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Isolation and Identification of *Clostridium difficile* Using ChromID *C. difficile* Medium Combined With Gram Staining and PRO Disc Testing: A Proposal for a Simple Culture Process

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Background: ChromID *C. difficile* agar (CDIF; bioMérieux, France), a chromogenic medium, allows for the isolation and identification of *Clostridium difficile* strains within 24 hr regardless of pretreatment of stool specimens with heat or alcohol shock. In the present study, we designed and evaluated a simple procedure for the implementation *C. difficile* cultures using CDIF medium in a tertiary hospital setting.

Methods: We designed a simple protocol for untreated stool specimens using CDIF medium followed by Gram staining and PRO disc (PRO disc K1532B, Key Scientific Products, USA) testing for the identification of *C. difficile* in colonies produced on CDIF agar. A total of 1,402 prospectively collected stool specimens from patients with suspected *C. difficile* infection were tested. The protocol was evaluated by phenotypic or molecular identification of *C. difficile* using Vitek 2 ANC card (bioMérieux) or 16S rDNA/*tpi* gene sequencing, respectively.

Results: Of 1,402 stool specimens, 650 isolates were cultured in CDIF. Overall, 235 (36.2%, 235/650) strains could be presumptively identified as *C. difficile* by using Gram staining and PRO disc testing. Of those, 231 (98.3%, 231/235) isolates were confirmed as true *C. difficile* by molecular assays.

Conclusions: The use of CDIF combined with Gram staining and PRO disc testing of untreated stool specimens would allow for isolation and accurate identification of *C. difficile* strains and would be advantageous in reducing the multistep process for *C. difficile* culture.

Key Words: *Clostridium difficile*, Culture, Isolation

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INTRODUCTION

Clostridium difficile causes infectious nosocomial diarrhea. There are various test methods for the diagnosis of *C. difficile* infection, including enzyme immunoassays (EIA) for toxin detection, glutamate dehydrogenase testing, anaerobic toxigenic culture, cell culture neutralization assays, and nucleic acid amplification methods. Of these, anaerobic toxigenic culture has been ac-

cepted as the standard because culture is the most sensitive method [1].

There are various selective culture media for *C. difficile*. Since cycloserine cefoxitin fructose agar (CCFA) was developed by George *et al*. [2], modifications with taurocholate or lysozyme have been proposed to improve sensitivity [3-5]. Several studies reported that pretreatment of stool with heat or alcohol shock can enhance the sensitivity of culture [6, 7] because only spore-forming organisms can survive this procedure, thus eliminating the growth of other, non-spore forming fecal organisms. Once colonies are isolated on selective media, tests for toxigenicity, such as cell culture cytotoxin neutralization, toxin EIA, or PCR, should be performed.

Recently, a chromogenic medium for *C. difficile*, chromID *C. difficile* agar [CDIF]), has been developed commercially [8, 9]. According to the manufacturer, CDIF can detect and identify β-glucosidase-producing *C. difficile* strains within 24 hr based on the presence of grey-to-black colonies with irregular or smooth borders. In addition, CDIF medium can be used to isolate *C. difficile* both with and without the use of alcohol-shock treatment. However, when treated stool specimens are used, additional biochemical or molecular tests on the colonies are not recommended. In addition, colonies produced on CDIF agar should not be used with an automated analyzer for identification, such as with the VITEK 2 ANC identification card. If these procedures are performed following culture in CDIF medium, the stool specimens need to be inoculated onto another anaerobic culture medium before identification of *C. difficile* or testing for the presence of *C. difficile* toxin.

To grow *C. difficile* cultures using CDIF medium in our laboratory, we needed to develop a simple procedure using untreated stool specimens. Therefore, we designed and evaluated a simple procedure for the implementation *C. difficile* cultures using CDIF medium in a tertiary hospital setting.

METHODS

1. Study design

Ethical approval was not required because this study was for clinical laboratory testing that was not subject to human subjects review. We designed a protocol for culturing *C. difficile* in CDIF medium without pretreating the specimen, followed by Gram staining and PRO disc (PRO disc K1532B, Key Scientific Products, Round Rock, TX, USA) testing [10] for the identification of *C. difficile* in colonies produced on CDIF agar. To confirm the utility of this protocol, we prospectively collected a total of 1,402 stool specimens from patients with suspected *C. difficile* infection from November 2011 to March 2012 at Samsung Medical Center in Seoul, Korea. On arrival at the clinical microbiologic laboratory, these specimens were immediately tested by using the *C. difficile* toxin A & B immunoassay (CDAB; Vidas CDAB assay, bioMérieux, Marcy-l'Etoile, France) and inoculated into CDIF. In addition to Gram staining and PRO disc testing, the cultured isolates were subjected to automated analysis by using the Vitek 2 ANC kit (bioMérieux) and 16S rDNA sequencing for the identification of *C. difficile*. Fig. 1 presents a schematic overall process for confirmation of our protocol.

2. *C. difficile* toxin A & B immunoassay

CDAB is an automated enzyme-linked fluorescent immunoassay (ELFA). For the assay, 200 µL of stool specimen was processed with 1 mL of sample diluent, followed by centrifugation at 12,000 g for 5 min. Next, 300 µL of supernatant was added to the sample well of the CDAB kit. The results were reported as negative, equivocal, or positive, according to fluorescence intensity.

3. Culture, isolation, and phenotypic identification of *C. difficile*

Stool sampleswithout pretreatment were inoculated into CDIF medium in an anaerobic workstation, and incubated at 35°C for 24-48 hr. All colonies suspected to be *C. difficile* (grey-to-black colonies with irregular or smooth borders) were initially Gram stained. Additionally, production of prolineaminopeptidase by colonies was tested by using a PRO disc [10]. Moreover, when CDAB was negative or equivocal, but colonies were positive and *C. difficile* was suspected, stool specimens were inoculated onto a universal anaerobic culture medium (Brucella agar medium) and then identified with a Vitek 2 ANC card (bioMérieux).

4. Detection of *C. difficile* toxin genes by PCR

All possible isolates of *C. difficile* (culture-positive, gram-positive rods, and PRO disc-positive) were examined for *tcdA* (toxin A), *tcdB* (toxin B), and triose phosphate isomerase genes (*tpi*; a *C. difficile*-specific internal fragment of a housekeeping gene) by PCR as previously described [11]. DNA was isolated from 3-5 *C. difficile* colonies by using a MagNA Pure LC Total Nucleic Acid Isolation kit (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's protocol. Of the PCR results, only *tpi*+, *tpi*+/*tcdA*+/*tcdB*-, or *tpi*+/*tcdA*-/*tcdB*+ results were confirmed by another PCR method, which was performed as previously described [12], in which, to detect the *tcdA* gene, a deletion in the 3′ end of the *tcdA* gene, and the *tcdB* gene, NK2/NK3, NK9/

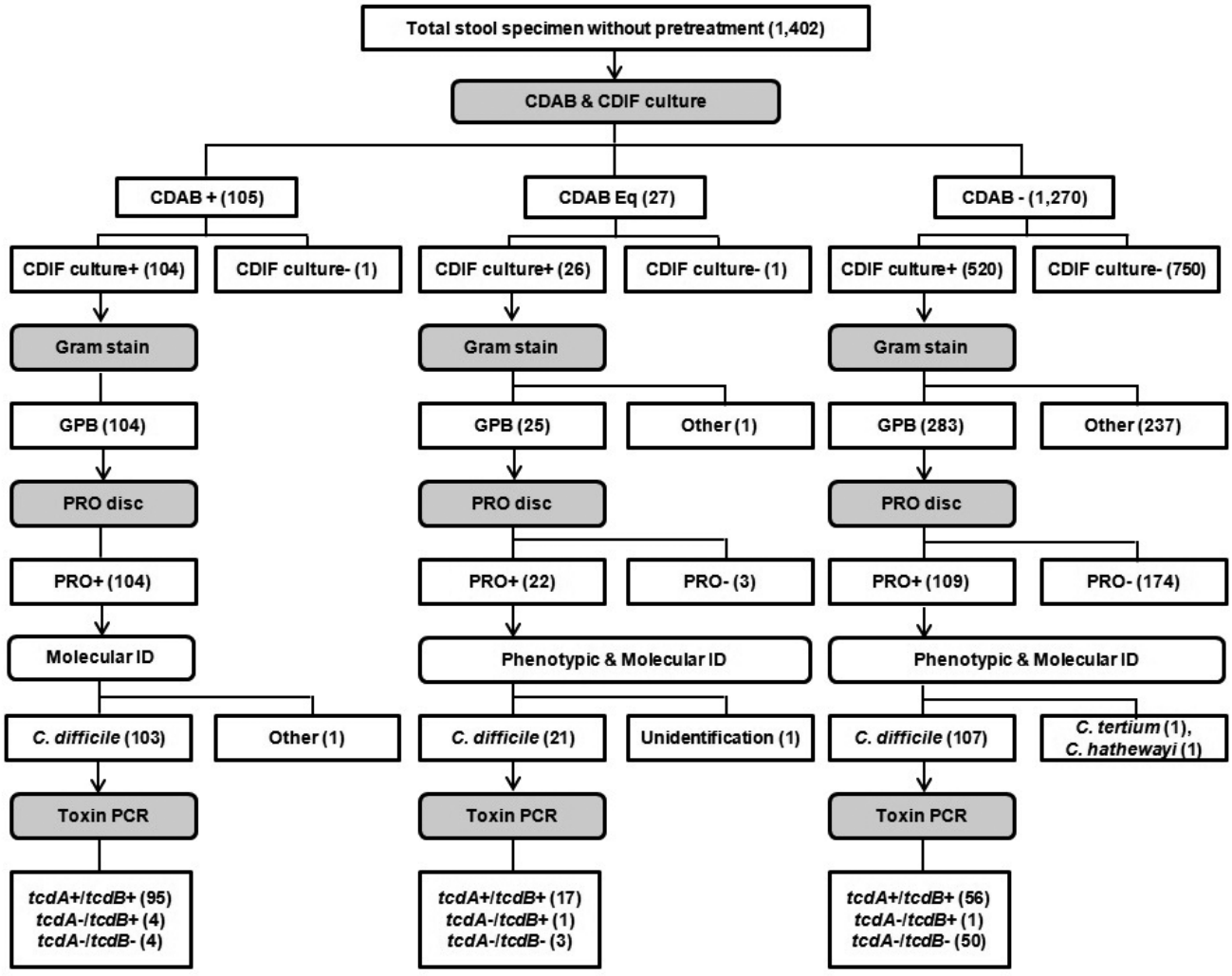

Fig. 1. Study design and results for the *C. difficile* culture using CDIF medium. Our proposed protocol for toxigenic *C. difficile* culture using CDIF medium depicted a gray-colored rectangle with rounded corners.
Abbreviations: CDIF, chromID *C. difficile* agar (bioMérieux, Marcy-l'Etoile, France); CDAB, Vidas CDAB assay (bioMérieuxe); +, positive; -, negative; Eq, equivocal; GPB, gram-positive bacilli; PRO, PRO disc K1532B (Key Scientific Products, Round Rock, TX, USA).

NK11, and NK104/NK105 primers were used.

5. Molecular identification of *C. difficile* using 16S rDNA or *tpi* gene sequencing

For the identification of *C. difficile*, CDAB-negative or CDAB-equivocal, CDIF culture-positive, gram-positive bacilli, and PRO-positive strains were confirmed by 16S rDNA and/or *tpi* gene sequencing (with the *tpi* gene-positive amplicons). A 16S rDNA fragment was amplified with the primers 4F (5′-TTGGAGAGTTTGATCCTGGCT-3′), 534R (5′-TACCGCGGCTGCTGGCAC-3′), 27F (5′-AGAGTTTGATCMTGGCTCAG-3′), and 801R (5′-GGCGTGGACTTCCAGGGTATCT-3′). PCR was performed by using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The amplification profile was 94°C for 5 min, followed by 32 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 7 min. The 16S rDNA or *tpi* amplicons were purified and sequenced by an ABI Prism 3730*xl* Genetic Analyzer (Applied Biosystems).

6. Statistical analysis

Statistical analysis was performed by using MedCalc software version 15.2.2 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2015). We calculated positive predictive value (PPV) with 95% confidence intervals (CI, Clopper-Pearson 'exact' method) of our protocol designed, in which detection rate was not considered.

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Table 1. Results of Gram staining and PRO disc testing in CDAB-equivocal or CDAB-negative and CDIF culture-positive strains

Gram stain	N of isolates recovered					
	CDAB-equivocal (N=26)		CDAB-negative (N=520)		Total (N=546)	
	PRO-	PRO+	PRO-	PRO+	PRO-	PRO+
GNB	0	1	131	1	131	2
GNB, GNC	0	0	3	0	3	0
GNB, GPC	0	0	54	0	54	0
GNB, YSC	0	0	1	0	1	0
GNC	0	0	2	0	2	0
GPB	3	22	14	107	17	129
GPB, GNB	0	0	103	2	103	2
GPB, GNB, GPC	0	0	47	0	47	0
GPB, GNC	0	0	2	0	2	0
GPB, GPC	0	0	8	0	8	0
GPC	0	0	40	0	40	0
GPC, GNC	0	0	1	0	1	0
GPC, YSC	0	0	1	0	1	0
Unknown	0	0	3	0	3	0

Abbreviations: CDAB, Vidas CDAB assay (bioMérieux, Marcy-l'Etoile, France); CDIF, chromID *C. difficile* agar (bioMérieux); GNB, gram-negative bacilli; GNC, gram-negative cocci; GPC, gram-positive cocci; YSC, yeast form; GPB, gram-positive bacilli.

RESULTS

A total of 1,402 prospectively collected stool specimens were tested and 650 (46.4%) were positive by CDIF culture (Fig. 1). Of those, 104 subjects (16% of CDIF-positive cases; 7.4% of total cases) were CDAB- and culture-positive, and 103 cases (99.0% of both CDAB and CDIF culture-positive cases; 7.3% of total cases) were identified as *C. difficile* by Gram staining, PRO disc testing, and *tpi* sequencing. Of the 103 *C. difficile* isolates, PCR of the *C. difficile* toxin genes, *tcdA* and/or *tcdB*, was positive in 99 (96.1%) of the cases: 95 cases (92.2%) were both *tcdA*- and *tcdB*-positive, four cases (3.9%) were only *tcdB*-positive, and four cases (3.9%) were *tcdA*- and *tcdB*-negative.

Of the 26 CDIF culture-positive but CDAB-equivocal cases, gram-positive bacilli were observed in 25 (96.2%) cases (Fig. 1 and Table 1). Using the PRO disc, we identified 22 positive cases. Of these, 11 isolates were identified as *C. difficile* by using Vitek 2 ANC card (Table 2). Six cases could not be identified and three cases could not be differentiated as *C. difficile*, *C. bifermentans*, or *C. sporogenes*. Remaining two cases were identified as *C. bifermentans* by using Vitek 2 ANC card. However, of the 22 cases that were CDAB-equivocal, gram-positive, and PRO disc-positive, 21 cases (84.6% of CDAB-equivocal and CDIF culture-positive cases; 1.5% of total cases) were confirmed as *C.*

Table 2. Identification of CDAB-equivocal or CDAB-negative, CDIF culture-positive, gram-positive bacilli-positive, and PRO disc-positive strains

Identification by Vitek 2	N of isolates identified								
	CDAB-equivocal (N=22)			CDAB-negative (N=109)			Total (N=131)		
	Vitek 2	Identification by molecular assays*		Vitek 2	Identification by molecular assays*		Vitek 2	Identification by molecular assays*	
		C. difficile	Others		*C. difficile*	Others		*C. difficile*	Others
Unidentified	6	5	1[†]	16	15	1[‡]	22	20	2[†,‡]
Clostridium difficile	11	11[§]	0	40	40[§]	0	51	51[§]	0
Clostridium difficile/Clostridium bifermentans/Clostridium sporogenes	3	3	0	23	23	0	26	26	0
Clostridium difficile/Clostridium bifermentans/Clostridium subterminale	0	0	0	6	6	0	6	6	0
Clostridium clostridioforme	0	0	0	4	3	1[§]	4	3[‡]	1[‖]
Clostridium bifermentans	2	2	0	18	18	0	20	20	0
Bacteroides ovatus	0	0	0	1	1	0	1	1	0
Clostridium sporogenes/Clostridium aerofaciens/Eggerthella lenta	0	0	0	1	1	0	1	1	0

*Molecular identification of *C. difficile* was performed by using 16S rDNA and/or *tpi* sequencing; [†]One isolate could not be identified by molecular assays; [‡]One isolate was identified as *C. tertium* by 16S rDNA sequencing; [§]Strains identified as *C. difficile* by using Vitek2 were confirmed by the *tpi* gene; [‖]One isolate was identified as *C. hathewayi* by 16S rDNA sequencing.

difficile by direct sequencing of the 16S rDNA or *tpi* genes. Of the 21 strains of *C. difficile*, 17 (81.0%) strains were positive for *tcdA* and *tcdB*, one (4.8%) was positive for only *tcdB*, and three (14.3%) were negative for both *tcdA* and *tcdB*.

Of the 520 specimens that tested negative by CDAB but positive by CDIF culture, gram-positive bacilli were observed in 121 cases (23.3%) and gram-positive bacilli mixed with other bacteria were observed in 162 cases (31.2%); overall gram-positive bacilli were 283 cases (54.4%) (Fig. 1 and Table 1). Among these, 109 tested positive with the PRO disc. Only 40 of these 109 cases were identified as *C. difficile* using the Vitek 2 ANC card, while 107 cases (20.6% of CDAB-negative and CDIF culture-positive cases; 7.6% of total cases) were identified as *C. difficile* by using 16S rDNA or *tpi* sequencing (Table 2). Of the 107 strains of *C. difficile*, 57 (53.3%) were positive for *C. difficile* toxin; 56 (52.3%) were positive for both *tcdA* and *tcdB*, and one (0.9%) was positive for only *tcdB*.

Overall, of the 650 CDIF culture-positive strains, 235 (36.2%) could be presumptively identified as *C. difficile* by using Gram staining and PRO disc testing without the use of a commercial identification kit or molecular assay. Of these, 231 (35.5%) strains were confirmed as *C. difficile* using phenotypic and molecular identification methods, therefore, PPV of Gram staining and PRO disc testing for identification of *C. difficile* was 98.3% (231/235, 95% CI 95.7-99.5%). Of the 231 total *C. difficile* strains, 174 (75.3%) had either *tcdA* and/or *tcdB* genes.

DISCUSSION

Several studies have evaluated the performance of CDIF agar in comparison with other selective media for the recovery of *C. difficile* [8, 9, 13, 14]. Perry *et al*. [8] reported that 99.6% (235/236) of *C. difficile* isolates were recovered on CDIF medium within 24 hr, while *Clostridium difficile* selective agar (CLO; bioMérieux) showed 74.6% (176/236) recovery of *C. difficile* isolates within 48 hr when untreated stool specimens were used. Another study using untreated stool samples reported that the sensitivities of CDIF at 24 and 48 hr were 74.1% and 87%, respectively, and the sensitivities in CLO medium and TCCA medium (taurocholate cycloserine cefoxitin agar) medium at 48 hr were 85.2% and 70.4%, respectively [9]. In addition, this study demonstrated that there was more abundant endogenous flora on TCCA medium after 48 hr of incubation (59.1%) than on CDIF medium after 24 hr of incubation (3.9%) or 48 hr of incubation (15%). Boseiwaqa *et al*. [14] reported that the concentration of vegetative cells on CDIF was significantly higher than on CCFA containing 0.1% sodium taurocholate medium at 24 and 48 hr, but that the spore count did not significantly differ.

In the present study, the overall recovery rate of *C. difficile* using CDIF medium was 35.5% because of the low selectivity (20.6%, 107/520) for *C. difficile* from CDAB-negative but CDIF-positive specimens (Fig. 1). Our results were different from the previous results [8, 15]; it might be due to the pretreatment of specimens in the previous studies. Therefore, in the absence of pretreatment, a new method to identify *C. difficile* in CDAB-negative stool specimens is necessary.

Our study showed that Gram staining and PRO disc testing of the colonies produced on CDIF could identify *C. difficile* with 98.3% PPV (95% CI 95.7-99.5%). However, sensitivity, specificity, and negative predictive value of our proposed procedure were not analyzed, because negative results by PRO disc testing were not confirmed using other phenotypic and molecular identification methods based on the previous study, in which PRO disc testing has 100% sensitivity for identification of *C. difficile* [10]. In addition, when Gram staining and PRO disc testing steps were skipped, highly selective isolation of *C. difficile* was possible with specimens that tested CDAB-positive or CDAB-equivocal, and with CDIF culture-positive specimens (95.4%, 124/130) (Fig. 1). Therefore, if tests for toxigenicity, such as detecting the *tpi* gene, are incorporated into the protocol to accurately identify and confirm the presence of *C. difficile*, Gram staining and PRO disc testing could be eliminated in the cases of CDAB-positive/equivocal and CDIF culture-positive specimens.

In conclusion, we demonstrated that the use of CDIF combined with Gram staining and PRO disc testing could accurately identify *C. difficile* strains from untreated stool specimens. In addition, our protocol for *C. difficile* culture reduces the multistep process which consists of pretreatment and identification procedures for *C. difficile*, especially considering that the inoculation onto another nonselective anaerobic culture medium is needed to identify *C. difficile* using an automated analyzer or to perform the *C. difficile* toxin testing when treated stool specimens on CDIF are used. Our protocol may benefit laboratories that use CDIF medium for *C. difficile* culture.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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