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

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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 diagnosis 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.²⁴ 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 Use of these assays offer high sensitivity and specificity compared to tox-
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.8-11 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 Cdiffix 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. Ninety-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 (CTDA) 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 \textit{C. difficile} isolates. AdvanSure CD Real-Time PCR Kit (LG Life Science, Seoul, Korea) was used for the detection of \textit{tcdA} and \textit{tcdB} production from pathogenicity locus (PaLoc)-harboring toxigenic \textit{C. difficile}, and Illumigene \textit{C. difficile} DNA amplification assay (Meridian Bioscience, Cincinnati, OH, USA) for the detection of \textit{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 \textit{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.

RESULTS

Positive rates of test methods
Positive rate, detection rate of toxin producing \textit{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 \textit{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 \textit{C. difficile} at 24 and 48 h
Table 2 compares the abilities of the three culture media to detect isolates of \textit{C. difficile} at 24 h and 48 h. From retrospective specimens, chromID showed the highest detection rate of \textit{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 \textit{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 \textit{C. difficile} and Sensitivity (%) of Tests Methods**

|                      | Retrospective (n=99) | Prospective (n=190) | Total (n=289) | Sensitivity |
|----------------------|----------------------|---------------------|---------------|-------------|
| Toxin assay (EIA)    |                      |                     |               | 55.1        |
| Positive             | 7                    | 6                   | 22            | 21          | 29          | 27          |               |
| True positive*       |                      |                     |               |             |             |             |               |
| 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 \textit{C. difficile}; BAP, blood agar plate; CCFA, cycloserine-cefoxitin-fructose-egg yolk agar; \textit{C. difficile}, \textit{Clostridium difficile}.

*Isolates of \textit{C. difficile} demonstrating toxin producing ability.
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 gerninant 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,19 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.

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