Detection of *Clostridium difficile* Toxin A and B Genes from Stool Samples of Thai Diarrheal Patients by Polymerase Chain Reaction Technique

SIRIPAN WONGWANICH, MSc*, SIRIPORN RUGDEEKHA, MSc*,
PINTIP PONGPECH, PhD**, CHERTSAK DHIRAPUTRA, MD, BSc***

Abstract

The prevalence of *Clostridium difficile* isolated from stools of Thai adult patients with suspected antibiotic-associated diarrhea (AAD) was 18.64 per cent.

The recovery rate of toxin genes (*tcdA* and *tcdB*) by polymerase chain reaction (PCR) from stool samples yielded almost the same compared to the recovery rate of the toxin detection by enzyme immunoassay (EIA), which were 44.9 per cent and 46.7 per cent, respectively. Correlation of toxin gene detection by PCR and toxin detection by EIA was 90.6 per cent. All but one stool sample, the *tcdA* gene was detected together with the *tcdB* gene. Both genes were always detected together from *tox* gene-positive strains.

Although, there were some discrepancy results for certain samples, the direct PCR-based-detection of *C. difficile tox* genes in stool samples seems to be the appropriate method for the diagnosis of *C. difficile* diarrhea. The PCR assay should be a recommended technique to be used routinely in laboratories. Further optimization of the technique to increase the sensitivity of the PCR assays is still needed.

However, a quantitative isolation of the organism from stools of suspected antibiotic-associated diarrhea (AAD) or antibiotic-associated colitis (AAC) patients may give some evidence for clinicians in hospitals who cannot perform PCR-based or EIA-based techniques, since 48.6 per cent of the isolates were demonstrated as toxigenic strains.

Key word : *Clostridium difficile*, Diarrhea, Polymerase Chain Reaction

**WONGWANICH S, RUGDEEKHA S,
PONGPECH P, DHIRAPUTRA C
J Med Assoc Thai 2003; 86: 970-975**

* National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi 11000,
** Deparment of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330,
*** Deparment of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.

Toxin producing *Clostridium difficile* (*C. difficile*) is a causative agent of pseudomembranous colitis, antibiotic-associated diarrhea (AAD) and antibiotic-associated colitis (AAC)[1]. Two types of toxins produced by *C. difficile* responsible for causing the diseases[2,3], are toxin A (enterotoxin) and toxin B (cytotoxin). Not all strains can produce two toxins, but most toxigenic strains produce both toxins.

At the present time, the clinical diagnosis of the disease is based on the demonstration of the *C. difficile* toxins in stools. The gold standard technique for the toxin detection is tissue culture assay[4]. This method is time consuming and can produce up to 30 per cent false-negative results due to many factors, such as degradation of the toxins by proteases in stools, the dilution technique used in the detection method, and inclusion of low toxin producing strains[5,6]. Although, immunological approaches, latex agglutination tests or enzyme immunoassays have also been used for the toxin detection in stool samples[7,8], both techniques are costly, time consuming and cumbersome. Recently, molecular biology techniques, particularly the determination of both toxin genes (*tox* genes) using PCR technique, have been developed[9-12].

The PCR technique for the direct detection of *C. difficile tox* genes in the stool of Thai patients suspected of *C. difficile* diarrhea, a comparative study using toxin detection by EIA and *tox* gene detection by PCR method was performed.

MATERIAL AND METHOD

Stool samples

Two hundred and eighty-four diarrheal stools used in the present study were collected from individual admitted patients with suspected *C. difficile* AAD at Siriraj Hospital, Bangkok, Thailand from January 2000 to May 2001. Two hundred and ninety stools from other government and private hospitals were sent to the Anaerobic Bacteria Laboratory at the National Institute of Health (NIH), Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand from March 2000 to May 2001.

Isolation and identification of *C. difficile*

Isolation and identification of *C. difficile* from the stools were performed according to the standard procedure as recommended in Wadworth Anaerobic Bacteriology Manual, fifth edition, 1997[13].

DNA extraction

DNA extraction from all the culture-positive stools was performed using a commercial system (QIAamp DNA stool mini kit, QIAGEN, USA). DNA extraction from toxigenic and nontoxigenic strains of *C. difficile* was also performed as described by Wongwanich et al, 2000[14].

Amplification of toxin A and B genes extracted from bacteria and stools

The eluted samples were used directly for amplification. Internal fragments of toxin A and B genes were amplified as described by Wongwanich et al, 2000. Tag DNA polymerase was used according to the manufacturer's protocol (Promega, USA). NK2 and NK3 were used for toxin A fragment amplification (Kato et al, 1991), and YT17 and YT 18 were used for toxin B fragment amplification (Gumerlock et al, 1993) (Table 1). PCR condition was at 95°C for 20 sec, 55°C for 30 sec, and 60°C for 2 minutes (35 cycles).

Detection of *C. difficile* toxin A and B in stool specimens by EIA

The detection of *C. difficile* toxin A and B of 107 culture-positive and 50 culture-negative stool samples was performed according to the manufactuer's instruction (Premier Cytoclone A+B EIA, Meridian, USA).

RESULTS

The 107 *C. difficile* isolates were obtained from 574 (18.64%) diarrheal stools. Fifty-two of 284 (18.31%) were isolated from the stools of the patients admitted to Siriraj Hospital, and 55 of 290 (18.97%) from NIH.

Table 1. Sequence of the PCR primers.

Gene	Primer	Sequence (5′→3′)	Product
TcdA	NK2	CCC AAT AGA AGA TTC AAT ATT AGG CTT	252
	NK3	GGA AGA AAA GAA CTT CTG GCT CAC TCA GGT	
TcdB	YT17	GGT GGA GCT TCA ATT GGA GAG	399
	YT18	GTG TAA CCT ACT TTC ATA ACA CCA G	

Comparison of the recovery of the *C. difficile tox* genes from the stools and isolates by PCR and toxins by EIA is shown in Table 2. The *tox* A and B genes were recovered from 48 (44.9%) stool samples. Toxin A and toxin B were identified from 50 of the 107 (46.7%) stool samples. The result of both *tox* genes and toxins was recovered in all positive samples except in one stool sample in which only *tox* B gene was recovered. Neither *tox* gene nor toxin was identified from 53 of the 107 (49.5%) stools. Among the 107 isolates from the stools, the *tox* genes were identified in only 52 (48.5%) of them.

Correlation result between the detection of toxins by both PCR and EIA techniques was 97 of 107 (90.6%) of the stool samples.

Fifty-two of 107 (46.8%) isolates were toxigenic strains. Fourteen isolates showed no *tox* gene while *tox* genes were recovered in the stools where these strains were isolated. The number of *tox* gene-negative isolates was increased by 2, when only the EIA technique was used. Fifteen toxin producing strains of the 107 (14.0%) were recovered from the stools in which neither *tox* gene nor toxin was found. Eighteen of the 107 (16.8%) *tox* gene-positive isolates were recovered from the same *tox* gene-negative stools. Fifty culture-negative stools were also studied, and one of them was positive *tox* genes.

DISCUSSION

PCR asssay has been used to identify toxigenic strains of *C. difficile* and *tox* genes directly from the stools of diarrheal patients by several investigators[15-17].

The primer sets used for the detection of *C. difficile* toxin A and B genes were the NK3-NK2 primers as recommended by Kato et al, 1991[9] and YT18-YT17 by Gumerlock et al, 1993[11]. Gumerlock showed the high sensitivity of this pair of primers in the detection of toxin B gene of *C. difficile* (only 1 pg of DNA) and demonstrated the amplified product of 399 bp fragment.

It was found that there was a high correlation (90.6%) between the results of isolation of *tox* genes and toxin A and toxin B from the stool samples.

Fourteen (13.1%) of non toxigenic isolates were observed from the *tox* genes- positive stools. Borriello and Honour has shown that the non-toxigenic strains could be isolated along with toxigenic strains from the same stools of individual patients [18]. Therefore, there was a mixed population of toxigenic and nontoxigenic *C. diffcile* strains in one stool specimen, and there was a possibility that only non toxigenic isolate was selected. Kelly et al, also demonstrated that the limitation of the anaerobic culture was the inability to distinguish toxigenic strains from non toxigenic strains[19]. This indicated that more than one strain of *C. difficile* could be found in one patient.

On the contrary, 18 (16.8%) of toxigenic strains were isolated from the *tox* gene negative stools. The major problems for the failure of the direct detection of the *tox* genes in stool specimens may be the presence of PCR inhibitory substances in the speci-

Table 2. Comparison of the recovery of the *C. difficile tox* genes or toxins from the stools and isolates by PCR and EIA.

Stool sample		Isolate	No. of sample with indicated results	%
Tox genes	Toxins	*Tox* genes		
+	+	+	31	28.97
+	+	-	13	12.15
+	-	+	2	1.87
+	-	-	1	0.93
+*	-	+	1	0.93
-	+	+	3	2.8
-	+	-	3	2.8
-	-	+	15	14.02
-	-	-	38	35.51
Total = 48	Total = 50	Total = 52	Total = 107	

tox genes = toxin A and toxin B genes, toxins = toxin A and toxin B.
* positive only *tox* B gene

mens, and the presence of DNA-degrading enzymes from numerous gut bacteria and ingested foods[20]. Although the sensitivity of the PCR technique allowed the detection of as few as 10 cells of *C. difficile* from a total of 10^{10}-10^{11} bacterial cells present in 1 g in stools[21], specimen processing is a crucial step in PCR assays in order to remove and inactivate substances that inhibit PCR assays[22].

An unexpected result was found, one stool sample recovered only the *tox* B gene but the isolates, from the same specimen yielded *tcdA* and *tcdB* genes, even though the test was repeated twice. Degradation of only the *tox* A gene could be the explanation.

Handling of stool specimens including transportation and processing should also be considered, even though there was only one *tox* gene-positive result from 50 culture-negative stools.

To validate the PCR technique, toxin detection by ELA was also performed. The results of *tox* genes and toxin detection by PCR and EIA detection directly from stools were not concordant in only 9 (8.4%) stool samples. Failure of the toxin detection could be that the amount of toxin in stool specimens was too small due to the degradation by proteases in the feces during transportation to the laboratories in toxin-negative cases[23,24]. On the other hand, the positive toxin detection alone, without positive gene amplification, may be the cross reaction of *C. sordellii* toxin in stools in toxin-positive cases, since this organism produces two toxins which are very similar to toxins A and B of *C. difficile*[25].

The primers used in the present study may have some limitations to detect certain genotypes, because the recent gene sequence of *tcdA* and *tcdB* in the GenBank database showed primer mismatches (accession no. X92982 and AJ011301; Ref. and unpublished data). In order to increase the sensitivity of the PCR, additional sets of primers that can amplify the new variants of *toxin* A and B genes should be included, as this PCR assay is a cheap and convenient tool in the diagnosis of *C. difficile*-related diarrhea.

This is the first study in Thailand using the PCR technique in the detection of *C. difficile* toxin A and B genes directly from stool specimens of adult patients with suspected AAD. The results have shown that it is possible to recommend PCR assay as an appropriate technique used routinely in laboratories. Since the technique is easy to perform and provide rapid and reliable results, the modified methods for removal or inactivation of PCR inhibitors in fecal specimens and improvement of sensitivity and specificity of primers should be considered.

However, quantitative isolation of the organism from the stools of suspected AAD or AAC patients may help clinicians in hospitals who can not perform PCR-based or EIA-based techniques, since about half of the isolates were demonstrated to be toxigenic strains.

ACKNOWLEDGEMENT

This study was partly supported by the Ministry of University Affairs.

(Received for publication on July 29, 2003)

REFERENCES

1. Lyerly DM, Krican HC, Wilkins TD. *Clostridum difficile* : Its disease and toxins. Clin Microbiol Rev 1988; 1: 1-18.
2. Bartlett JG. *Clostridium difficile*: History of its role as an enteric pathogen and the current state of knowledge about the organism. Clin Infect Dis 1994; 18 (Suppl 4); 265-72.
3. Fluit AC, Wolfhagen MJ, Verdonk GP, et al. Non-toxigenic strains of *Clostridium difficile* lack the genes for both toxin A and B. J Clin Microbiol 1991; 29: 2666-7.
4. Pothoulakis C, LaMont, JT. *Clostridium difficile* colitis and diarrhea. Gastroenterol. Clin North Am 1993; 22: 623-37.
5. Cleary RK. *Clostridium difficile*-associated diarrhea and colitis. Dis Colon Rectum 1998; 41: 1435-49.
6. Alonso R, Munoz C, Gros S, et al. Rapid detection of toxigenic *Clostridium difficile* from stool samples by a nested PCR of toxin B gene. J Hosp Infect 1999; 41: 145-9.
7. Laughon BE, Viscidi RP, Gdovin SL, Yolken RH, Bartlett JG. Enzyme immunoassays for detection of *Clostridium difficile* toxins A and B in fecal specimens. J Infect Dis 1984; 149: 781-8.
8. Lyerly DM, Sullivan NM, Wilkins TD. Enzyme-linked immunosorbent assay for *Clostridium difficile* toxin A. J Clin Microbiol 1983; 17: 72-8.
9. Kato N, Ou CY, Kato H, et al. Identification of toxigenic *Clostridium difficile* by polymerase chain reaction. J Clin Microbiol 1991; 29: 33-7.
10. Wren BW, Clayton CL, Tabaqchali S. Rapid identification of toxigenic *Clostridium difficile* by polymerase chain reaction. Lancet 1990; 335: 423.
11. Gumerlock PH, Tang YT, Weiss JB, Silva J. Specific detection of toxigenic strains of *Clostridium difficile* in stool specimens. J Clin Microbiol 1993; 31: 507-11.
12. Kato H, Kato N, Watanabe K, et al. Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. J Clin Microbiol 1998; 36: 2178-82.
13. Summanen P, Baron EJ, Citron DM, et al. Wadworth anaerobic bacteriology manual, fifth edition, Los Angeles, California, Star Publishing Company: 1997: 95-102.
14. Wongwanich S, Pongpech P, Dhiraputra C, Huttayananont S, Sawanpayalert P. Characteristics of *Clostridium difficile* strains isolated from asymptomatic individuals and from diarrheal patients. Clin Infect Dis 2001; 7: 438
15. Boondeekhun HS, Gurtler V, Odd ML, Wilson VA, Mayall BC. Detection of *Clostridium difficile* enterotoxin gene in clinical specimens by the PCR. J Med Microbiol 1993; 38: 384-7.
16. Kuhl SJ, Tang YT, Navarro L, Gumerlock PH, Silva J Jr. Diagnosis and monitoring of *Clostridium difficile* infections with the polymerase chain reaction. Clin Infect Dis 1993; 16 (Suppl 4); 234-8.
17. Kato N, Ou CY, Kato H, et al. Detection of toxigenic *Clostridium difficile* in stool specimens by the PCR. J Infect Dis 1993; 167: 455-8.
18. Borriello SP, Honour P. Concomitance of cytotoxigenic and noncytotoxigenic *Clostridium difficile* in stool specimens. J Clin Microbiol 1983; 18: 1006.
19. Kelly CP, Polhoulakis C, LaMont JT. *Clostridium difficile* colitis. N Engl J Med 1994; 330: 257-62.
20. Greenfield L, White TJ. Sample preparation methods. *In* Persing DH, Smith TF, Tenover FC, and White TJ. (ed). Diagnostic molecular microbiology: Principles and application. American society for Microbiology, Washington DC, ASM Press: 1993: 122-37.
21. Gumerlock PH, Tang YJ, Meyers FJ, Silva J. Use of the polymerase chain reaction for the specific and direct detection of *Clostridium difficile* in human feces. Rev Infect Dis 1991; 13: 1053-60.
22. Monteiro L, Bonnemaison D, Vekris A, et al. Complex polysaccharides as PCR inhibitors in Feces: *Helicobactor pylori* model. J Clin Microbiol 1997; 35: 995-8.
23. Knoop FC, Owens M, Crocker IC. *Clostridum difficile*: Clinical disease and diagnosis. Clin Microbiol Rev 1993; 6: 251-65.
24. Merz CS, Kramer C, Forman M, et al. Comparison of four commercially available rapid enzyme immunoassays with cytotoxin assay for detection of *Clostridium difficile* toxin(s) from stool specimens. J Clin Microbiol 1994; 32: 1142-7.
25. Lyerly DM, Nevilie LM, Evans DT, et al. Multicenter evaluation of the *Clostridium difficile* TOX A/B test. J Clin Microbiol 1998; 36: 184-90.

การตรวจหายีนส์สร้างสารพิษชนิด A และ B ของ *Clostridium difficile* ในตัวอย่างอุจจาระของผู้ป่วยไทยที่เป็นโรคอุจจาระร่วง โดยใช้เทคนิค Polymerase Chain Reaction

ศิริพรรณ วงศ์วานิช, วทม, ศิริพร รักดีแช, วทม*,*
*พิณทิพย์ พงษ์เพ็ชร, ปรด**, เชิดศักดิ์ ธีระบุตร, พบ, วทม****

อัตราการตรวจพบเชื้อ *Clostridium difficile* ร้อยละ 18.64 ในผู้ป่วยไทยที่เป็นโรคอุจจาระร่วงที่สงสัยว่าเกิดจากสาเหตุที่ได้รับการรักษาด้วยยาปฏิชีวนะ เมื่อเปรียบเทียบผลการตรวจหายีนส์สร้างสารพิษชนิด A และ B ของเชื้อ *C. difficile* จากตัวอย่างอุจจาระโดยตรงด้วยเทคนิค Polymerase Chain Reaction (PCR) และ วิธี Enzyme Immunoassay (EIA) พบว่าให้ผลบวกใกล้เคียงกันคือร้อยละ 44.9 และ 46.7 ตามลำดับ และทั้งสองวิธีให้ผลสอดคล้องกันร้อยละ 90.6 ตัวอย่างอุจจาระทั้งหมดที่ศึกษาแต่ยกเว้นเพียง 1 ตัวอย่าง ตรวจพบยีนส์สร้างสารพิษทั้งสองชนิดของเชื้อ *C. difficile* และตรวจพบยีนส์ทั้งสองในสายพันธ์เชื้อ *C. difficile* ที่แยกพบด้วย

ถึงแม้ว่ามีอุจจาระบางตัวอย่างให้ผลไม่คงที่ แต่การใช้วิธี PCR ตรวจหายีนส์สร้างสารพิษของเชื้อ *C. difficile* ในตัวอย่างอุจจาระโดยตรง ก็น่าจะเป็นวิธีที่เหมาะสมนำไปใช้ในงานประจำของห้องปฏิบัติการเพื่อตรวจวินิจฉัยโรคอุจจาระร่วงที่มีสาเหตุจาก *C. difficile* โดยจำเป็นต้องมีการปรับเทคนิค PCR ให้สมบูรณ์และมีความไวมากยิ่งขึ้น

อย่างไรก็ตามการตรวจแยกหาเชื้อ *C. difficile* จากอุจจาระผู้ป่วยที่สงสัยว่าเป็นโรคอุจจาระร่วงที่เกี่ยวกับการได้รับยาปฏิชีวนะ (antibiotic-associated diarrhea และ antibiotic-associated colitis) จะเป็นข้อมูลสำคัญที่แพทย์นำไปใช้ประกอบการรักษาในโรงพยาบาลที่ยังไม่มีการนำวิธี PCR หรือ EIA มาใช้ โดยตรวจพบว่า *C. difficile* ที่แยกพบร้อยละ 48.6 เป็นสายพันธ์ที่ก่อโรคได้เพราะมียีนส์สร้างสารพิษ

คำสำคัญ : *Clostridium difficile*, โรคอุจจาระร่วง, Polymerase Chain Reaction

ศิริพรรณ วงศ์วานิช, ศิริพร รักดีแช,
พิณทิพย์ พงษ์เพ็ชร, เชิดศักดิ์ ธีระบุตร
จดหมายเหตุทางแพทย์ ฯ 2546; 86: 970-975

* สถาบันวิจัยวิทยาศาสตร์สาธารณสุข, กรมวิทยาศาสตร์การแพทย์, กระทรวงสาธารณสุข, นนทบุรี 11000
** ภาควิชาจุลชีววิทยา, คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย, กรุงเทพ ฯ 10330
*** ภาควิชาจุลชีววิทยา, คณะแพทย์ศาสตร์ศิริราชพยาบาล, มหาวิทยาลัยมหิดล, กรุงเทพ ฯ 10700