolates were typed into 47 different PCR ribotypes other than type 027, and were negative for the LAMP assay with the exception of two isolates. The sensitivity and specificity of the LAMP method for identification of PCR ribotype 027 were 100 % and 98 %, respectively. The LAMP assay detecting *slpA-gc8* is a reliable tool for the identification of PCR ribotype 027 *C. difficile*. This simple and rapid method will contribute to detection of the hypervirulent strain.

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INTRODUCTION

Clostridium difficile is well known as an important cause of health-care-associated infection. The emergence of a *C. difficile* strain characterized as restriction endonuclease analysis (REA) type BI, PFGE type NAP1 and PCR ribotype 027 has been reported to be responsible in part for the epidemic in North America and Europe in the past decade (Kuijper *et al.*, 2008; Loo *et al.*, 2005; MacCannell *et al.*, 2006; McDonald *et al.*, 2005; Warny *et al.*, 2005). In addition, it has been documented that patients infected with this strain are more likely to develop severe disease (Barbut *et al.*, 2007; Goorhuis *et al.*, 2007). Since the epidemic by PCR ribotype 027 threatens to extend to the rest of the world including Asia (Gerding, 2010), early recognition of the emergence of the strain is valuable (Goorhuis *et al.*, 2007), especially in the countries or areas where the strain is not currently epidemic.

In previous studies, a system for typing *C. difficile* by sequencing the gene encoding the surface-layer protein A (*slpA*) was established and evaluated (Kato *et al.*, 2010; Killgore *et al.*, 2008). These reports documented that the typing results exhibited a high concordance with those obtained by PCR ribotyping; *slpA* sequence type gc8 corresponded to PCR ribotype 027 (Kato *et al.*, 2010; Killgore *et al.*, 2008). In the present study, we established a simple method for identification of PCR ribotype 027 by loop-mediated isothermal amplification (LAMP) detecting the *slpA* gene of *slpA* sequence type gc8 strains (*slpA-gc8*).

Abbreviations: CDT, binary toxin; LAMP, loop-mediated isothermal amplification; REA, restriction endonuclease analysis.

The GenBank/EMBL/DDBJ accession numbers (*slpA* sequence type) for the *slpA* genes reported in this study are AB249986 (gc8-01), AB257285 (gc8-02), AB257286 (gc8-03), AB461839 (gc8-05) and AB461840 (gc8-06).

METHODS

Bacterial strain. A total of 107 *C. difficile* isolates were examined. The reference strains of serogroups A (ATCC 43594), C (ATCC 43596), F (ATCC 43598), G (ATCC 43599) and H (ATCC 43600) (Delmée *et al.*, 1986) were obtained from the American Type Culture Collection. The 59 clinical *C. difficile* isolates used in this study were isolated from various hospitals in Japan. A collection of 42 isolates used for a previous study including strain US42 (REA type BI/PFGE type NAP1/PCR ribotype 027; Killgore *et al.*, 2008) and strain UMCG12(3) (PCR ribotype 078; Goorhuis *et al.*, 2008) were tested as well.

PCR detecting the toxin genes. The non-repeating and repeating sequences of the toxin A gene (*tcdA*) were amplified by PCR with primer sets NK3–NK2 (Kato *et al.*, 1991) and NK9–NK11–NKV011 (Kato *et al.*, 1998, 1999), respectively. The isolates were identified as toxin A-negative when PCR with the primer set NK3–NK2 was negative (Kato *et al.*, 1991). When PCR by the NK3–NK2 primer set was positive and PCR by the NK9–NK11–NKV011 primer set generated an amplification product of approximately 1200 bp in size, the isolates were identified as toxin A-positive. When PCR by NK3–NK2 was positive and the product by PCR with NK9–NK11–NKV011 was approximately 700 bp in size, the isolates were identified as toxin A-negative (Kato *et al.*, 1998, 1999). When the isolates were positive for PCR by NK3–NK2 but the PCR product generated by NK9–NK11–NKV011 was neither approximately 1200 bp nor 700 bp in size, toxin production by the isolates was determined. The presence of the non-repeating sequences of the toxin B gene (*tcdB*) was examined by PCR using primer set NK105–NK104 (Kato *et al.*, 1998). PCR detection of the gene encoding the binding component of binary toxin (CDT) was performed as previously described (Stubbs *et al.*, 2000).

Determination of toxin production by isolates. *C. difficile* was cultured anaerobically in brain–heart infusion broth (Becton

Dickinson) for 5 days. Toxin A was detected by an enzyme immunoassay kit, *C. difficile* toxin A test (Oxoid). Toxin B was detected by a culture assay using Vero cells and *C. difficile* goat anti-toxin B serum was used for the neutralization test (Kato *et al.*, 1998).

Typing of isolates. Typing of isolates by sequencing *slpA* was carried out as previously described (Kato *et al.*, 2010). Isolates were assigned to different major types when they had 20 or more amino acid differences, and to subtypes (01–06) when they had fewer than 20 such differences. PCR ribotyping was performed by the modified methods of Stubbs and others as previously described (Kato *et al.*, 2010; Stubbs *et al.*, 1999).

Detection of *slpA-gc8* by LAMP. For DNA extraction for LAMP, *C. difficile* was inoculated on Brucella agar supplemented with vitamin K_1 and haemin (Kyokuto Pharmaceutical) for 1–7 days anaerobically. A single colony was suspended in 50 µl TES (50 mM Tris hydrochloride, pH 8.0; 5 mM EDTA; and 50 mM NaCl). The suspension was heated at 95 °C for 15 min and then centrifuged at 15 000 ***g*** for 2 min, and the resultant supernatant was used as a template for the LAMP assay. The six primers used for the LAMP were derived from the *slpA* gene of strain US42, which was identified as REA type BI, PFGE type NAP1, PCR ribotype 027 and *slpA* sequence type gc8-01 (GenBank accession no. AB249986) (Kato *et al.*, 2010; Killgore *et al.*, 2008). The outer primers used were gc8002a-F3 (5′-GCTCTCCAGCAGAGGGAG-3′) and gc8002-B3 (5′-AGTTCCA-TCAACTAAACCAAAC-3′); the inner primers were gc8002b-FIP (5′-TTGGAGCTGTATTTTTTGCTCCCGCAATAAAAGTAGCTACAAG-TAG-3′) and gc8002-BIP (5′-GTCTATGTCAGATGTATTTGATAC-ATAAGTTTCACAGCAGTTTCAGT-3′); and the loop primers were gc8002-FL (5′-TACCAGCATTTTCGACTTCACC-3′) and gc8002-BL (5′-GCTTTTACAGATTCA-3′). The LAMP reaction was performed using the Loopamp DNA amplification kit (Eiken Chemical) according to the manufacturer's instructions. Two microlitres of DNA template was added to a total volume of 25 µl buffer consisting of 5 pM of each of the outer primers, 40 pM of each of the inner primers and 20 pM of each of the loop primers. Amplification was performed at 62 °C for 60 min. The increased turbidity was monitored by a real-time turbidimeter, LA-320C (Eiken Chemical). The turbidity was calculated based on the ratio of light intensity (the intensity of light received by the photodiode/the emitted light intensity). The threshold of LAMP positive for the turbidimeter was defined as 0.1.

Determination of the sequences of *tcdC*. The sequences of *tcdC* were determined by sequencing the PCR product with primer set C1–C2 as described previously (Spigaglia & Mastrantonio, 2002). The primer set NK3–Lok1 (Braun *et al.*, 1996; Kato *et al.*, 1991) was used for sequencing *tcdC* of isolate DJNS 7-18. The sequences obtained were compared to those of strain VPI 10463 and strain US42 as well as to the sequences previously described (MacCannell *et al.*, 2006) and those in the GenBank database. The comparative analysis was performed by GENETYX-MAC version 12.2.3.

RESULTS

Typing results

Sequencing of the *slpA* gene resulted in 24 major types and 54 subtypes for the 107 isolates tested. Of the 107 isolates, 29 were typed as *slpA* sequence type gc8, and five different subtypes (*slpA* sequence types gc8-01, gc8-02, gc8-03, gc8-05 and gc8-06) were found among these 29 isolates (Table 1). Types gc8-01, gc8-02, gc8-03 and gc8-06 differed from each other by 1 nt, involving a single deduced amino acid. The *slpA* sequence of one isolate, DJNS 7-18, was found to differ from that of gc8-01 by eight deduced amino acids (*slpA* sequence type gc8-05). Among the 107 isolates, 48 different PCR ribotypes were identified. Of the 28 isolates belonging to gc8-01, gc8-02, gc8-03 or gc8-06, 27 were typed as PCR ribotype 027 (Fig. 1). One isolate of *slpA* sequence type gc8-01 (JND 10-61) was typed as a different PCR ribotype from PCR ribotype 027 (PCR ribotype 027r; Fig. 1), while its PCR banding pattern showed two band differences from that of 027. Isolate DJNS 7-18 and the remaining 78 isolates were typed into 46 different PCR ribotypes, banding patterns of which were distinct from that of PCR ribotype 027. The epidemiologically important PCR ribotypes 001, 002, 014, 017, 078, trf and smz (Barbut *et al.*, 2007; Goorhuis *et al.*, 2008; Kato *et al.*, 2010; Killgore *et al.*, 2008) were included in the 46 PCR ribotypes.

Detection of *slpA-gc8* by LAMP

slpA-gc8 was detected by LAMP in all 29 isolates of *slpA* sequence type gc8 including JND 10-61 and DJNS 7-18 (Fig. 2; Table 1). LAMP was performed on DNA extracted from strain US42, which was incubated for 1 or 7 days. The time for *C. difficile* culture to extract DNA did not affect the LAMP reaction. In a comparison of the LAMP results in 28 isolates identified as PCR ribotypes 027 and 027r (*slpA* sequence types gc8-01, gc8-02, gc8-03 and gc8-06), the amplification reaction in DJNS 7-18 (PCR ribotype tkm0718/*slpA* sequence type gc8-05) was slow (Fig. 2). LAMP was performed on 78 isolates of types other than *slpA* sequence type gc8 in a 90 min reaction, with negative results (Table 1). The sensitivity and specificity of the LAMP method for identification of PCR ribotype 027 were 100 % and 98 %, respectively.

slpA sequence type gc8 isolates recovered in Japan

Of seven *slpA* sequence type gc8 isolates recovered in Japan, six (subtypes gc8-01 and gc8-06) were toxin A-positive, toxin B-positive and CDT-positive. Sequencing analysis of the six isolates including JND 10-61 detected *tcdC* identical with that of strain US42 and published sequences (MacCannell *et al.*, 2006). Isolate DJNS 7-18 (subtype gc8-05) was toxin A-negative, toxin B-positive and CDT-positive. In the DJNS 7-18 isolate, PCR using the NK3–NK2 primer set was positive but no amplification was obtained by PCR using the NK9–NK11–NKV011 primer set. This isolate was tested by enzyme immunoassay for toxin A production, with a negative result, and toxin B was detected by a cell culture assay with a positive neutralization reaction. When the sequences of *tcdA* and *tcdC* were compared to those registered in the GenBank database, DJNS 7-18 had deletions in *tcdA* and *tcdC*; the deletions were the same in location and size as those of strain 8864 (GenBank accession no. AJ011301). Isolate DJNS 7-18 was

Table 1. Typing results and LAMP results for identification of PCR ribotype 027

slpA sequence major type	*slpA* sequence subtype	PCR ribotype	LAMP results	No. of isolates tested from:			Total no. of isolates studied
				Japan	North America and Europe*	ATCC	
gc8	gc8-01	027	+	4	16		20
	gc8-01	027r†	+	1			1
	gc8-02	027	+		2		2
	gc8-03	027	+		4		4
	gc8-05	tkm0718	+‡	1			1
	gc8-06	027	+	1			1
Other types§			−	52	21	5	78
Total				59	43	5	107

*A collection of 42 isolates used for a previous study (Killgore *et al.*, 2008) and strain UMCG12(3) (PCR ribotype 078; Goorhuis *et al.*, 2008).
†The banding pattern of JND 10-61 (PCR ribotype 027r) differed from that of type 027 by two bands.
‡The LAMP reaction in DJNS 7-18 was slow.
§A total of 78 isolates were typed into 23 *slpA* sequence major types, 49 *slpA* sequence subtypes and 45 PCR ribotypes.

recovered from a patient who suffered from pseudomembranous colitis in 2008.

DISCUSSION

In the present study, all PCR ribotype 027 isolates tested were successfully identified by LAMP assay detecting the *slpA-gc8* gene. The isolates typed as 47 different PCR ribotypes other than type 027 were all negative for LAMP with the exception of two isolates, JND 10-61 and DJNS 7-18. In JND 10-61, which was typed as *slpA* sequence gc8-01, the PCR ribotype pattern differed from that of type 027 by two bands and its *tcdC* sequence was the same as that of strain US42. More studies are needed to identify this isolate as a strain related to the hypervirulent strain. DJNS 7-18 was a toxin A-negative, toxin B-positive, CDT-positive isolate with the same deletions in *tcdA* and *tcdC* as those of strain 8864 (Lyerly *et al.*, 1992; Rupnik *et al.*, 1998). Since strain 8864 was not available in the present study, PCR ribotyping and *slpA* sequence typing results for strain 8864 could not be compared to those for DJNS 7-18. DJNS 7-18 was unique among our collection of clinical isolates in Japan. Additional studies using more clinical isolates are warranted to determine the significance of isolates typed as *slpA* sequence type gc8 but not as PCR ribotype 027.

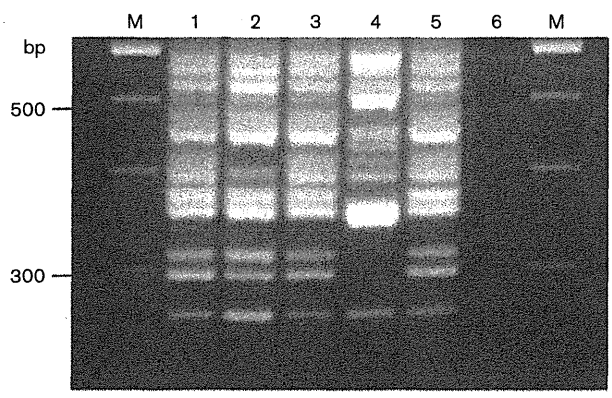

Fig. 1. PCR ribotype patterns of representative isolates typed as *slpA* sequence type gc8. Lanes: 1, DJNS 5-23 (*slpA* sequence type gc8-01); 2, JND 10-61 (gc8-01); 3, JND 8-64 (gc8-06); 4, DJNS 7-18 (gc8-05); 5, US42 (gc8-01); 6, negative control; M, 100 bp ladder as a molecular size marker. Strain US42 was used as the reference strain of PCR ribotype 027.

PCR ribotype 027 has been reported to be responsible for multiple outbreaks and a major portion of the increase in *C. difficile* infection rates in North America and Europe (Kuijper *et al.*, 2008; Pépin *et al.*, 2004; Loo *et al.*, 2005; McDonald *et al.*, 2005). It has been documented that patients infected with the PCR ribotype 027 strain are more likely to have severe disease and to have been specifically treated with metronidazole or vancomycin (Barbut *et al.*, 2007). Goorhuis *et al.* (2007) demonstrated that clear trends were observed for more severe diarrhoea, higher attributable mortality and more recurrences in patients infected with PCR ribotype 027 than in those infected with other types (Goorhuis *et al.*, 2007). The significant pathogenicity of this strain, such as robust toxin production or significantly more spore formation (Merrigan *et al.*, 2010; Warny *et al.*, 2005), may contribute to the severity and wide spread of *C. difficile* infection caused by the strain. These reports indicate that PCR ribotype 027 is more likely to cause outbreaks with high morbidity and mortality. Particularly in the countries or areas where the strain is not currently predominant, such as in Japan (Kato *et al.*, 2007; Sawabe *et al.*, 2007), earlier recognition of outbreaks caused by the strain will provide beneficial information for public health centres, leading to alerts of

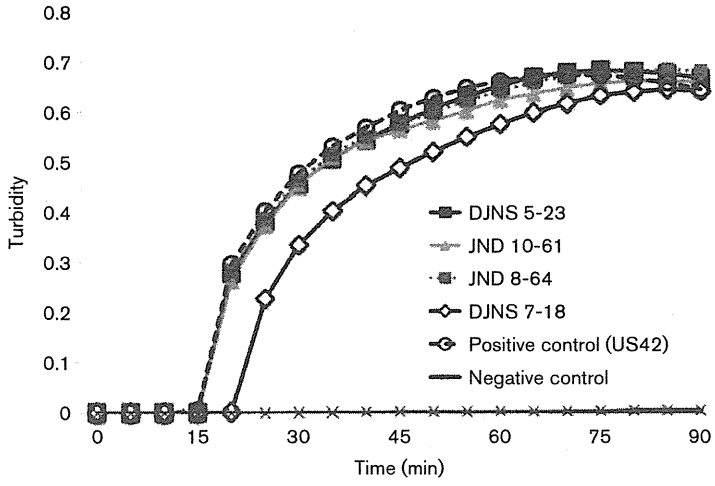

Fig. 2. Real-time turbidity of LAMP amplification products for representative isolates typed as *slpA* sequence type gc8. Strain US42 was used as a positive control.

the emergence of the strain to health-care facilities covered by the public centres. The LAMP assay detecting *slpA-gc8* may be valuable for monitoring trends in the prevalence of *C. difficile* infection caused by PCR ribotype 027. Since the LAMP method is simple to perform and does not require any special equipment, such as a thermal cycler or electrophoresis system, it is accessible to even small laboratories of local public health centres as well as of health-care facilities.

The LAMP assay detecting *slpA-gc8* proved to be a reliable tool for the identification of PCR ribotype 027 in the present study. The method may be applied to the direct detection of *slpA-gc8* from stool specimens without a culture step. In the present study, one stool specimen from a patient who suffered from pseudomembranous colitis caused by PCR ribotype 027 was available (Kato *et al.*, 2007); detection of *slpA-gc8* was performed on DNA extracted directly from the stool specimen as previously described (Kato *et al.*, 2005), with a positive result (data not shown). Further analysis using a large number of stool specimens is required to evaluate the method for the direct detection of PCR ribotype 027 strains from stool specimens.

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ORIGINAL ARTICLE

Evaluation of a simultaneous detection kit for the glutamate dehydrogenase antigen and toxin A/B in feces for diagnosis of *Clostridium difficile* infection

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Abstract Rapid detection kits for toxin A/B in feces are widely used as a diagnostic tool for *Clostridium difficile* infection (CDI). Their low sensitivity, however, has been considered a problem. In this study, we evaluated a new rapid diagnostic kit for simultaneous detection of the glutamate dehydrogenase (GDH) antigen and toxin A/B, *C. DIFF* QUIK CHEK COMPLETE. A total of 60 stool specimens from 60 patients with antibiotic-associated diarrhea were examined. Using *C. difficile* culture as the reference method, the GDH portion of this kit indicated a sensitivity, specificity, and negative predictive value of 100, 93.3, and 100%, respectively. The toxin A/B portion showed a sensitivity and specificity of 78.6 and 96.9%, respectively, compared to the culture results of toxin B-positive *C. difficile* (toxigenic culture). Of the 23 specimens that showed "dual positives" for GDH and toxin A/B, 22 were toxigenic culture positive, whereas *C. difficile* culture was negative in all the 28 specimens that showed "dual negatives" for GDH and toxin A/B. Of the nine "GDH-positive and toxin A/B-negative" specimens, six exhibited positive results by toxigenic culture. Results showing "dual positives" and "dual negatives" for GDH and toxin A/B can be reported as "true positive" and "true negative," respectively, whereas additional testing for confirmation, such as toxigenic culture, is required for specimens with discrepant results. Diagnostic algorithms, utilizing the simultaneous detection kit for GDH and toxin A/B as an initial screening test, may be useful for accurate and efficient diagnosis of CDI as well as the control of healthcare-associated infections.

Keywords *Clostridium difficile* infection · Glutamate dehydrogenase · Toxin A · Toxin B · Laboratory diagnosis · *Clostridium difficile* culture

Introduction

Clostridium difficile is a major causative agent of antibiotic-associated diarrhea and colitis. It can lead to a variety of clinical manifestations ranging from mild diarrhea to severe forms of intestinal illness including pseudomembranous colitis, ileus, toxic megacolon, and bowel perforation. *C. difficile* is a spore-bearing obligate anaerobe and is resistant to oxygen or antiseptics, depending on its spore status, thus making it possible for the pathogen to reside in the hospital environment for long periods and ultimately to cause healthcare-associated infections [1–4].

Toxins A and B have been well documented as major virulence factors of *C. difficile*. Toxin A-positive, toxin B-positive (A^+B^+) strains as well as toxin A-negative, toxin B-positive (A^-B^+) strains are known to cause diarrhea and colitis, and detection of toxin B in feces is important for the diagnosis of *C. difficile* infection (CDI). The traditional gold standard is a cytotoxin assay that detects the cytotoxicity of

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toxin B in fecal eluate by using cell culture, but this assay requires special equipment and techniques, making it unfeasible in many clinical laboratories [3, 4]. Rapid detection tests for toxin A/B by enzyme immunoassay (EIA) are widely used because of their ease of use and lower labor costs, but these tests appear to have a low sensitivity, which leads to concern about false-negative results [5–7]. Polymerase chain reaction (PCR) tests that detect the toxin B gene in stool samples may be rapid and more sensitive than EIA-based toxin detection, but it would be difficult for many ordinary clinical laboratories to employ these as routine diagnostic tests because acquisition of the instrument requires an initial large expenditure [7]. *C. difficile* culture followed by detection of a toxin-producing isolate (toxigenic culture) is considered the most sensitive diagnostic method. However, the turnaround time of at least 2–3 days until reporting of the final results is too long, and performing toxigenic culture on all the stool specimens submitted for *C. difficile* testing is labor intensive [8, 9].

Rapid detection tests for glutamate dehydrogenase (GDH), a cell-wall protein of *C. difficile*, have also been utilized as a diagnostic tool for CDI, although they provide no information about the toxigenicity. In contrast to the GDH detection tests by latex agglutination assay, those by EIA show a high sensitivity and negative predictive value, making them useful for rapid screening of *C. difficile* strains in feces. To make an accurate and efficient diagnosis of CDI, researchers have proposed testing algorithms, such as one that consists of an initial screening by EIA-based GDH detection, a rapid toxin detection test for GDH-positive specimens, and a final confirmatory test for GDH-positive/toxin-negative specimens by a cytotoxin assay, toxigenic culture, or PCR [3, 4, 8, 9].

Recently, an EIA-based rapid combination test kit for GDH and toxin A/B in feces, the *C. DIFF* QUIK CHEK COMPLETE (TECHLAB, Blacksburg, VA, USA) (*C. DIFF* COMPLETE), has become available in countries outside Japan and appears to be a promising aid in improving the accuracy and efficiency of CDI diagnosis [10–12]. In this study, we evaluated the *C. DIFF* COMPLETE in comparison with the other diagnostic methods including *C. difficile* toxigenic culture, a GDH detection test [ImmunoCard *C. difficile* (Meridian Bioscience, USA) (ImmunoCard)], and a toxin A/B detection test [TOX A/B QUIK CHEK (TECHLAB, USA) (TOX A/B)].

Materials and methods

Sample collection

A total of 60 fecal specimens were collected from 60 patients, who were hospitalized at Tokyo Metropolitan Geriatric Hospital (Tokyo, Japan) between October 2009 and January 2010 and diagnosed as having antibiotic-associated diarrhea (33 men and 27 women; age range, 54–100 years; mean, 82.9 years). All the patients consented to have their specimens used in this study. All samples examined were stored at 4°C for <72 h until *C. difficile* testing was performed.

Detection of the GDH and toxin A/B in fecal specimens

Simultaneous detection of the GDH and toxin A/B by *C. DIFF* COMPLETE, GDH detection by ImmunoCard, and toxin A/B detection by TOX A/B were performed in accordance with the manufacturers' instructions.

C. difficile culture

For *C. difficile* culture, fecal specimens were inoculated onto cycloserine–cefoxitin–mannitol agar (CCMA) (Nissui Pharmaceutical, Tokyo, Japan) and incubated for 48 h at 35°C under anaerobic conditions. The presence of the toxin A and toxin B genes of the recovered isolates was determined by PCR assay as previously described [13, 14]. For specimens that were "GDH-positive and toxin A/B-negative" by *C. DIFF* COMPLETE and "positive" by *C. difficile* culture, an additional *C. DIFF* COMPLETE test was performed on the isolates. Isolated colonies cultured on GAM agar (Nissui Pharmaceutical) for 48 h at 35°C under anaerobic conditions were suspended in the dilution buffer with turbidity equivalent to the McFarland standard no. 4 and tested by *C. DIFF* COMPLETE in accordance with the manufacture's protocol for fecal specimen testing.

Statistical analyses

As a significance test, the chi-square test with Yates' correction was performed. The significance level was set at $P < 0.05$.

Results

Clostridium difficile was isolated from 30 of the 60 fecal specimens. The GDH antigen portion of the *C. DIFF* COMPLETE test showed positive results in all 30 culture-positive specimens and negative results in 28 of the 30 culture-negative specimens, implying 100% sensitivity and 93.3% specificity. The negative predictive value was 100%. ImmunoCard exhibited positive results in 24 of the 30 culture-positive specimens and negative results in the remaining 36 specimens, indicating that its sensitivity and specificity were 80.0% (vs. *C. DIFF* COMPLETE, $P < 0.05$) and 100%, respectively (Table 1).

Table 1 Comparison of performance results for *C. DIFF* COMPLETE (GDH portion) and ImmunoCard compared to *Clostridium difficile culture*

		C. difficile culture		Sensitivity	Specificity	PPV	NPV	Correlation
		Positive	Negative	%	%	%	%	%
C. DIFF COMPLETE (GDH portion)	Positive	30	2	100 (30/30)	93.3 (28/30) *	93.8 (30/32) **	100 (28/28) **	96.7 (58/60) **
	Negative	0	28					
ImmunoCard	Positive	24	0	80.0 (24/30)	100 (30/30)	100 (24/24)	83.3 (30/36)	90.0 (54/60)
	Negative	6	30					

PPV, positive predictive value; NPV, negative predictive value

* $P < 0.05$, chi-square test with Yates' correction

** Not significant, chi-square test with Yates' correction

The results of toxin A/B detection by *C. DIFF* COMPLETE showed a correlation of 91.7% (55/60) with those by TOX A/B (data not shown). Compared with the culture results of toxin B-positive (i.e., A^+B^+ or A^-B^+) *C. difficile* (toxigenic culture), the sensitivity and specificity of the toxin A/B portion of *C. DIFF* COMPLETE were 78.6 and 96.9%, respectively, whereas those of TOX A/B were 71.4 and 93.8%, respectively (Table 2).

Of the 30 *C. difficile* isolates recovered from stool specimens, 19, 9, and 2 isolates were A^+B^+, A^-B^+, and toxin A-negative, toxin B-negative (A^-B^-) strains, respectively. Of the 19 fecal specimens from which A^+B^+ strains were isolated, 16 and 14 specimens exhibited positive results in the toxin A/B portion of *C. DIFF* COMPLETE and TOX A/B, respectively. Similarly, of the nine fecal specimens from which A^-B^+ strains were isolated, six specimens each exhibited positive results in respective assays, and of the two fecal specimens from which A^-B^- strains were isolated, one specimen gave a positive result in both tests (results of *C. DIFF* COMPLETE are shown in Table 3).

C. DIFF COMPLETE showed "dual positives" for GDH and toxin A/B in 23 fecal specimens. Toxin B-positive *C. difficile* strains were isolated from 22 of the 23 specimens, indicating a positive predictive value of 95.7% (an A^-B^- strain was isolated from the remaining 1 specimen). *C. DIFF* COMPLETE showed "dual negatives" for GDH and toxin A/B in 28 fecal specimens. All were negative for *C. difficile* culture, implying a negative predictive value of 100%. Of the nine specimens that showed "GDH-positive and toxin A/B-negative" results by *C. DIFF* COMPLETE, seven were positive for *C. difficile* culture (Table 3). By an additional *C. DIFF* COMPLETE test on the isolates, all the six toxin B-positive isolates (three A^+B^+ and three A^-B^+ isolates) showed "GDH-positive and toxin A/B-positive" results, whereas the one A^-B^- isolate indicated a "GDH-positive and toxin A/B-negative" result (data not shown).

Discussion

In this study, we evaluated *C. DIFF* COMPLETE, a new EIA-based rapid diagnostic tool for simultaneous detection of GDH and toxin A/B in a single device.

Using *C. difficile* culture as a reference, the GDH portion of the *C. DIFF* COMPLETE had a sensitivity of 100% (30/30), which was significantly higher than the sensitivity of 80% (24/30) observed in the ImmunoCard test. GDH detection in the *C. DIFF* COMPLETE test is mediated by anti-GDH polyclonal antibodies immobilized on the membrane (capture antibodies) and enzyme-labeled anti-GDH monoclonal antibodies (detector antibodies), whereas the ImmunoCard test utilizes anti-GDH polyclonal antibodies as both capture and detector antibodies. Such differences might contribute to the high sensitivity and specificity of GDH detection in *C. DIFF* COMPLETE.

For two specimens that were GDH positive by *C. DIFF* COMPLETE but culture negative, we cannot exclude the possibility of false-negative results of *C. difficile* culture. However, it was more strongly suspected that the GDH detection gave false-positive results because they exhibited negative results in all the other tests performed (i.e., GDH detection by ImmunoCard, toxin A/B detection by *C. DIFF* COMPLETE and TOX A/B), and neither of the two

Table 2 Comparison of performance results for *C. DIFF* COMPLETE (toxin portion) and TOX A/B compared to toxigenic culture

		A^+B^+/A^-B^+[a] *C. difficile* culture		Sensitivity %	Specificity %	PPV %	NPV %	Correlation %
		Positive	Negative					
C. DIFF COMPLETE (toxin portion)	Positive	22	1	78.6 (22/28)	96.9 (31/32)	95.7 (22/23)	83.8 (31/37)	88.3 (53/60)
	Negative	6	31	**	**	**	**	**
TOX A/B	Positive	20	2	71.4 (20/28)	93.8 (30/32)	90.9 (20/22)	78.9 (30/38)	83.3 (50/60)
	Negative	8	30					

PPV, positive predictive value; NPV, negative predictive value

** Not significant, chi-square test with Yates' correction

[a] A^+B^+, toxin A-positive, toxin B-positive; A^-B^+, toxin A-negative, toxin B-positive

Table 3 Results of the *C. DIFF* COMPLETE test and *C. difficile culture*

C. DIFF COMPLETE detecting		*C. difficile* culture			
		Positive			Negative
GDH	Toxin A/B	A^+B^+[a]	A^-B^+	A^-B^-	
Positive	Positive[b]	16[c]	6	1	0
Positive	Negative	3	3	1	2
Negative	Positive	0	0	0	0
Negative	Negative[b]	0	0	0	28
Total		19	9	2	30

[a] A^+B^+, toxin A-positive, toxin B-positive; A^-B^+, toxin A-negative, toxin-B-positive; A^-B^-, toxin A-negative, toxin B-negative

[b] A positive predictive value of "GDH-positive and toxin A/B-positive" results and a negative predictive value of "GDH-negative and toxin A/B-negative" results were 95.7% (22/23) and 100% (28/28), respectively

[c] Number of stool specimens

patients had received vancomycin or metronidazole for CDI before sample collection.

The toxin A/B portion of *C. DIFF* COMPLETE was able to detect both A^+B^+ and A^-B^+ strains, and had a detection rate of 78.6% (22/28) compared with the results of toxigenic culture, which was slightly higher than the rate of 71.4% (20/28) observed in the TOX A/B test (the difference was not statistically significant). Two A^+B^+ strains showed positive results in *C. DIFF* COMPLETE but negative results in TOX A/B, whereas the detection rate for A^-B^+ strains was the same for both tests (six of the nine specimens indicated positive results in each test).

Rapid detection EIA tests for toxin A/B have been reported to lack sufficient sensitivity [5–7]. In the present study, the toxin A/B portion of *C. DIFF* COMPLETE showed a relatively low sensitivity (78.6%), whereas the GDH portion indicated a sensitivity of 100%. Recent studies on *C. DIFF* COMPLETE have also described a low sensitivity of the toxin A/B portion (61.1–78.3%), in contrast to the high sensitivity of the GDH portion (97.6–100%) [10–12]. The specificity of toxin A/B detection by *C. DIFF* COMPLETE was high (96.9%), as reported in the articles described above (99.2–100%). Moreover, our study revealed a negative predictive value of 100% in GDH detection. Given such excellent sensitivity and negative predictive value of the GDH portion and the high specificity of the toxin A/B portion, using *C. DIFF* COMPLETE as the first-line screening test for CDI is considered reasonable.

Toxin B-positive *C. difficile* strains were isolated from 22 of the 23 fecal specimens that showed "dual positives" for GDH and toxin A/B by the *C. DIFF* COMPLETE test, implying a positive predictive value of 95.7%. The remaining 1 specimen from which an A^-B^- strain was isolated might have contained both toxin B-positive and A^-B^- strains, because TOX A/B as well as *C. DIFF* COMPLETE exhibited positive results for toxin A/B in this specimen. *C. difficile* culture was negative in all the 28 specimens that showed "dual negatives" for GDH and toxin A/B by the *C. DIFF* COMPLETE test, indicating a negative predictive value of 100%. Thus, results showing "dual positives" for GDH and toxin A/B strongly suggest "true positive," i.e., the presence of toxin B-positive

C. difficile strains in feces, whereas results showing "dual negatives" imply "true negative."

When the *C. DIFF* COMPLETE test shows "GDH-positive and toxin A/B-negative" results, it cannot be concluded that the nontoxic (A^-B^-) strain of *C. difficile* exists in feces. However, the GDH-positive result may alert clinicians to the possibility of the presence of toxigenic organisms as a result of the false-negative result for toxin A/B. Performing confirmatory tests only for the specimens with discrepant results (not for the dual-positive and dual-negative specimens) would alleviate the burden imposed on clinical microbiology laboratories and help improve the efficiency of accurate CDI diagnosis. Confirmatory testing by PCR on stool samples allows a rapid, simple, and accurate diagnosis [10–12], but the initial expenditure for purchase of the instrument is considerable. A cytotoxin assay may be also impracticable for many clinical laboratories because of the need for special equipment and techniques. Some researchers evaluated a combination of a toxin detection kit and *C. difficile* culture, in which the toxigenicity of a culture isolate was retested with the toxin detection kit [15, 16]. Such a diagnostic strategy may be feasible in many clinical institutions, although several days are required before results are reported.

C. difficile is important as a pathogen that frequently causes healthcare-associated infections. In the present study, 28 inpatients were diagnosed as having CDI during the study period of approximately 3.5 months. It is strongly suspected that some strains spread among these patients, although typing analysis on recovered isolates was not performed in this study. Diagnostic algorithms, utilizing the *C. DIFF* COMPLETE as an initial screening test, may contribute to the infection control of CDI by allowing us to make more accurate and rapid diagnosis of the infection.

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