Studies of Metabolites in Diarrheal Stool Specimens Containing \textit{Shigella} Species by Frequency-Pulsed Electron Capture Gas-Liquid Chromatography

J. B. BROOKS,\textsuperscript{1,*} M. T. BASTA,\textsuperscript{2} AND A. M. EL KHOLY\textsuperscript{2}

Biotechnology Branch, Division of Bacterial Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333,\textsuperscript{1} and Biomedical Research Center for Infectious Diseases, Cairo, Egypt\textsuperscript{2}

Received 22 October 1984/Accepted 18 December 1984

Eleven diarrheal stool specimens and 10 control stool specimens from Cairo, Egypt, were studied by frequency-pulsed electron capture gas-liquid chromatography (FPEC-GLC). Four cases involving \textit{Shigella sonnei}, three cases involving \textit{Shigella boydii}, and four cases involving \textit{Shigella flexneri} were studied. The aqueous stools were centrifuged, extracted with organic solvents, and derivatized to form specific electron-capturing derivatives of carboxylic acids, alcohols, and amines. Analyses were performed on high-resolution glass columns with an instrument equipped with an extremely sensitive electron capture detector that is specific for the detection of electron-capturing compounds. The diarrheal stools studied had specific FPEC-GLC profiles and contained metabolic markers that readily distinguished between the \textit{Shigella} spp. studied and \textit{Escherichia coli} producing heat-stable or heat-labile enterotoxins. \textit{S. sonnei} stools contained hexanoic acid, 2-hydroxy-4-methylmethiobutyric acid, and some unidentified alcohols that distinguished this organism from other enteric pathogens. \textit{S. boydii} produced an acid that was unique for this species, and \textit{S. flexneri} produced alcohols that could be used to distinguish between it and other enteric organisms. The FPEC-GLC profiles obtained during this study were also very different from those reported earlier for \textit{Clostridium difficile} and rotavirus. This study presents further evidence that the selectivity and sensitivity of FPEC-GLC techniques can be used to rapidly identify causative agents of diarrhea and detect physiological changes that occur in the gut during the course of diarrheal illness.

Specific causative agents of diarrheal disease are often not apparent even after elaborate testing has been done. Part of this diagnostic problem lies in the fact that either no pathogens are isolated or, particularly in developing countries, several potential causative agents are found. Since diarrheal disease accounts for a significant number of deaths among infants in developing countries (3, 11, 12) and presents diagnostic problems throughout the world (6, 8, 9, 11), new methods are needed both for rapid diagnosis and to study the physiological changes in metabolism that occur in the gut during the course of diarrheal illness.

Frequency-pulsed electron capture gas-liquid chromatography (FPEC-GLC) techniques have been developed that are capable of detecting specific metabolic changes in diarrheal stool specimens with a high degree of accuracy (3, 6). FPEC-GLC analysis can be used to identify metabolites that are characteristic of diarrheal disease caused by \textit{Clostridium difficile}, \textit{Escherichia coli} strains that produce heat-stable and heat-labile (LT) toxin, and rotavirus. These techniques have also been extensively used to study normal stool specimens (6), and it has been established that FPEC-GLC profiles of normal stool specimens are consistent enough to permit the detection of disease processes by observing changes in metabolites that occur in the gut during the disease process (3, 6).

A definite need exists to continue and expand the study of diarrheal diseases by FPEC-GLC. Enteric organisms are an important pathogenic group because of their involvement in a number of diarrheal diseases. Since \textit{Shigella} ranks as the second-most common cause of travelers diarrhea (7), we decided to broaden the scope of earlier research begun on the enteric organisms (3) to include \textit{Shigella} spp. Through the analysis of diarrheal stools, we hope to establish FPEC-GLC metabolic profiles and markers that can be used both for rapid identification of the causative agent of disease and to further expand our knowledge of the metabolic changes that occur in the gut during the course of diarrheal illness.

(This study represents a portion of the work conducted by M. T. Basta at the Centers for Disease Control in fulfillment of a Ph. D. degree from the University of Cairo.)

MATERIALS AND METHODS

Stool specimens from 11 patients with diarrhea and 10 control specimens from infants with diarrhea, but from which causative agents were not isolated, were studied by FPEC-GLC. In addition, the FPEC-GLC profiles obtained from the \textit{Shigella}-containing stools were compared with those of normal stools reported previously (6). The diarrheal cases consisted of the following: three cases of \textit{S. boydii}, four cases of \textit{S. flexneri}, and four cases of \textit{S. sonnei}. The samples were collected from infants less than 2 years of age in Tamoooh, a village near Cairo, Egypt, by personnel from the Biomedical Research Center for Infectious Diseases, Cairo. The specimens were frozen until analysis by FPEC-GLC. Identification of the causative agent was accomplished by the use of the Roche Entero-Tube (10), for which a previously described biochemical scheme (3) is employed.

The stool specimens were extracted, derivatized, and analyzed by FPEC-GLC on packed and capillary columns as described previously (3).

\textsuperscript{*} Corresponding author.
FIG. 1. FPEC-GLC chromatograms of trichloroethanol (TCE)-derivatized acidic chloroform extracts of stools. The type organism responsible for the diarrheal disease is labeled in the figure. (D) Control specimen. Analysis was made on an OV-101 glass column (inner diameter, 2 mm; length, 7.6 m). The letter C followed by a number indicates a saturated carboxylic acid, with the number of carbon atoms indicated by the number. The use of a colon between two numbers indicates unsaturation. R, Reagent; I.S., internal standard; I, iso; PAA, phenylacetic acid.

RESULTS

Figure 1 shows the results obtained by FPEC-GLC analysis of trichloroethanol-derivatized acidic chloroform extractions of diarrheal stools. Figure 1A is a typical FPEC-GLC profile from patients with diarrheal disease caused by S. sonnei. One of the key metabolites produced by this organism was hexanoic acid (C₆). The short-chain carboxylic acids C₂ through C₅, present in all normal stools (Fig. 1D), were in some cases either totally or partially absent (Fig. 2A–C).

Figure 2 also illustrates the degree of reproducibility obtained from FPEC-GLC analysis of separate stools from patients with S. sonnei diarrhea. Peak C₆ has been blackened for easy observation.

Figure 1B shows the results obtained by FPEC-GLC analysis of diarrheal stools from which S. boydii was isolated. The short-chain acids found in normals stools were present, and a key acid metabolite (blackened peak designated 1) was determined by electron impact and chemical ionization mass spectrometry to be esterified with trichlo-
roethanol and to have a molecular weight of 246. The degree of reproducibility obtained by FPEC-GLC analysis of stools containing *S. boydii* for carboxylic acids is demonstrated in Fig. 3A–C.

Figure 1C and 4A–C show the results obtained by FPEC-GLC analysis for carboxylic acids of diarrheal stools from which *S. flexneri* was isolated. The short-chain acids C₂ through C₃ were, in general, absent or greatly reduced in stools from patients infected by this organism (Fig. 4A–C).

No unique carboxylic acids were detected in stools containing *S. flexneri*.

Figure 5A–C shows the FPEC-GLC profiles obtained by diesterification with heptafluorobutyric anhydride–ethanol of acidic (third extraction) ethyl ether extractions for hydroxy acids. Chromatogram A shows the profile obtained from analysis of stools from which *S. sonnei* was isolated. Peaks labeled C, D, and H were considered to be important metabolites useful for identification of this species. Peak D
has been tentatively identified by mass spectrometry (spectra to be presented elsewhere) as 2-hydroxy-4-methylmethiobutyric acid. *S. flexneri* (Fig. 5B) and *S. boydii* (Fig. 5C) did not produce detectable hydroxy acids in stool. Compare chromatograms B and C against the control stool analysis for hydroxy acids (chromatogram D). Figure 6 shows the degree of reproducibility found in the FPEC-GLC analysis of heptafluorobutryric anhydride–ethanol derivatives of stools from which *S. sonnei* was isolated. It is evident from the data presented (Fig. 5 and 6) that 2-hydroxy-4-methylmethiobutyric acid may be a key metabolite useful for identification of *S. sonnei* diarrhea.

Figure 7 shows the FPEC-GLC results obtained by esterification of chloroform extracts of diarrheal stools with heptafluorobutryric anhydride for detection of hydroxy compounds or alcohols. It might be noted that chloroform-extractable hydroxy acids are occasionally detected in the analysis. Figure 7A shows the type of FPEC-GLC profile obtained from analysis of stools taken from patients with diarrhea caused by *S. sonnei*. Peaks B, E, and G are
blackened for easy observation and to indicate importance. Peaks B, F, and G, were not detected in the FPEC-GLC analysis of diarrheal stools positive for *S. flexneri* (Fig. 7B) and *S. boydii* (Fig. 7C). However, peaks labeled I and II (chromatograms B and C) were found in diarrheal stools from which *S. flexneri* (Fig. 7B) and *S. boydii* (Fig. 7C) were isolated, but they were not found in cases involving *S. sonnei* (Fig. 7A) or the controls (Fig. 7D). Figure 8A–C shows the excellent reproducibility of alcohol metabolites produced by *S. sonnei*.

**DISCUSSION**

The family *Enterobacteriaceae* contains several important causative agents of diarrhea. *E. coli* strains producing heat-stable and heat-labile enterotoxins rank first and *Shigella* spp. rank second among the causative agents of travelers diarrhea (7). Data collected from the study of *E. coli* strains that produce heat-stable and heat-labile toxins (3) and the study presented here indicate that FPEC-GLC analysis of stools from patients with diarrhea can yield information that
have shown these compounds to be unique for \textit{S. sonnei}. Some of the most important metabolic markers in diarrheal stools from patients infected with \textit{S. boydii} were the carboxylic acid designated peak 1, the presence of the short-chain carboxylic acids C\textsubscript{2} through C\textsubscript{5}, which were unaffected by the diarrhea, and production of two alcohol peaks designated I and II. These two alcohols were also produced by \textit{S. flexneri}. \textit{S. flexneri} did not produce any

![FIG. 5. FPEC-GLC chromatograms of heptafluorobutyric anhydride-ethanol (HYD)-derivatized acidic diethyl ether extracts of stools. The type organisms responsible for the diarrhea are labeled in the figure. (A) Control stool. The internal standard (I.S.) was 2-hydroxy-isovaleric acid. Peak D has been tentatively identified as 2-hydroxy-4-methylmethiobutyric acid. Lac, Lactic acid; R, reagent.](http://jcm.asm.org/)

![FIG. 6. FPEC-GLC chromatograms of heptafluorobutyric anhydride-ether (HYD)-derivatized acidic diethyl ether extracts of stools. (A–C) Analysis of diarrheal stools from patients infected with \textit{S. sonnei}; (D) chromatogram of a control stool. The figure illustrates the reproducibility of hydroxy acid metabolites detected during the study. Lac, Lactic acid; R, reagent; I.S., internal standard.](http://jcm.asm.org/)
unique or characteristic carboxylic acids or hydroxy acids, and it should be noted that the short-chain carboxylic acids C3 through C5, found in all normal stools, were missing. These data clearly demonstrate the use of FPEC-GLC in the analysis of metabolic products present in diarrheal stool for differentiation of enteric disease caused by E. coli that produces heat-stable or -labile toxin (3), S. sonnei, S. boydii, and S. flexneri. Other studies which will be presented elsewhere show that many of the metabolites detected in vivo are also detected in vitro. The possible physiological effects of these metabolites on the host, if any, remain to be investigated.

LITERATURE CITED


