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Identification of *Clostridium difficile* in Stool Specimens by Culture-Enhanced Gas-Liquid Chromatography

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We have developed a sensitive and specific method for the identification of *Clostridium difficile* in stool specimens based on the detection of metabolic breakdown products of the organism by gas-liquid chromatography after incubation of stool samples in a selective broth medium containing cefoxitin. Use of this approach to test samples from two different populations of patients at separate medical centers showed this method to be superior to plate cultures or cytotoxin testing alone for both populations. The combined results from the two patient populations showed that 225 of 226 confirmed isolates were identified correctly, resulting in a sensitivity of 99.6% and a specificity of 99.0%. This method eliminates the delay caused by subculturing for tests requiring a pure isolate. The culture phase amplifies even low numbers of *C. difficile* in fecal samples (due to low in vivo concentrations or delayed transport) and thus increases sensitivity. Other advantages include the ability to detect *C. difficile* in the mixed flora of the stool and the ability of most clinical laboratories to use this procedure. Given the complexities of the detection of *C. difficile* toxins and the increasing importance of this organism as a nosocomical agent, culture-based methods remain the preferred approach to screening and routine workup for cases of diarrhea.

Previous studies have shown that *Clostridium difficile*-induced enteric disease requires four interrelated variables: a source of the organism, antibiotic exposure, toxin production, and a mature gut (12, 16). Although much progress has been made during the last decade in our understanding of antibiotic-associated diarrhea, many aspects of this complication and the role of *C. difficile* are still under active investigation (10). The spectrum of clinical illness ranges from antibiotic-associated diarrhea (AAD) to colitis (AAC) to pseudomembranous colitis (PMC). *C. difficile* has emerged as an important agent of nosocomial infections mainly owing to its ease of transmission and the extensive use of antibiotics in hospitalized patients (19, 20, 27).

Several aspects of *C. difficile*-associated disease remain unclear. Infants are found to be colonized with toxigenic strains but usually do not exhibit symptoms (28, 29). The roles of as many as two toxins and a motility-altering factor produced by *C. difficile* have not been fully defined in the causation of clinical illness, and patients with severe diarrhea have been found to be infected with *C. difficile* strains which are cytotoxin negative (13).

These aspects have prompted us to develop a more reliable and sensitive culture-based method for the detection and identification of *C. difficile* in stool specimens to facilitate future studies on the nosocomial transmission and causation of *C. difficile*-associated disease. Our approach is based on the detection of unique metabolic breakdown products of *C. difficile* by simple gas-liquid chromatography (GLC) after a period of incubation in a cefoxitin-containing selective broth in order to maximize *C. difficile* numbers and increase sensitivity. The reliability of this method in terms of its specificity and sensitivity was tested in two separate patient populations at the University Hospital (UH) and at Harborview Medical Center (HMC).

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MATERIALS AND METHODS

Specimen source (UH). Stool samples from 746 consecutive specimens received from adult in- and outpatients at UH, Seattle, Wash., were tested for the presence of *C. difficile*. In contrast to the patients at HMC, all the UH patients had a tentative diagnosis of AAD, AAC, or unspecified diarrhea.

Specimen source (HMC). All adult patients (428) enrolled in one general medicine ward at a county hospital (HMC, Seattle, Wash.) during an 11-month prospective study were tested for the presence of *C. difficile* in their stools, regardless of clinical symptoms (19). Cultures were done for all patients within 48 h of admission, every 2 to 5 days while on the study ward, and at discharge.

Microbiologic culturing. Patient stool samples or rectal swabs were inoculated by swab onto commercial cefoxitin-cycloserine-fructose-agar obtained from Prepared Media (at UH) or Difficile Agar plates (Prepared Media Laboratories, Tualatin, Oreg.) as recommended by guidelines for *C. difficile* isolation (1, 18, 25). The plates were incubated at 35°C in a GasPak jar (BBL Microbiology Systems) and examined at 48 h for characteristic growth (7, 9). The quality of all lots of commercial medium was tested with a positive control (a standard *C. difficile* strain) and a negative control (*Clostridium sporogenes* at UH and *C. perfringens* at HMC). Swabs were also rotated in 1 ml of sterile saline, which was then injected into 18 ml of Becton Dickinson (BD) supplemental peptone broths (Becton Dickinson, Rutherford, N.J.) to which cefoxitin (39 μg/ml) had been added. Samples of concentrated cefoxitin solution (0.5 ml) were stored frozen and thawed just prior to use due to the instability of cefoxitin at 25°C. Pure sodium taurocholate (0.1%) was added to the BD broths in the HMC study (31). A Vaspar-plugged vent was inserted in the top to allow gas diffusion while maintaining anaerobiasis. The tubes were incubated at 35°C for at least 48 h. The differences in the methods in these two

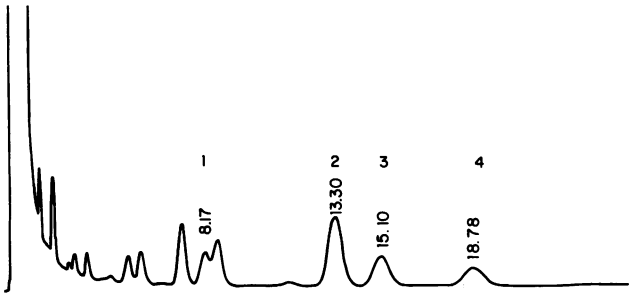

FIG. 1. GLC chromatogram of BD broth supernatant from *C. difficile* culture, showing retention times (in minutes). Peaks are numbered as follows: 1, pre-isovaleric acid; 2, phenylacetic acid; 3, isocaproic acid; 4, hydrocinnamic acid.

centers reflect the accepted methods at the time and individual judgments by the investigators.

GLC procedure. After the BD broth was incubated for 48 h, a methylated extract was made as outlined previously (11). A 1-ml amount of the well-mixed BD broth was removed and added to 2 ml of methanol and 0.4 ml of 50% sulfuric acid and heated at 56°C for 30 min. After heating, 1 ml of water and 0.5 ml of chloroform were added to the extract, and the contents were mixed by inverting the tube gently 20 times. A 5-μl sample of the bottom chloroform layer was injected into the chromatograph. The chromatograph was a Hewlett Packard 5830-A with a flame ionization detector, and nitrogen was used as the carrier gas. The column was stainless steel (6 ft by 0.25 in. outer diameter [ca. 2 m by 1 cm]) packed with 10% SP-1000–1% H_3PO_4 on Chromosorb W/AW 100/120 mesh. The operating conditions were 145°C column temperature and a carrier gas flow rate of 120 ml/min.

The chloroform layer of the methylated sample contains the fatty acids to be assayed. If *C. difficile* is present, four distinctive peaks will be observed (Fig. 1). The first peak emerges in about 8 min and is not completely separated from isovaleric acid (termed pre-isovaleric); the second peak emerges at 13 min (phenylacetic acid); the third peak emerges at 15 min (isocaproic acid); and the fourth peak arrives at 18 min (hydrocinnamic acid). Phenylacetic and hydrocinnamic acids are not volatile acids and will not be seen in a nonmethylated sample. Isocaproic acid, although a volatile fatty acid, "bleeds" through, so all peaks of interest are in the methylated samples. The identification of the phenylacetic and hydrocinnamic acid peaks was confirmed by mass spectrophotometry, but the pre-isovaleric peak remains unidentified. Both studies used the identical GLC procedure.

Confirmational identification. Additional tests were done on purified isolates to confirm the identification as *C. difficile*. UH isolates were confirmed by using the API 20A Identification Strip for Anaerobes (Analytab Products, Plainsville, N.Y.). Isolates from HMC were confirmed by using RapID-ANA System Minipanels (Innovative Diagnostic Systems, Inc., Atlanta, Ga.), which utilized 18 biochemical tests (2).

Cytotoxin assays. Stool samples were collected for cytotoxin assays to be performed at a reference laboratory (Children's Orthopedic Hospital, Seattle, Wash.) by the procedure described by Willey and Bartlett except that human embryonic tonsil (fibroblasts) were used as the tissue cell layer (8, 30). The specificity was confirmed by neutralization of the cytopathic effect with antisera against *C. sordellii*. One hundred and seventy stool samples (UH) were assayed for cytotoxin upon physician request. All 110 culture-positive (HMC) patients with available stool specimens were assayed for cytotoxin, regardless of symptoms.

TABLE 1. Comparison of GLC technique and commercial kits for the identification of *C. difficile* in stool samples

GLC broth result	Confirmed result[a]	No. of samples	
		UH patient population[b] (n = 746)	HMC patient population[c] (n = 428)
C. difficile positive	*C. difficile* positive[c]	113	112
	C. difficile negative	5	4
C. difficile negative	*C. difficile* positive	1	0
	C. difficile negative	627	312

[a] Confirmed with API 20A strips or with RapID-ANA panels.
[b] All patients at UH with diarrhea.
[c] Patients at HMC with diarrhea (38%) or asymptomatic carriers (62%).

Statistical analysis. The sensitivity, or the proportion of patients with *C. difficile* who had a positive GLC test, was calculated by the formula (TP/TP + FN) × 100, where TP were the true-positive samples and FN were the false-negative samples. The specificity, or the proportion of patients without *C. difficile* who had a negative GLC test, was calculated by the formula (TN/TN + FP) × 100, where TN were the true-negative samples and FP were the false-positive samples. The positive predictive value (PV+) of a GLC test was calculated by (TP/TP + FP) × 100, and the negative predictive value (PV–) was calculated by TN/TN + FN) × 100.

RESULTS

Detection of *C. difficile*. Of the 746 diarrheal stool samples submitted to the clinical laboratory at UH, 118 (15.8%) were positive for *C. difficile* by the initial GLC test (Table 1). Of the 118, 113 were confirmed by the API 20A Identification strips as *C. difficile*. The five BD broths (4.2%) falsely positive by GLC did not grow *C. difficile* upon subculture. Three of these five isolates were subsequently confirmed as *C. bifermentans*. All three of these isolates were cefoxitin sensitive and would not grow when inoculated back into BD broths with cefoxitin, raising the possibility that the initial specimens did not receive fresh cefoxitin. There was one false-negative sample (GLC broth negative, API positive) among the 746 specimens. The plate was *C. difficile* positive, but growth appeared to be delayed and the GLC at 48 h was negative.

Of the 428 patients from HMC, 116 (27.1%) were positive for *C. difficile* by the initial GLC procedure (Table 1). Of the 116, 112 were confirmed with the RapID-ANA panels as *C. difficile*, resulting in 4 (3.5%) false-positive results. The four false-positive broths were subcultured but did not yield *C. difficile*. The four isolates were identified as *C. innocuum*, *C. perfringens*, *C. paraputrificum*, and *Eubacterium limosum*. Pure isolates of *C. innocuum* and *C. paraputrificum* tested GLC negative upon subsequent chromatography.

Of the 112 *C. difficile* patients from HMC, 69 (61.6%) were asymptomatic carriers of *C. difficile*, 40 (35.7%) had diarrhea, 2 (1.8%) had AAC, and 1 (0.9%) had PMC. All the UH patients had diarrhea, but other clinical data were unavailable.

When the data from Table 1 were combined, 225 of 226 confirmed isolates were correctly identified by this GLC procedure, resulting in a sensitivity of 99.6%. There were

TABLE 2. Comparison of selective agar plate cultures and BD broth GLC methods for the detection of *C. difficile*: combined data for both patient populations

Plate result	No. of *C. difficile* results by GLC		
	Positive	Negative	Total
Positive	171	9	180
Negative	54	940	994
Total	225	949	1,174

one false-negative and nine (3.8%) false-positive results, for a specificity of 99.0%. In the combined populations, the PV+ for a GLC test was 96.1%, and the PV– was 99.9%. The predictive values of a GLC test were similar in the two populations: in the UH population, the PV+ was 95.8% (113 of 118) and the PV– was 99.8% (627 of 628); in the HMC population, the PV+ was 96.6% (112 of 116) and the PV– was 100% (312 of 312).

Plate-based isolation versus broth-based isolation. The advantage of additional culturing in a selective broth is evident upon comparison of the frequency with which *C. difficile* was isolated with just plates or just broth cultures. When the data from both patient sources were combined (Table 2), the selective broth detected a total of 225 strains (19.2%) which were subsequently identified as *C. difficile* of a total of 1,174 specimens. The selective plate cultures grew only 171 strains (14.6%) which were subsequently identified as *C. difficile* from the same total. Plate cultures also resulted in a higher frequency of false-positive (5.0%) and false-negative (5.4%) results. The sensitivity of the plate cultures was only 76%, and the specificity was 99.0%. An analysis of the nine isolates which were falsely positive on the selective plates proved that five were *C. innocuum*, one was *C. perfringens*, one was *C. paraputrificum*, one was *E. limosum*, and one was unidentifiable. Reliance on plate cultures as the sole means of *C. difficile* detection would have missed 54 (24%) of the true *C. difficile*-positive samples.

Cytotoxin. The effectiveness of cytotoxin as a screening tool for *C. difficile* was also investigated but showed a poor correlation between cytotoxin positivity and diarrhea (Table 3). Of the 45 samples positive by GLC at UH, only 29 were cytotoxin positive, resulting in a sensitivity for cytotoxin of 64% and a specificity of 98.4% (with culture techniques as the standard for detection of *C. difficile*).

For patients from HMC, of the 110 stool samples tested for cytotoxin, 43 were from patients with *C. difficile*-associated disease (diarrhea, colitis, or PMC) and 67 were from patients who were *C. difficile* positive but exhibited no diarrheal illness. Cytotoxin was present in 23 of the 43 diarrheal stool samples (53.5%) and 11 of the stool samples from asymptomatic carriers (16.4%) (Table 3). The sensitivity of the cytotoxin assay for *C. difficile* diarrhea was only 53.5%, and the specificity was 83.6%.

TABLE 3. Comparison of *C. difficile* cytotoxin detection and GLC results in clinical specimens from two sources[a]

Cytotoxin result	No. of results by GLC			
	UH patient population (170 samples)		HMC patient population (110 samples)	
	Positive	Negative	Positive, symptomatic patients	Positive, asymptomatic patients
Positive	29	2	23	11
Negative	16	123	20	56

[a] All patients at UH had diarrhea; HMC patients included 43 with diarrhea and 67 carriers without diarrhea (asymptomatic). No stool samples were available for two HMC patients.

DISCUSSION

The detection of *C. difficile* by the broth GLC method described in this study proved to be of high sensitivity (99.6%) and specificity (99.0%) and was consistently reliable for two distinct hospital populations as performed by two separate microbiology laboratories at different times. Other advantages of this GLC method are the relative speed of the results (within 48 h), the ability of the method to detect *C. difficile* in the milieu of stool flora, and the ability of most clinical laboratories to use this procedure. Potvliege et al. used GLC to detect isocaproic acid produced by *C. difficile* and reported a sensitivity of 61% and a specificity of 95% (24). Other researchers have used more complicated GLC procedures but did not achieve high sensitivity (3, 4, 17). Pepersack et al. used a more sensitive chromatograph and reported a sensitivity of 100% and a specificity of 92% (23). However, Levett used the same GLC method as Pepersack but did not find a decrease in the number of false-negative results associated with the use of isocaproic acid as the indicator for *C. difficile* (14). Reliance on only one fatty acid is unadvisable due to the number of clostridial species which produce isocaproic acid, including *C. sporogenes*, *C. bifermentans*, and *C. sordellii* (21).

The four fatty acids detected by this technique are metabolic end products of *C. difficile* resulting from growth in the BD medium regardless of whether the organism is in pure culture or mixed in with the rest of the fecal flora present within stool specimens. Other flora also produce metabolic end products, but these different end products do not interfere with the chromatogram reading of *C. difficile* peaks. It must be stressed that the success of this method is dependent upon the proper set-up of the specimen. Freshly thawed cefoxitin must be added or false-positive results may result. *C. bifermentans* produces similar fatty acid profiles but is cefoxitin susceptible and should not be a problem if the specimen is properly processed.

The main advantages of specimen inoculation into a selective broth followed by a period of growth are the higher frequencies of *C. difficile* detection and the ability to directly identify *C. difficile* in the broth culture by this GLC method. The BD broth cultures are more effective in detecting *C. difficile* than plate culturing alone. Of the total number of positive *C. difficile* specimens, only 76% were detected by plate cultures, whereas 99.6% were detected by using only the BD broths. The development of selective plates for the isolation of *C. difficile* has undergone modifications, including adjusting the concentration of antibiotics in the medium (15), using heat or alcohol shock to select for sporeforming bacteria (1), and diluting the initial stool sample before inoculation (30), but false-negative findings still result. In an attempt to increase the *C. difficile* detection rate, selective enrichment broths have been used (5, 7, 22, 26). The addition of 0.1% pure sodium taurocholate was found to stimulate spore germination and thus increase the yield if *C. difficile* was present in low concentrations (6, 31, 32). The use of both plate and broth cultures in this study resulted in high frequencies of *C. difficile* isolation.

Although its association with the most severe forms of AAC and PMC is high, cytotoxin screening has been found

to have variable predictive abilities for AAD (25) and was the least sensitive screening method in our study. Other researchers have found culture-positive, cytotoxin-negative situations in patients with *C. difficile* diarrhea (13, 16). In addition, patients receiving antibiotics but with no diarrhea have been found to be cytotoxin positive (25, 29). The results of our study confirm this lack of correlation between detection of cytotoxin and clinical illness in a general screening situation. Reliance on cytotoxin results alone would have missed 36 (40.9%) of the patients with *C. difficile*-associated diarrhea detected by GLC. More sensitive methods for the detection of all toxins of *C. difficile* may improve this correlation. In addition, 16% of the asymptomatic carriers of *C. difficile* had cytotoxin in the stool sample. In cases of serious AAC or PMC, cytotoxin assays will continue to be a valuable confirmatory test.

At present, detection of *C. difficile* by using only plate cultures or only cytotoxin assays does not seem justified, especially since the enrichment broth is readily available. It is recommended that isolation of *C. difficile* from stool specimens rely primarily on culturing techniques with selective enrichment broths (6, 22) and that detection of *C. difficile* be performed by GLC. This will allow prompt identification of *C. difficile* with the help of simple GLC procedures possible in most clinical laboratories and eliminates the delay caused by the need to subculture in order to test a pure isolate.

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