Original Article

http://dx.doi.org/10.3349/ymj.2014.55.4.994
pISSN: 0513-5796, eISSN: 1976-2437

Yonsei Med J 55(4):994-998, 2014

YMJ

Evaluation of a Chromogenic Culture Medium for the Detection of *Clostridium difficile*

John Jeongseok Yang,[1,2] You Sun Nam,[3] Min Jin Kim,[2] Sun Young Cho,[1] Eunkyung You,[1,2] Yun Soo Soh,[2] and Hee Joo Lee[1]

[1]Department of Laboratory Medicine, School of Medicine, Kyung Hee University, Seoul;
Departments of [2]Medicine and [3]Biomedical Science, Kyung Hee University Graduate School, Seoul, Korea.

Received: July 31, 2013
Revised: November 6, 2013
Accepted: November 28, 2013
Corresponding author: Dr. Hee Joo Lee, Department of Laboratory Medicine, School of Medicine, Kyung Hee University, 23 Kyungheedae-ro, Dongdaemun-gu, Seoul 130-872, Korea.
Tel: 82-2-958-8672, Fax: 82-2-958-8609
E-mail: leehejo@khmc.or.kr

· The authors have no financial conflicts of interest.

Purpose: *Clostridium difficile* (*C. difficile*) is an important cause of nosocomial diarrhea. Diagnostic methods for detection of *C. difficile* infection (CDI) are shifting to molecular techniques, which are faster and more sensitive than conventional methods. Although recent advances in these methods have been made in terms of their cost-benefit, ease of use, and turnaround time, anaerobic culture remains an important method for detection of CDI. **Materials and Methods:** In efforts to evaluate a novel chromogenic medium for the detection of *C. difficile* (chromID CD agar), 289 fecal specimens were analyzed using two other culture media of blood agar and cycloserine-cefoxitin-fructose-egg yolk agar while enzyme immunosorbent assay and polymerase chain reaction-based assay were used for toxin detection. **Results:** ChromID showed the highest detection rate among the three culture media. Both positive rate and sensitivity were higher from chromID than other culture media. ChromID was better at detecting toxin producing *C. difficile* at 24 h and showed the highest detection rate at both 24 h and 48 h. **Conclusion:** Simultaneous use of toxin assay and anaerobic culture has been considered as the most accurate and sensitive diagnostic approach of CDI. Utilization of a more rapid and sensitive chromogenic medium will aid in the dianogsis of CDI.

Key Words: *Clostridium difficile*, chromogenic, culture

INTRODUCTION

Clostridium difficile infection (CDI) is a frequent and important cause of nosocomial diarrhea, especially in developed countries. Accurate and prompt diagnosis is an important aspect for monitoring and controlling of outbreaks, which tend to be more severe and fulminant.[1] Toxin detection is most commonly achieved by cost-efficient enzyme immunosorbent assays (EIAs); however, their positive predictive value depends on the prevalence of CDI, and false negative results due to low sensitivity is a limitation for their use in the diagnosis of CDI.[2-4]

Polymerase chain reaction (PCR) assays are being favored for their high sensitivity. Also, FDA approved, commercial ready-to-use PCR assays are currently available.[4-7] These assays offer high sensitivity and specificity compared to toxi-

genic culture although their cost-effectiveness has been questioned.[3,6]

Since no single test is sufficient for the diagnosis of CDI due to the individual limitations of each method, algorithmic approaches are being suggested instead of stand-alone tests. Anaerobic stool culture still plays an important role as a confirmatory test in these suggested algorithms for a more prompt and accurate diagnosis of CDI.[8-13] Anaerobic stool culture remains an important tool in regions of East Asia including Korea and mainly for epidemiologic surveillance which requires isolation of the causative organism.[14] To improve the time-consuming aspect of anaerobic culture, a new chromogenic culture medium for the isolation of toxigenic *C. difficile* (chromID CD agar, bioMerieux, Craponne, France) has been introduced, and has shown to facilitate the rapid and effective detection of CDI within 24 hours.[15] The ability of chromID to detect toxigenic strains of *C. difficile* within 24–48 hours is a notable improvement from conventional anaerobic stool culture media. As use of anaerobic culture, in addition to confirmation of toxin production, improves the accuracy of diagnosis, the use of culture is encouraged.[16,17]

A one-step selective and differential agar-based assay enabling the detection and isolation of active toxin producing *C. difficile* directly from stool samples, namely the Cdifftox plate assay, has also been introduced.[18] The use of glucosyltransferase activities of the A and B toxins is a novel approach which deserves further extensive evaluation whether molecular assays for detection of toxigenic strains could be replaced by this one-step assay.

The aims of this study are to evaluate three culture media of chromID, cycloserine cefoxitin-fructose-egg yolk agar (CCFA) and blood agar plates (BAP) of their recovery rate, detection rate of toxin producing *C. difficile* and the 24 h detection rate of toxin producing *C. difficile*, respectively.

This study was approved by Kyung Hee University Hospital Institutional Review Board (KMC IRB 1316-06) and was partially presented as an abstract at the 53rd Annual Meeting of the Korean Society for Laboratory Medicine (P-113) as a poster presentation.

MATERIALS AND METHODS

Study design and stool specimens

Stool samples requested for analysis of *C. difficile* toxin using EIA assay of VIDAS (bioMérieux, Marcy-l'Étoile, France) were selected for evaluation. A total of 289 specimens were collected from two different periods. Ninty-nine frozen retrospective samples were collected from October 2010 to February 2011, and 190 fresh samples were collected from August 2011 to October 2011 at a tertiary care university hospital in Korea. An analysis of the retrospective samples was done at the time of initial request for EIA in fresh condition using VIDAS assay. Thawed samples which had been stored at -70°C were analyzed again for this study using chromID, CCFA, BAP media and PCR assay.

Fresh specimens were stored at room temperature if they were to be plated within 2 h of collection; otherwise, they were kept at 4°C for two days until processing. All stool specimens were processed with ethanol in advance to exclude non-spore-forming contaminating flora. Aliquots were inoculated into culture media at amount of 40 uL.

Toxin assay

VIDAS *C. difficile* A & B (bioMérieux, Lyon, France) was used for the qualitative detection of toxins A and B from stool specimens, as instructed by the manufacturer. All specimens in this study were requested for *Clostridium difficile* toxin assay (CDTA) by clinicians for suspected CDI.

Culture media, anaerobic stool culture, and colony identification

ChromID was used as pre-poured plates supplied from bioMérieux. The media for comparison, CCFA (Komed, Seongnam, Korea) and BAP (Hangang, Gunpo, Korea) were also used as pre-poured plates. Following anaerobic culture at 37°C for 24 h, the samples were inspected for no longer than 30 min for colony identification then cultured anaerobically for an additional 24 h. Anaerobic atmosphere was generated using GasPak EZ Anaerobe Pouch System (Becton Dickinson and Company, Franklin Lakes, NJ, USA).

Colony identification was performed by experienced laboratory personnel, considering only those samples with the typical morphology of *C. difficile* as positive. Typical morphology of *C. difficile* was gray to black colony with smooth or irregular border as seen from chromID (Fig. 1). For BAP and CCFA, typical gray-brown flat colonies with a characteristic odor were suspected as *C. difficile*.

All colonies from three culture media were initially processed by Gram staining and observed under light microscopy. Through Gram staining and API Rapid ID 32A (bioMérieux Inc., Durham, NC, USA) were colonies recovered from culture media identified as *C. difficile*.

PCR assays

In addition to EIA, two molecular assays were used in this study to confirm the presence of toxigenic *C. difficile* isolates. AdvanSure CD Real-Time PCR Kit (LG Life Science, Seoul, Korea) was used for the detection of *tcdA* and *tcdB* production from pathogenicity locus (PaLoc)-harboring toxigenic *C. difficile*, and Illumigene *C. difficile* DNA amplification assay (Meridian Bioscience, Cincinnati, OH, USA) for the detection of *tcdA* gene, respectively. All tests were conducted according to the manufacturers' instructions. Isolated colonies from anaerobic stool culture were used for PCR assays in this study.

Interpretation of test results

In order to compare the performance of each culture media, gram positive isolates with negative toxin production results were considered only culture positive for *C. difficile*.

Statistical analyses

All statistical analyses were carried out using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). McNemar's test was used to compare the agreement of three culture media. Statistical significance was determined at p value of <0.05.

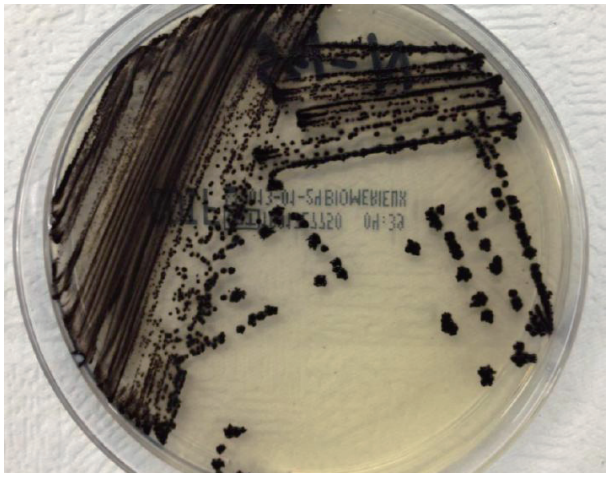

Fig. 1. Culture of *Clostridium difficile* from stool specimen showing characteristic black colonies after 24 h.

RESULTS

Positive rates of test methods

Positive rate, detection rate of toxin producing *C. difficile*, and sensitivity of test methods are shown in Table 1. From the 99 retrospective specimens, chromID, BAP and CCFA were able to recover 26, 17, and 5 isolates. And from the 190 prospective specimens, 47, 32, and 15 isolates of *C. difficile* were recovered by chromID, BAP and CCFA.

Toxin assay using VIDAS showed positive results in 7 and 22 specimens from retrospective and prospective specimens. The results of PCR assays from retrospective and prospective specimens were positive in 14 and 36 specimens by Illumigene, and 13 and 34 specimens by Advansure. In total, chromID recovered 73 isolates, followed by 49 by BAP and 20 by CCFA. The sensitivities from the total number of specimens were 97.9%, 71.4%, and 30.6% for chromID, BAP and CCFA, respectively.

Detection of *C. difficile* at 24 and 48 h

Table 2 compares the abilities of the three culture media to detect isolates of *C. difficile* at 24 h and 48 h. From retrospective specimens, chromID showed the highest detection rate of *C. difficile* at 24 h; detecting all 12 true positives at 24 h while BAP and CCFA showed detection rates of 75.0% and 8.3%, respectively. Two additional toxigenic *C. difficile* isolates were recovered by CCFA after 48 h, although its overall performance of 25% after 48 h was comparably lower than chromID and BAP.

From the results of fresh, prospective specimens, sensitivities at 24 h were 91.9% by chromID, 64.9% by BAP and 21.6% by CCFA. Two more true positives were detected by chromID at 48 h showing 97.3% sensitivity at 48 h.

Table 1. Positive Rate, Detection Rate of Toxin Producing *C. difficile* and Sensitivity (%) of Tests Methods

	Retrospective (n=99)		Prospective (n=190)		Total (n=289)		Sensitivity
	Positive	True positive*	Positive	True positive	Positive	True positive	
Toxin assay (EIA)	7	6	22	21	29	27	55.1
Culture							
ChromID	26	12	47	36	73	48	98.0
BAP	17	10	32	25	49	35	71.4
CCFA	5	3	15	12	20	15	30.6

EIA, enzyme immunosorbent assays; chromID, chromogenic agar for *C. difficile*; BAP, blood agar plate; CCFA, cycloserine-cefoxitin-fructose-egg yolk agar; *C. difficile*, *Clostridium difficile*.

*Isolates of *C. difficile* demonstrating toxin producing ability.

Table 2. Time Dependent Detection Rate of Three Culture Media and Sensitivity (%) at 24 Hours

Culture	Retrospective (n=99)			Prospective (n=190)		
	24 hrs	48 hrs	Sensitivity (24 hrs)	24 hrs	48 hrs	Sensitivity (24 hrs)
ChromID	12/12	12/12	100%	34/37	36/37	91.9%
BAP	9/12	10/12	75.0%	24/37	25/37	64.9%
CCFA	1/12	3/12	8.3%	8/37	12/37	21.6%

chromID, chromogenic agar for *C. difficile*; BAP, blood agar plate; CCFA, cycloserine-cefoxitin-fructose-egg yolk agar; *C. difficile, Clostridium difficile.*

Another true positive was detected by BAP at 48 h, and sensitivity at 48 h was 67.6%. CCFA was able to detect four more true positives, 32.4% was its sensitivity at 48 h.

When the three culture media were compared against each other, chromID and BAP had the highest agreement (kappa=0.73, $p<0.01$), while BAP and CCFA had less agreement (kappa=0.41, $p<0.01$) and chromID and CCFA had the least agreement (kappa=0.36, $p<0.01$).

DISCUSSION

From our comparison of three culture media, the newly introduced chromID showed excellent performance in identification of toxin producing *C. difficile*. ChromID has been previously described as a novel chromogenic agar containing enzyme substrate which is hydrolyzed as a black colony is generated.[15,19] This selective agar for *C. difficile* is based on supplementation of taurocholate and undisclosed selective mixture while inclusion of a more suitable germinant is accountable for superior number of colonies recovered. ChromID is also advantageous as black colonies are in contrast with its clear background.[15] It is notable that the ability to detect most toxigenic *C. difficile* isolates, regarded as true positives in this study, within 24 h is an important aspect when utilizing anaerobic stool culture in clinical practice. Although acknowledging that still a considerable amount of delay exist using culture compared to EIA or PCR assays, improvement of culture is a favorable aspect.

Our findings are consistent with those of a previous report showing that chromID displayed superior performance in detecting toxigenic *C. difficile*. While currently suggested algorithmic approaches are based on culture media other than chromID,[10] the performance of these algorithms could be enhanced by the use of chromID in both terms of time and accuracy.

The limitations of our study are that the specimens were obtained at a single-center study with a limited number of positive *C. difficile* cases in which the prevalence of CDI may differ from other environmental settings. While the performances of BAP and CCFA were considerably lower than chromID, they may not have been the best selective culture media currently available for comparison. It has been reported that the detection rate on CCFA could benefit from a spore germination enhancer; we suspect its absence as a possible factor for the comparably lower detection rate of CCFA in this study.[20] Additionally, the overall detection rates were lower than previous reports using anaerobic culture.[15] A likely explanation for this is that the number of specimens included in this study was considerably lower, prevalence rates and epidemiology may have been different from other institutions.

A prominent feature of chromID is its chromogenicity, which makes the use and interpretation of this medium relatively easy. While gray-black colonies on chromID are suggestive of a *C. difficile* isolate, not all gray-black colonies are *C. difficile*, nor are colonies of other colors necessarily non-*C. difficile*.[15] Possible confusion may originate from the chromogenicity of chromID; therefore, careful interpretation of colonies is required, while its use by skilled experienced personnel is clearly a benefit. The results from this study is consistent with the manufacturer's claims that higher detection rate within 24 h can be achieved by chromID.

As the changing epidemiology of this common nosocomial pathogen requires more attention, improved diagnostic approaches are in need. A recent study adds the use of chromogenic medium for hospital environmental screening,[21] as contamination of *C. difficile* spore correlates with high frequency of CDI.[22] The incorporation of a novel chromogenic culture medium into suggested diagnostic algorithms could aid both in the diagnosis and epidemiologic study of toxigenic *C. difficile* by increasing the detection rate.

ACKNOWLEDGEMENTS

This work was supported by a grant from Kyung Hee University (KHU-20120797).

The authors thank bioMerieux for providing chromID used in this study.

REFERENCES

1. Kuijper EJ, Barbut F, Brazier JS, Kleinkauf N, Eckmanns T, Lambert ML, et al. Update of Clostridium difficile infection due to PCR ribotype 027 in Europe, 2008. Euro Surveill 2008;13:18942.
2. Alcalá L, Sánchez-Cambronero L, Catalán MP, Sánchez-Somolinos M, Peláez MT, Marín M, et al. Comparison of three commercial methods for rapid detection of Clostridium difficile toxins A and B from fecal specimens. J Clin Microbiol 2008;46:3833-5.
3. Tenover FC, Baron EJ, Peterson LR, Persing DH. Laboratory diagnosis of Clostridium difficile infection can molecular amplification methods move us out of uncertainty? J Mol Diagn 2011;13:573-82.
4. Sloan LM, Duresko BJ, Gustafson DR, Rosenblatt JE. Comparison of real-time PCR for detection of the tcdC gene with four toxin immunoassays and culture in diagnosis of Clostridium difficile infection. J Clin Microbiol 2008;46:1996-2001.
5. Doing KM, Hintz MS. Prospective evaluation of the Meridian Illumigene™ loop-mediated amplification assay and the Gen Probe ProGastro™ Cd polymerase chain reaction assay for the direct detection of toxigenic Clostridium difficile from fecal samples. Diagn Microbiol Infect Dis 2012;72:8-13.
6. Peterson LR, Manson RU, Paule SM, Hacek DM, Robicsek A, Thomson RB Jr, et al. Detection of toxigenic Clostridium difficile in stool samples by real-time polymerase chain reaction for the diagnosis of C. difficile-associated diarrhea. Clin Infect Dis 2007;45:1152-60.
7. Peterson LR, Mehta MS, Patel PA, Hacek DM, Harazin M, Nagwekar PP, et al. Laboratory testing for Clostridium difficile infection: light at the end of the tunnel. Am J Clin Pathol 2011;136:372-80.
8. Fenner L, Widmer AF, Goy G, Rudin S, Frei R. Rapid and reliable diagnostic algorithm for detection of Clostridium difficile. J Clin Microbiol 2008;46:328-30.
9. Knetsch CW, Bakker D, de Boer RF, Sanders I, Hofs S, Kooistra-Smid AM, et al. Comparison of real-time PCR techniques to cytotoxigenic culture methods for diagnosing Clostridium difficile infection. J Clin Microbiol 2011;49:227-31.
10. Novak-Weekley SM, Marlowe EM, Miller JM, Cumpio J, Nomura JH, Vance PH, et al. Clostridium difficile testing in the clinical laboratory by use of multiple testing algorithms. J Clin Microbiol 2010;48:889-93.
11. Schmidt ML, Gilligan PH. Clostridium difficile testing algorithms: what is practical and feasible? Anaerobe 2009;15:270-3.
12. Swindells J, Brenwald N, Reading N, Oppenheim B. Evaluation of diagnostic tests for Clostridium difficile infection. J Clin Microbiol 2010;48:606-8.
13. Wilcox MH, Planche T, Fang FC, Gilligan P. What is the current role of algorithmic approaches for diagnosis of Clostridium difficile infection? J Clin Microbiol 2010;48:4347-53.
14. McDonald LC, Killgore GE, Thompson A, Owens RC Jr, Kazakova SV, Sambol SP, et al. An epidemic, toxin gene-variant strain of Clostridium difficile. N Engl J Med 2005;353:2433-41.
15. Perry JD, Asir K, Halimi D, Orenga S, Dale J, Payne M, et al. Evaluation of a chromogenic culture medium for isolation of Clostridium difficile within 24 hours. J Clin Microbiol 2010;48:3852-8.
16. Blossom DB, McDonald LC. The challenges posed by reemerging Clostridium difficile infection. Clin Infect Dis 2007;45:222-7.
17. Cho SY, Nam YS, Kim MJ, Suh JT, Lee HJ, Kim H. Detection of Clostridium difficile as a routine diagnosis: comparison of real-time PCR and enzyme immunoassay. Am J Clin Pathol 2012;137:494-5.
18. Darkoh C, Dupont HL, Kaplan HB. Novel one-step method for detection and isolation of active-toxin-producing Clostridium difficile strains directly from stool samples. J Clin Microbiol 2011;49:4219-24.
19. Eckert C, Burghoffer B, Lalande V, Barbut F. Evaluation of the chromogenic agar chromID C. difficile. J Clin Microbiol 2013;51:1002-4.
20. Foster NF, Riley TV. Improved recovery of Clostridium difficile spores with the incorporation of synthetic taurocholate in cycloserine-cefoxitin-fructose agar (CCFA). Pathology 2012;44:354-6.
21. Hill KA, Collins J, Wilson L, Perry JD, Gould FK. Comparison of two selective media for the recovery of Clostridium difficile from environmental surfaces. J Hosp Infect 2013;83:164-6.
22. Wilcox MH, Fawley WN, Wigglesworth N, Parnell P, Verity P, Freeman J. Comparison of the effect of detergent versus hypochlorite cleaning on environmental contamination and incidence of Clostridium difficile infection. J Hosp Infect 2003;54:109-14.