Clostridium difficile 감염 진단을 위한 *C. difficile* 정량배양 평가

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Evaluation of Quantitative culture of *Clostridium difficile* From Fecal Specimens for the Diagnosis of *C. difficile*-associated Disease

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Background: C. difficile-associated diarrhea (CDAD), the most frequently identified cause of nosocomial diarrhea, results from the overgrowth of cytotoxin (toxin B)-producing strains. The aim of this study was to evaluate the quantitative culture of *Clostridium difficile* to improve the laboratory diagnosis of CDAD.

Methods: The quantitative culture and cytotoxin gene results were evaluated based on the findings of colonoscopy and/or histology of the biopsy specimens.

Results: Among the 402 specimens with cytotoxin-positive isolates, 301 (74.9%) contained $\geq 10^6$ CFU/mL of *C. difficile*. Nine (60%) of the 15 pseudomembranous colitis patients yielded $\geq 10^6$ CFU/mL of toxigenic isolate. The proportion of cytotoxin gene-positive isolates was higher in the specimens with $\geq 10^6$ CFU/mL of *C. difficile* than in those with 10^2-$<10^3$ CFU/mL (86.5% vs. 66.7%).

Conclusions: Quantitative culture may aid in the interpretation of toxigenic *C. difficile* culture results, and reduce false positivity, thus avoiding unnecessary treatment.

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Key words : *Clostridium difficile, C. difficile*-associated diarrhea, pseudomembranous colitis, quantitative culture, cytotoxin

INTRODUCTION

C. difficile-associated diarrhea (CDAD), the most frequently identified cause of nosocomial diarrhea[1], results from the overgrowth of cytotoxin (toxin B)-producing strains. Toxigenic culture of *C. difficile* is considered to be the most

sensitive test for diagnosis of the disease[2], but it is not highly specific as some non-infected persons[3] and many hospitalized patients carry both toxigenic and non-toxigenic *C. difficile* in their intestine. The use of vancomycin to treat CDAD is considered to be one of significant factors to increase in vancomycin-resistant enterococci[4, 5]. More specific methods for the diagnosis of CDAD may reduce the use of vancomycin and, consequently, the emergence of vancomycin-resistant enterococci[6, 7].

Quantitative and semi-quantitative cultures are used in routine diagnostic microbiology, to improve the specificity of the results, in such specimens as expectorated sputum and voided urine, which inevitably contain contaminated potential pathogens[8]. The use of a quantitative method is

also recommended for the culture of *C. difficile* from fecal specimens[9], but its clinical significance is not known. The aim of this study was to evaluate whether the quantitative culture of *C. difficile* can improve the specificity of the results.

MATERIALS AND METHODS

Loose stool specimens were obtained in the period from 1997 to 1999 from inpatients suspected of having CDAD, either pseudomembranous colitis (PMC) or antibiotic-associated diarrhea (AAD). A 0.1-mL amount of a serial 10-fold dilution of the specimens in a thioglycollate medium without dextrose or indicator (Becton Dickinson, Cockeysville, Md., USA) was spread onto a prereduced, amphotericin B-supplemented CCFA plate[10]. After 48-h incubation in an anaerobic chamber (Forma Scientific, Marrieta, OH, USA), suspected *C. difficile* colonies were counted and identified using the ATB 32A system (bioMerieux sa, Marcy-l' Etoile, France). The number of colonies was expressed per mL of original stool specimen. The detection limit of *C. difficile* in this study was 10^2 CFU/mL.

Cytotoxin gene of the isolate was detected by PCR, which was performed using the Mastercycler gradient (Eppendorf, Hamburg, Germany), with 1 μL each of oligonucleotide primers, designed by Gumerlock et al.[11], 1 μL of heat extracted template, AccuPower PCR PreMix (Bioneer Co., Daejeon, Korea) containing 1 U of Taq DNA polymerase. The reaction conditions were: 35 cycles of denaturation at 93 ℃ for 30 seconds, annealing at 58℃ for 30 seconds, and extension at 72℃ for 30 seconds.

Colonoscopy and histologic examination of the biopsy specimens were also performed in 97 suspected CDAD patients, and for these patients, the colonoscopic and histologic findings were compared to the culture results, retrospectively.

RESULTS

The culture of 2,701 specimens yielded 495 (18.3%) unduplicated isolates of *C. difficile*, and of these, 402 (81.2%) were cytotoxin-gene positive (Table. 1). The count of both cytotoxin gene-negative and -positive isolates was in the range $102-\geq 10^6$ CFU/mL. However, among the cytotoxin-positive isolates, 3.2% contained 10^2-$<10^4$ CFU/mL, while 74.9% contained $\geq 10^6$ CFU/mL. Among the cytotoxin-negative isolates, 8.6% contained 10^2-$<10^4$ CFU/mL, while 50.5% contained $\geq 10^6$ CFU/mL. The proportion of cytotoxin gene-positive isolates was higher in the specimens with $\geq 10^6$ CFU/mL of *C. difficile* than in those with 10^2-$<10^3$ CFU/mL (86.5% vs. 66.7%) (Fig. 1).

Table 1. Results of *C. difficile* quantitative culture of fecal specimen from CDAD-suspected patient by cytotoxin gene-positive and-negative strains

Cytotoxin gene	No. (%) of specimens with *C. difficile* (CFU/mL)					Total
	10^2-$<10^3$	10^3-$<10^4$	10^4-$<10^5$	10^5-$<10^6$	$\geq 10^6$	
Positive	4 (1.0)	9 (2.2)	30 (7.5)	58 (14.4)	301 (74.9)	402 (100)
Negative	2 (2.1)	6 (6.5)	16 (17.2)	22 (23.7)	47 (50.5)	93 (100)
Total	6 (1.2)	15 (3.0)	46 (9.3)	80 (16.2)	348 (70.3)	495 (100)

Table 2. Isolation frequency of cytotoxin gene-positive and-negative strains of *C. difficile* from stool specimens according to the patients' diagnosis

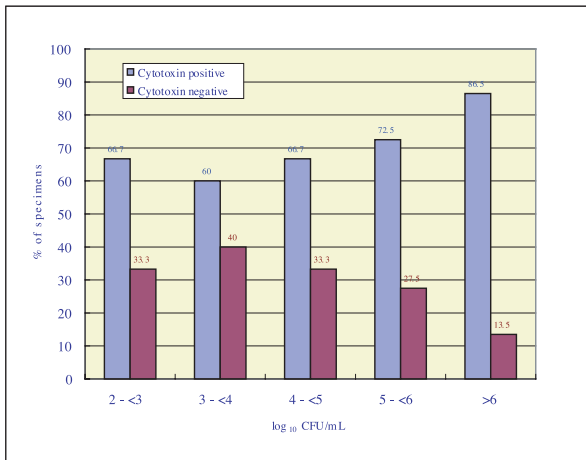| Diagnosis * (No. of patients)	(No. of patients) *C. difficile* (CFU/mL):				No growth
	Cytotoxin gene(+)		Cytotoxin gene(-)		
	10^4-10^5	$\geq 10^6$	10^4-10^5	$\geq 10^6$	($<10^2$)
Pseudomembranous colitis (15)	1^2	9	0	0	5^2
Colitis (60)	1	4	1	2	52
No abnormal finding (22)	2	4	0	1	15
Total (97)	4	17	1	3	72

* By colonoscopy/histologic findings.

Fig. 1. Proportion of cytotoxin-positive *C. difficile* by number of the organisms in the stool specimen.

Among the 97 patients who were examined by colonoscopy and/or histology of biopsy specimens, 15 were diagnosed as having PMC and 60 had findings of colitis, but for the remaining 22, no abnormalities were found (Table 2). Among the specimens from the 15 patients diagnosed as having PMC, 10 yielded cytotoxin gene-positive *C. difficile*: one with 10^4 CFU/mL, and 9 with $\geq 10^6$ CFU/mL. *C. difficile* was not isolated from the specimens of the remaining 5 PMC patients.

Among the specimens from the 60 patients with findings of colitis, the numbers of specimens which yielded 10^4 CFU/mL and $\geq 10^6$ CFU/mL of cytotoxin gene-positive isolates were only 1 and 4, respectively. Three (5%) patients yielded cytotoxin gene-negative isolates and the remaining 52 (86.7%) were negative for *C. difficile* culture. In the 22 patients with no abnormal colonoscopic or histologic findings, 2 (9.1%) and 4 (18.2%) yielded 10^4-10^5 CFU/mL and $\geq 10^6$ CFU/mL of cytotoxin gene-positive isolates, respectively.

DISCUSSION

C. difficile, a major cause of AAD and PMC, is the most frequently identified cause of hospital-acquired diarrhea. CDAD can be treated with oral vancomycin, metronidazole or antibiotics withdrawal[12]. There is some controversies about which detection method is optimal for the diagnosis of CDAD[13]. In regards with the bacteriological method, the culture has been a mainstay in the laboratory diagnosis of CDAD and is essential for the epidemiologic study of nosocomial isolates[14].

However, the culture alone often leads to misdiagnosis of CDAD, because normal adults may carry *C. difficile* in their intestine, but, in this case, their number was reported to be only $\leq 10^2$ CFU/mL[3]. It is well recognized that both PMC and many cases of AAD are due to an overgrowth of cytotoxin-producing *C. difficile*[2], but an evaluation based on quantitative studies are not known. In this study, it was shown that the number of cytotoxin gene-positive *C. difficile* in 99% of the stool of suspected CDAD patients was $\geq 10^3$ CFU/mL (Table 1). In fact, 89.3% of the samples had a count of $\geq 10^5$ CFU/mL. This result supports the notion that an enrichment culture is not necessary for the diagnosis of CDAD. In fact, an enrichment culture may detect asymptomatic carriers, resulting in potentially misleading indications to unnecessary vancomycin therapy.

In our study, the quantitative culture showed some merit to distinguish patients with toxigenic strains from those with nontoxigenic ones (Fig. 1). It may be natural that nontoxigenic isolates can also attain high numbers when there is antimicrobial pressure, as their antibiotic resistance may not be different from that of the toxigenic strains.

C. difficile infection may be associated with a wide spectrum of severity, ranging from mild diarrhea, through severe disease with watery diarrhea, to sometimes fatal PMC[15]. Colonoscopic findings are highly specific, but not sensitive enough for the diagnosis of PMC, because the lesion may be difficult to find in some patients, depending on the site and extent of the lesion[16]. Histological examination also has some limitations, because the biopsy specimens cannot be taken from appropriate site and the findings, in the case of mild CDAD, are not specific[17]. Therefore, diagnosis of CDAD is generally based both on clinical findings and on the presence of cytotoxin-producing *C. difficile* in the stool[18].

Hence, it may seem illogical to evaluate the results of the *C. difficile* culture based on the findings of colonoscopy and histologic examination. However, the reason of comparing the two findings in this study was to determine any difference in the positive rate and number of *C. difficile*. It was reported that the stool of patients with PMC contained 10^7 CFU/mL or more of *C. difficile*[2]. In this study, the number of cytotoxin gene-positive *C. difficile* in the stool of PMC patients was also high, $\geq 10^6$ CFU/mL. One specimen, which yielded only 10^4 CFU/mL of *C. difficile*, and another sample which did not yield any *C. difficile* growth, were taken from two PMC patients after the initiation of anti-*C. difficile* treatment. This implies that prior treatment might suppress the growth of *C. difficile*, partially or completely. Specimen collection, before initiation of antimicrobial therapy, is important in the culture of *C. difficile*, as it is in the case of other bacterial cultures.

It was surprising that the specimens of 5 of the 15 patients in this study, who were diagnosed as having PMC by colonoscopy or microscopy, did not yield *C. difficile*, considering that the organism is associated with 90-100% of cases of PMC[1]. The specimens did not yield growth of *Salmonella* and *Shigella* spp. Although the possibility may be low, other infectious organisms, such as *C. perfringens, Staphylococcus aureus, Klebsiella oxytoca*, or *Candida* species, which have not been examined, may have caused the intestinal lesion[19].

Among the 60 patients who were initially suspected of having CDAD and subsequently categorized as having colitis, based on colonoscopy and histology, only 5 patients yielded cytotoxin gene-positive *C. difficile*. The fact that the vast majority of the suspected CDAD patients did not have the disease indicates the importance of toxigenic culture for the diagnosis of CDAD[17]. It is known that only 10%-20% of all cases of AAD are caused by *C. difficile* and most of the clinically mild AAD cases are due to functional disturbances of intestinal carbohydrate or bile acid metabolism, to the allergic and toxic effects of antibiotics on intestinal mucosa, or to the pharmacological effects on motility[20].

In light of this study, the presence of the organism in very large numbers may represent a useful factor in the interpretation of the culture results. We suggest that the number of *C. difficile* be reported quantitatively by CFU/mL or, at least semi-quantitatively, when a quantitative culture was not performed[9].

The absence of cytotoxin gene-positive *C. difficile* in many specimens collected from suspected CDAD patients indicates the importance of using a culture method, in order to reduce unnecessary therapy. The detection of a high number of cytotoxin gene-positive *C. difficile* by quantitative culture of stool specimens may increase the specificity of the culture.

요 약

배 경 : *Clostridium difficile* 유발 설사 (CDAD)는 입원 환자 설사의 가장 흔한 원인이며, cytotoxin 생성 *C. difficile*에 의해 발생한다. 본 연구에서는 CDAD 진단을 위한 *C. difficile* 정량배양의 유용성을 평가하고자 하였다.

방 법 : 대장내시경과 조직생검 소견을 기준으로 *C. difficile* cytotoxin 유전자와 정량배양결과를 비교 평가하였다.

결 과 : Cytotoxin 양성 402 검체 중 301 (74.9%) 검체에서 분리된 *C. difficile* 균수는 106 CFU/mL 이상이었고, 위막성 대장염 환자 15명중 9명(60%)의 검체에서는 cytotoxin 양성 균수가 106 CFU/mL 이상이었다. Cytotoxin 양성 균주의 비율은 *C. difficile* 수가 10^2-10^3 CFU/mL인 검체에서는 66.7%이었고, 10^6 CFU/mL이상인 검체에서는 86.5%로 더 높았다.

결 론 : *C. difficile* 정량배양이 이 세균 감염 판단에 도움이 되고, CDAD 진단 위양성을 감소시켜서 불필요한 치료를 예방할 수 있다고 판단되었다.

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