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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 $p < 0.05$

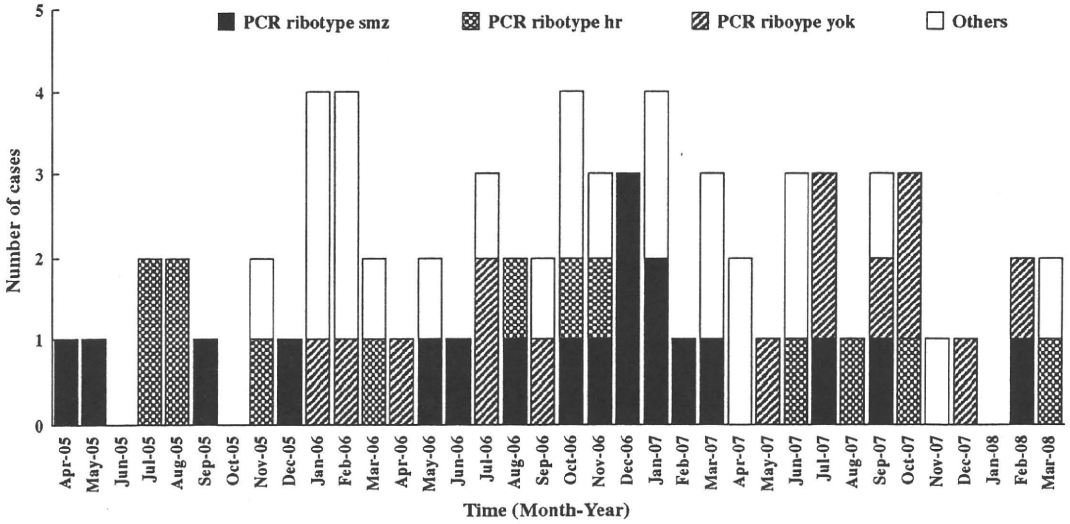

Fig. 1 Numbers of patients with *Clostridium difficile* infection and the distribution of polymerase chain reaction (PCR) ribotypes, with numbers of cases shown in *parentheses*: PCR ribotypes *smz* (19), *yok* (14), *hr* (13), and *others; others* includes PCR ribotype fr (2), gc0578 (1), j52 (2), km0403 (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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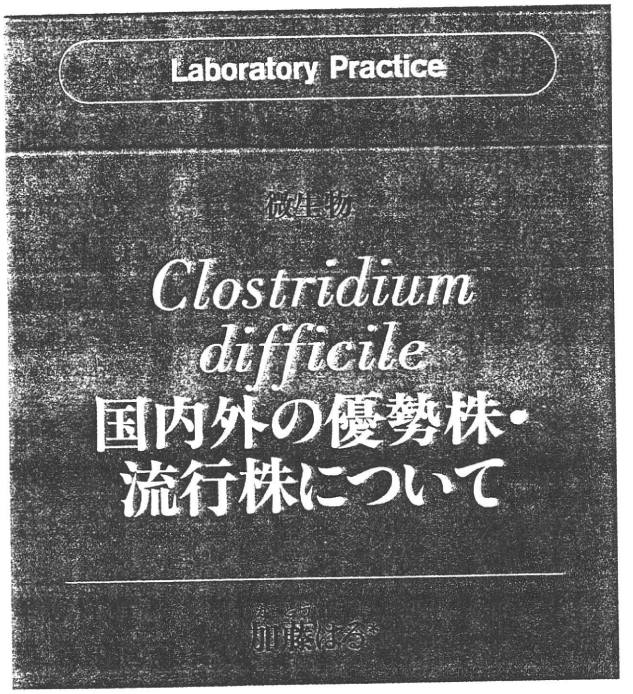

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Clostridium difficile 感染症とタイピング法について

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，

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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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