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Comparison of Traditional Gas Chromatography (GC), Headspace GC, and the Microbial Identification Library GC System for the Identification of *Clostridium difficile*

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Three gas chromatography (GC) methods were compared for the identification of 52 clinical *Clostridium difficile* isolates, as well as 17 non-*C. difficile Clostridium* isolates. Headspace GC and Microbial Identification System (MIS) GC, an automated system which utilizes a software library developed at the Virginia Polytechnic Institute to identify organisms based on the fatty acids extracted from the bacterial cell wall, were compared against the reference method of traditional GC. Headspace GC and MIS were of approximately equivalent accuracy in identifying the 52 *C. difficile* isolates (52 of 52 versus 51 of 52, respectively). However, 7 of 52 organisms required repeated sample preparation before an identification was achieved by the MIS method. Both systems effectively differentiated *C. difficile* from non-*C. difficile* clostridia, although the MIS method correctly identified only 9 of 17. We conclude that the headspace GC system is an accurate method of *C. difficile* identification, which requires only one-fifth of the sample preparation time of MIS GC and one-half of the sample preparation time of traditional GC.

Clostridium difficile has been recognized as a significant enteric pathogen since 1977 (2), being implicated in a spectrum of disease ranging from antibiotic-associated diarrhea to pseudomembranous colitis (4, 5). Laboratory diagnosis classically requires isolation of the organism on selective media and identification of cytotoxin in fecal supernatants, using tissue cell cultures, with observation of a cytopathic effect that is neutralized by the addition of a *C. difficile* or *C. sordellii* antiserum (1, 13). Culture confirmation of *C. difficile* from isolated colonies usually requires traditional gas chromatography (GC) techniques. These are time-consuming and specialized processes for a potential pathogen which may be carried in up to 10 to 20% of hospitalized patients (5). Hence, a faster and more efficient method of identification of *C. difficile* is desirable.

Gas-liquid chromatography of the short-chain fatty acids produced by bacteria as a result of carbohydrate metabolism is a well-established method of anaerobe identification (3, 8, 14–17). Headspace GC, which involves the sampling of vapor above heated 24-h broth cultures, could provide an especially useful method for the identification of *C. difficile* since the tedious solvent extraction procedures of traditional GC methods are avoided, while a highly characteristic fatty acid profile amenable to qualitative interpretation is maintained (9–12). A newer GC method, the Microbial Identification System (MIS), utilizes the longer-chain fatty acids found in the bacterial cell wall after 24 h of growth in broth as the basis of identification. MIS automated software automatically assigns these identifications based on reference data obtained from the Virginia Polytechnic Institute (VPI) (8). Using traditional GC of solvent-extracted short-chain fatty acids as the reference method, we compared the identities of 52 clinical *C. difficile* isolates by headspace GC and MIS. In addition, 17 non-*C. difficile* clostridia were included as a measure of each system's specificity.

(This work was presented in part at the 90th Annual Meeting of the American Society for Microbiology, Anaheim, Calif. [6].)

MATERIALS AND METHODS

Bacterial strains. Fifty-two organisms isolated from clinical specimens on cycloserine-cefoxitin-fructose agar were identified as *C. difficile* based on characteristic colonial morphology, pungent odor, and Gram stain (13). Ten non-*C. difficile* clostridia were clinical isolates identified previously by a combination of morphology, traditional GC, and biochemical assays. The non-*C. difficile* clostridia also included several strains obtained from the American Type Culture Collection. These were *C. histolyticum* 19401, *C. perfringens* 3624 and 13124, *C. sordellii* 9714, and *C. sporogenes* 19404. Two proficiency strains from the College of American Pathologists, *C. tertium* and *C. perfringens*, were also included.

Traditional GC. PYG broth (Scott Laboratories, Fiskeville, R.I.) was inoculated with the isolate to be tested and allowed to incubate at 37°C for 24 h. The broth culture was then extracted by the method of Thomann and Hill (16). One milliliter of the broth was combined with 0.2 ml of 50% H_2SO_4, 0.4 g of NaCl, and 2 ml of methyl-*t*-butyl ether (MTBE) in borosilicate tubes, stoppered, shaken vigorously with venting, and then centrifuged at 250 × *g* for 30 s. The top layer was removed and dried over 0.25 g of 3A 1/16 pellet molecular sieve (Union Carbide, South Plainfield, N.J.) for 10 min. One microliter of this extracted ether phase was injected onto an HP5890A (Hewlett-Packard, Avondale, Pa.) GC equipped with a flame ionization detector containing a Carbowax column (30 m by 0.25 mm [internal diameter]) with a film thickness of 0.25 μm (J&W Scientific, Folsom, Calif.). The injector port temperature was 270°C and the

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detector was at 250°C. The column oven temperature was programmed from an initial temperature of 120 to 200°C at 8°C/min, followed by a temperature ramp from 200 to 230°C at 30°C/min. The temperature was held at 230°C for 5 min. The carrier gas was helium flowing at 1 ml/min, and a split ratio of 50:1 was used.

Headspace GC. Three milliliters of the 24-h PYG (Scott Laboratories) broth culture was combined with 1.5 g of NaCl, 0.6 ml of 50% H_2SO_4, and 0.03 ml of 1:100 *n*-butanol in water as an internal standard in a 10-ml glass autosampler vial. Standards were processed in the same manner, with 3 ml of a fatty acid mixture (Supelco, Inc., Bellefonte, Pa.) containing the pertinent carboxylic acids replacing the broth culture. The samples were sealed and incubated for 20 min in the 80°C headspace sampler (HP 19395A; Hewlett-Packard). One milliliter of the headspace from each sample was then automatically injected into a Carbowax column (the same as the one used in the traditional GC described above) installed in an HP 5890 GC. The injector temperature was 225°C, and the flame ionization detector was maintained at 250°C. Chromatographic separation was achieved with a temperature program initially at 120°C to a final temperature of 200°C at 8°C/min. This final temperature was held for 2 min.

MIS GC. Samples were processed by using the MIS instruction manual (7). All reagents used were purchased from the recommended vendors. Two 10-ml tubes of PYG-Tween 80 (Carr-Scarborough Microbiologicals, Stone Mountain, Ga.) were inoculated with each organism. The tubes were incubated for 24 h at 37°C and then centrifuged for 25 min at 4°C and 5,800 × *g*. Supernatants were decanted, followed by aspiration of the small volume of remaining fluid. Pellets from pairs of tubes containing the same isolate were resuspended in 1.5 ml of 0.7% $MgSO_4$, combined, and recentrifuged by the parameters listed previously. To the final pellet was added 1 ml of saponification reagent (NaOH-MeOH) followed by vortexing and 30 min in a boiling water bath. Two milliliters of methylation reagent (1 ml of HCl-MeOH and 1 ml of H_2SO_4-MeOH) was added, and the tubes were vortexed and heated at 80°C for 10 min, followed by rapid cooling in a water bath at 25°C. Hexane-MTBE reagent (1.25 ml) was added, followed by 10 min of end-over-end mixing on a rotating mixer. The H_2O (bottom) phase was then removed with a Pasteur pipette and discarded. A base wash followed, using 3 ml of dilute NaOH-NaCl and 5 min of end-over-end mixing. The upper two-thirds of the MTBE phase was removed with a Pasteur pipette and placed in an autosampler vial with an aluminum crimp cap. Chromatography was performed on a Hewlett-Packard 5890 capillary column GC running version 1.0 of the VPI broth-based anaerobe library on MIS automated software.

RESULTS

Traditional GC. A representative chromatogram for *C. difficile* is shown in Fig. 1. All 52 *C. difficile* samples exhibited this characteristic pattern of peaks, which included butyric, isovaleric, valeric, and isocaproic acids. Lesser amounts of other fatty acids were also present, but did not interfere with qualitative interpretation. The non-*C. difficile* clostridia were easily differentiated from *C. difficile*, as they did not produce the same variety or abundance of fatty acids. *C. sporogenes* came closest to mimicking the *C. difficile* pattern, but lacked a definitive valeric acid peak.

Headspace GC. A representative chromatogram for *C. difficile* is shown in Fig. 2. The correlation in qualitative fatty acid profiles between traditional GC and headspace GC was excellent. However, headspace GC generally gave a cleaner base line and produced a more distinctive J-shaped configuration of the four fatty acid peaks of interest, with isocaproic acid dominating, followed by a much lesser amount of valeric acid and then slightly increasing amounts of isovaleric and butyric acids (these peaks together form the J shape). The majority of non-*C. difficile* clostridia yielded very few fatty acid peaks and were therefore distinctly different. However, *C. bifermentans*, *C. innocuum*, *C. sordellii*, and *C. sporogenes* did share at least three fatty acid peaks with *C. difficile* (Table 1). Notably, significant valeric acid production was lacking in these strains, allowing their differentiation from *C. difficile*. Furthermore, the characteristic distribution of fatty acids for *C. difficile* did not appear among these strains.

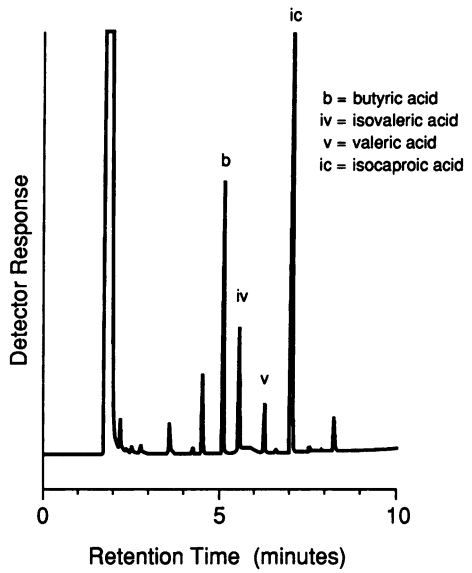

FIG. 1. Typical chromatograph for *C. difficile* by traditional GC. Note the prominent solvent front and dominant peaks of isocaproic, valeric, isovaleric, and butyric acids, which together are characteristic of *C. difficile*.

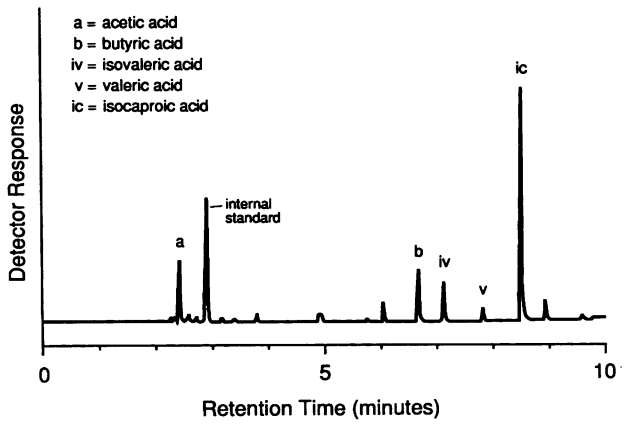

FIG. 2. Typical chromatograph for *C. difficile* by headspace GC. Here, the prominent J-shaped configuration composed of isocaproic, valeric, isovaleric, and butyric acids is seen. Also note the lack of a solvent front and cleaner baseline compared with traditional GC.

TABLE 1. Overall results with and comparison of the three GC methods

Organism (no.)	Dominant traditional GC peaks[a]	Dominant headspace GC peaks	No. of MIS/VPI identifications
C. difficile (52)	b, iv, v, ic	b, iv, v, ic	51/52 *C. difficile* 1/52 *C. bifermentans*
C. bifermentans (2)	a, iv, ic	a, b, iv, ic	2/2 *C. bifermentans*
C. butyricum (2)	a, b	a, b	1/2 *C. butyricum* 1/2 *C. bifermentans*
C. histolyticum (1)	a	a	1/1 *C. tetani*
C. innocuum (1)	b	b, iv, ic	1/1 *Peptostreptococcus*
C. perfringens (5)	a, b	a, b	4/5 *C. perfringens* 1/5 *C. botulinum*
C. septicum (2)	a, b (weak)	a, b (weak)	1/2 *C. cochlearium* 1/2 *C. chauvoei*
C. sordellii (2)	a, ic	a, b, iv, ic	1/2 *C. sordellii* 1/2 *C. botulinum*
C. sporogenes (1)	b, iv, ic	a, b, iv, ic	1/1 *C. sporogenes*
C. tertium (1)	a, b (weak)	a, b (weak)	1/1 *Lactobacillus*

[a] a, Acetic acid; b, butyric acid, ic, isocaproic acid; iv, isovaleric acid; v, valeric acid.

MIS GC. While a representative chromatogram for *C. difficile* (Fig. 3) is included for the sake of comparison, this method did not use our qualitative chromatographic interpretation, but, rather, utilized a computer software program to assign an identification based on comparison between the chromatogram and its reference file from VPI. Of 52 *C. difficile* isolates, 51 were correctly identified; however, 7 of 52 required multiple trials before a definitive match with MIS software was achieved. In these seven instances, we were unable to achieve an automatic identification on the first run (usually because of difficulty in achieving an adequate total peak area in the chromatograms with slow-growing organisms) and the library software was unable to make an identification. These organisms were rerun with increased sample amounts until we obtained any definitive type of identification (with an adequate total peak area). The single misidentified *C. difficile* was named *C. bifermentans*. The MIS system had difficulty in correctly identifying many non-*C. difficile* clostridia, with only 9 of 17 organisms being classified properly. The system was more successful in identifying *C. bifermentans* and *C. perfringens*, but performed poorly in identifying *C. innocuum* as *Peptostreptococcus* and *C. tertium* as *Lactobacillus* (Table 1).

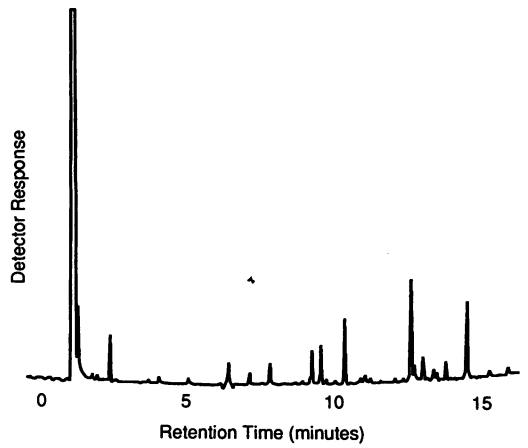

FIG. 3. Typical chromatogram for *C. difficile* by the MIS/VPI GC method. This pattern is derived from cell wall fatty acids and is quantitated by a computer for comparison with its software library of *C. difficile* chromatographs.

Reproducibility. All strains were analyzed twice by traditional GC (in separate extraction and analysis runs), and 25 organisms were run two or more times with headspace GC. The stability of the peak locations was within 0.5%, and the qualitative shape of the peak patterns was unchanged in each run. For the MIS analysis, a subgroup of 10 strains of *C. difficile* was run two or more times and all discrepant results (10 non-*C. difficile* clostridia strains) were run at least three times (separate extraction and analysis runs). In all but one run on this subgroup of organisms the MIS made the same identification each time. In the case of a *C. innocuum* strain the MIS procedure alternately identified the organism as *Peptostreptococcus* or as *C. innocuum*. In either case the other identification type was listed as the first alternative by the library software.

DISCUSSION

In identifying the 52 *C. difficile* isolates, both headspace GC and MIS/VPI GC were of approximately equivalent accuracy when compared with the standard GC extraction method. However, a very crucial difference arose in regard to sample processing time. Headspace GC required only one-fifth of the preparation time of MIS and one-half of the time of traditional GC sample preparation for a typical batch of 15 organisms. Further, the hazards of extraction solvents such as MTBE, which is utilized in both the traditional GC and MIS methods, are avoided with the headspace method.

Although the headspace technique requires minimal sample preparation, a certain level of expertise similar to that required for interpretation of traditional GC chromatograms is necessary. However, this interpretation is facilitated by the presence of a distinctive and consistent fatty acid profile for *C. difficile*. While 4 of the 17 non-*C. difficile* clostridia did share at least three fatty acid peaks with *C. difficile* (Table 1), the distinctive pattern was nevertheless unique to *C. difficile*. Larsson and Holst (9) examined several anaerobes by headspace GC, including six species of clostridia, and found that *C. difficile* and *C. sporogenes* most closely resembled each other in terms of fatty acid profiles. In specifically comparing 38 strains of *C. difficile* and 9 of *C. sporogenes* by headspace GC, Larsson and co-workers (10) concluded that

C. sporogenes had a more dominant isovaleric peak but produced lesser amounts of butyric and valeric acids than did *C. difficile*, consistent with our own findings. It should also be noted that Larsson et al. were working with 96-h PYG cultures, whereas ours were incubated for only 24 h prior to evaluation.

In contrast, the MIS system provides a clear-cut statement of identification, but requires extensive sample preparation. Both systems are conducive to large sample batches. Both GC methods also effectively differentiated non-*C. difficile* clostridia from *C. difficile*; nevertheless, the MIS system incorrectly identified 8 of the 17 non-*C. difficile* strains.

Thus, we conclude that headspace GC is a faster, simpler method for the identification of *C. difficile* than either traditional GC or the MIS system, with apparently equivalent accuracy. We found the current MIS system and VPI library to have significant limitations for the identification of anaerobic gram-positive bacilli in the typical clinical microbiology environment. Since the completion of this study, MIS has updated its broth-based VPI anaerobe library. This may improve the level of accuracy of the system for non-*C. difficile* clostridia, although the complicated extraction procedure remains the same. By comparison, the use of headspace GC has the potential of simplifying the identification of *C. difficile* isolates in clinical laboratories.

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