Journal of Chromatography, 430 (1988) 209–221
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4262

STUDIES OF METABOLITES IN DIARRHEAL STOOL SPECIMENS POSITIVE FOR *KLEBSIELLA, SERRATIA,* AND *PROTEUS* spp. BY FREQUENCY-PULSED ELECTRON-CAPTURE GAS CHROMATOGRAPHY

J.B. BROOKS*

Analytical Chemistry Laboratory, Meningitis and Special Pathogens Branch, Division of Bacterial Diseases, Center for Infectious Diseases, Centers for Disease Control, 1600 Clifton Road, Atlanta, GA 30333 (U.S.A.)

and

M.T. BASTA* and A.M. EL KHOLY

Biomedical Research Center for Infectious Diseases, P.O. Box 426, Cairo (Egypt)

(First received October 13th, 1986; revised manuscript received April 18th, 1988)

SUMMARY

Diarrheal stools from infants from which *Klebsiella pneumoniae, Serratia liquefaciens,* and *Proteus mirabilis* were isolated as possible causative agents of diarrhea were studied. These stools, along with control stool specimens which were collected from infants in the same village of Tamooh (near Cairo, Egypt), were analyzed by frequency-pulsed electron-capture gas chromatography (FPEC–GC). Watery stools and formed stools, to which distilled water was added, were centrifuged, and the supernatant was extracted with organic solvents and derivatized with specific functional group reagents to form electron-absorbing derivatives of carboxylic acids, hydroxy acids, alcohols, and amines. Results from the study showed distinct differences in FPEC–GC profiles of stools positive for *K. pneumoniae, S. liquefaciens,* and *P. mirabilis.* The major differences found were that diarrheal stools from which *K. pneumoniae* was isolated contained acetoin, a hydroxy acid-labeled peak F, and an unidentified amine, peak A. *S. liquefaciens* diarrheal stools had FPEC–GC profiles like the controls with the exception that an amine, peak A, was detected. The diarrhel stools containing *P. mirabilis* produced a distinct amine profile.

INTRODUCTION

Diarrheal disease has long been recognized in developing countries as a leading cause of morbidity and mortality in infants and young children. Using 1980 pop-

*Present address: The Hospital for Sick Children, Department of Bacteriology, 555 University Avenue, Toronto, Ontario 1156 1X8, Canada.

0378-4347/88/$03.50

ulation estimates, it was found that the total yearly morbidity and mortality from diarrheal disease for children under 5 years of age in Africa, Asia (excluding China), and Latin America were 0.774–1 billion episodes and 4.6 million deaths [1]. At present there are no specific means of preventing diarrheal diseases other than long-term programs of improved water supply and sanitation. In addition, diarrheal diseases present diagnostic problems in developing nations and throughout the world [2,3].

Before the recent study [2,4] there were no biochemical markers reported by which enterotoxigenic *Escherichia coli* that produces the heat-stable toxin (ST) could be distinguished from *E. coli* strains that produce the heat-labile toxin (LT) [5–8]. Therefore, the ability to recognize these strains has rested largely on the difficult demonstration of LT and ST enterotoxins. Moreover, there are the problems of failure to isolate a causative agent or isolation of more than one potential causative agents from a single specimen. Of equal concern to many investigators is the possible physiological effect that metabolites produced during the diarrheal state may have on the host. Some investigators have already suggested the possibility of pharmacological intervention in addition to rehydration therapy [9], and if toxic or physiologically active metabolites could be recognized, therapy might be developed that would counteract their effects.

The *Enterobacteriaceae* contain many important causative agents of diarrheal disease. Several members of this family have already been studied by frequency-pulsed electron-capture gas chromatographic (FPEC–GC) analysis of diarrheal stools [2,10,11]. The stools used in this study are a part of the stools obtained in previous studies [2,10,11], and all controls and specific tests applied to those studies apply to this study. Results from these studies indicate that FPEC–GC could be used effectively both as an instrument to aid in the rapid diagnosis of some enteric diarrheal disease and to study changes in metabolites that occur during the diarrheal disease state. Enterotoxigenic *E. coli* that produces ST and LT toxins along with *Shigella sonnei, S. boydii,* and *S. flexneri* strains were studied. FPEC–GC profiles or specific metabolic markers were detected in diarrheal stools containing these organisms that could be used to distinguish between the organisms. These FPEC–GC profiles were also different from those found in normal stools and diarrheal stools containing rotavirus and *Clostridium difficile* [11].

The purposes of this study were to point out important differences found in organisms that produce diarrheal disease studied by FPEC–GC thus far and to expand FPEC–GC studies of the family *Enterobacteriaceae*, specifically, to include *Proteus, Klebsiella,* and *Serratia* spp.

EXPERIMENTAL*

Fresh diarrheal stool specimens were collected within 48 h of onset, during active diarrhea, and before therapy, from infants less than 2 years of age. Stools from both the infants with diarrheal cases and ten similar age-matched controls

*Use of trade names is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

without diarrhea were obtained in Tamooh village near Cairo, Egypt, by personnel from the Biomedical Research Center for Infectious Diseases, Cairo, Egypt. A portion of the stool specimen was used to identify isolates using the enterotube (Roche, Nutley, NJ, U.S.A.) technique [2,12], which was based on the diagnostic scheme of Edwards and Ewing [13]. Methods used for detection of viral pathogens have been reported [2,11]. The remainder of the stool was frozen at −20°C until FPEC–GC analysis was performed. A total of 64 FPEC–GC analyses were performed on 21 stools. The only potential pathogens isolated were from five infant stools from which *Klebsiella pneumoniae* was isolated, three infant stools from which *Serratia liquefaciens* was isolated, and three cases from which *Proteus mirabilis* was isolated. FPEC–GC profiles were also compared with controls from previous studies [2,10,11].

The stool specimens were prepared for FPEC–GC analysis as follows: watery stool was used undiluted, loose stool was diluted 1:2 with distilled water, and formed stool was diluted 1:10 with distilled water. After mixing by shaking, the supernatant was centrifuged at 3920 *g* for 15 min. The stool supernatant fluid was placed in a 50-ml round-bottom centrifuge tube with a PTFE-lined screw cap. Nonanoic acid (3–15 nmol in 0.1 ml of distilled water, made basic to ca. pH 10 with sodium hydroxide to obtain solubility), 2-hydroxyisovaleric acid (2.68 mol in 0.1 ml of distilled water), and di-*n*-butylamine (1.19 mol in 0.4 ml of distilled water, made acidic to ca. pH 2 with sulfuric acid to increase solubility) were added to each sample as internal standards. Nonyl alcohol (400 nmol in 10 μl of chloroform) was added to each tube when an acidic extraction with chloroform was to be made for derivatization with heptafluorobutyric anhydride (HFBA) for the detection of alcohols.

Next, the samples were acidified to ca. pH 2 with 0.1 ml of 50% (v/v) sulfuric acid, mixed by shaking, and extracted with 20 ml of nanograde chloroform (Mallinckrodt, St. Louis, MO, U.S.A.) by shaking them for 5 min on a Burrell Wrist Action shaker at a setting of 10. To obtain amines, we made the residual aqueous phase basic (ca. pH 10) with 0.3 ml of 8 *M* sodium hydroxide and reextracted it with 20 ml of chloroform, as described for the acidic extraction. Then the residual aqueous phase from the basic extraction was reacidified to ca. pH 2 with 0.1 ml sulfuric acid and extracted with 20 ml of diethyl ether (Fisher reagent grade stabilized with butylated hydroxytoluene) to obtain the hydroxy acids. The acidic chloroform extracts were divided into two 10-ml portions. One portion was derivatized with trichloroethanol–HFBA (TCE-HFBA) to form TCE esters of carboxylic acids and HFBA esters of alcohols as described previously [14,16] and the second portion was derivatized with HFBA to detect alcohols and chloroform-soluble hydroxy acids. The basic chloroform extracts containing amines and the acidic ethyl ether extracts containing hydroxy acids were derivatized with HFBA–pyridine–methanol reagent to form amides and esters, respectively, as described previously [15–17]. After the TCE and HFBA derivatives of carboxylic acids, alcohols, and amines were prepared, the TCE derivatives were dissolved in 0.3 ml of xylene–ethanol (1:1), and both the alcohol and amine HFBA derivatives were dissolved in 0.1 ml of the same solvent. The HFBA derivatives of hydroxy acids were dissolved in 0.1 ml of ethyl acetate. A 1-μl injection was used for the analysis

of TCE derivatives and a 1.4-μl injection was used for other types of derivatives. The techniques for filling and cleaning the syringe have been described previously [16]. The GC conditions have been previously described [2,4].

RESULTS

FPEC–GC profiles of the ten control stools were consistent with earlier findings [2,11] and representatives are shown in Figs. 1–7.

Chromatographic results of the TCE esters of carboxylic acids extracted with chloroform under acidic conditions from diarrheal stool specimens culture-positive for *K. pneumoniae* (Fig. 1A, B, and C) and control stool (Fig. 1D) show that the stool specimens positive for *K. pneumoniae* contained significantly reduced amounts of the short-chain fatty acids propionic (C_3), isobutyric (IC_4), butyric (C_4), isovaleric (IC_5), and valeric (C_5) compared with the control. This reduction of the short-chain acids are more apparent when compared with chromatograms containing *P. mirabilis* (Fig. 5A, B, and C) and certain other enteric organisms [4].

Typical patterns obtained from analysis of derivatized acidic extractable ether-soluble hydroxy acids are shown in Fig. 2. Observe that the profile obtained from diarrheal stool specimens positive for *K. pneumoniae* (Fig. 2A) differed from the profiles of stool positive for *S. liquefaciens* (Fig. 2B) and control stool (Fig. 2C) in that the former profile contained peak F. Peak F was detected in four out of five stools positive for *K. pneumoniae*. Although the number of stools positive for *K. pneumoniae* were limited, the significance of peak F was confirmed by detecting it in vitro in the spent culture medium of *K. pneumoniae*. Nevertheless, further studies with larger sample size are needed to establish confidence in the profiles exhibited by *K. pneumoniae*. The FPEC–GC profile obtained from stool specimens positive for *S. liquefaciens* was essentially like the control (compare chromatograms B and C in Fig. 2).

Fig. 3 shows the type products detected in diarrheal stools from which *K. pneumoniae* was isolated. The acidic chloroform extracts of diarrheal and control stools were derivatized with HFBA–ethanol to detect alcohols and/or chloroform-soluble hydroxy acids. Acetoin was detected in all stools that contained *K. pneumoniae* isolates as shown in Fig. 3A, B, and C.

The identity of acetoin was confirmed by cochromatography with an authentic standard of acetoin and by the fact that, unlike most HFBA-derivatized hydroxy compounds, the ester decomposed after 24 h. Acetoin was missing in both control stools and diarrheal stools that were positive for *S. liquefaciens*.

The amine profiles detected from FPEC–GC analysis of HFBA-derivatized basic chloroform extracts of diarrheal stools positive for *K. pneumoniae* (Fig. 4A) and *S. liquefaciens* (Fig. 4B) were different from the profiles of control stool (Fig. 4C). A large portion of the blackened peak A was detected in all stools that were positive for *K. pneumoniae*, but only a relatively small portion (compare chromatograms A and B in Fig. 4) of this compound was found in all of the FPEC–GC profiles obtained from diarrheal stools positive for *S. liquefaciens* (Fig. 4B).

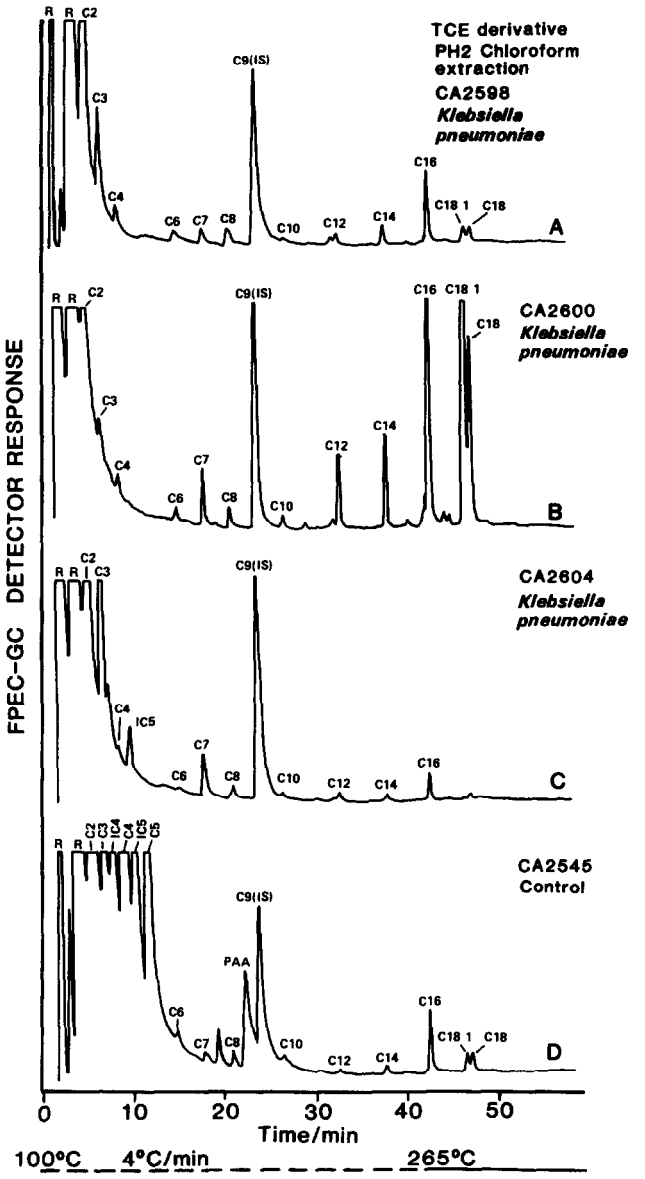

Fig. 1. Chromatograms of TCE-derivatized acidic chloroform extracts of stools positive for *K. pneumoniae* and control stool. Analyses were performed on a 7.3-m OV-101 packed column. The letter C followed by a number indicates a saturated carboxylic acid with the number of carbon atoms indicated by the number. The use of a colon between two numbers indicates unsaturation. I = iso; R = reagent; IS = internal standard C_9.

Peak A was not detected in control stools (Fig. 4C). Thus, peak A was the only significant metabolite detected in diarrheal stools containing *S. liquefaciens*.

Fig. 5 shows the FPEC–GC profiles of TCE esters of carboxylic acids obtained from acidic chloroform extracts of diarrheal stools positive for *P. mirabilis* (Fig.

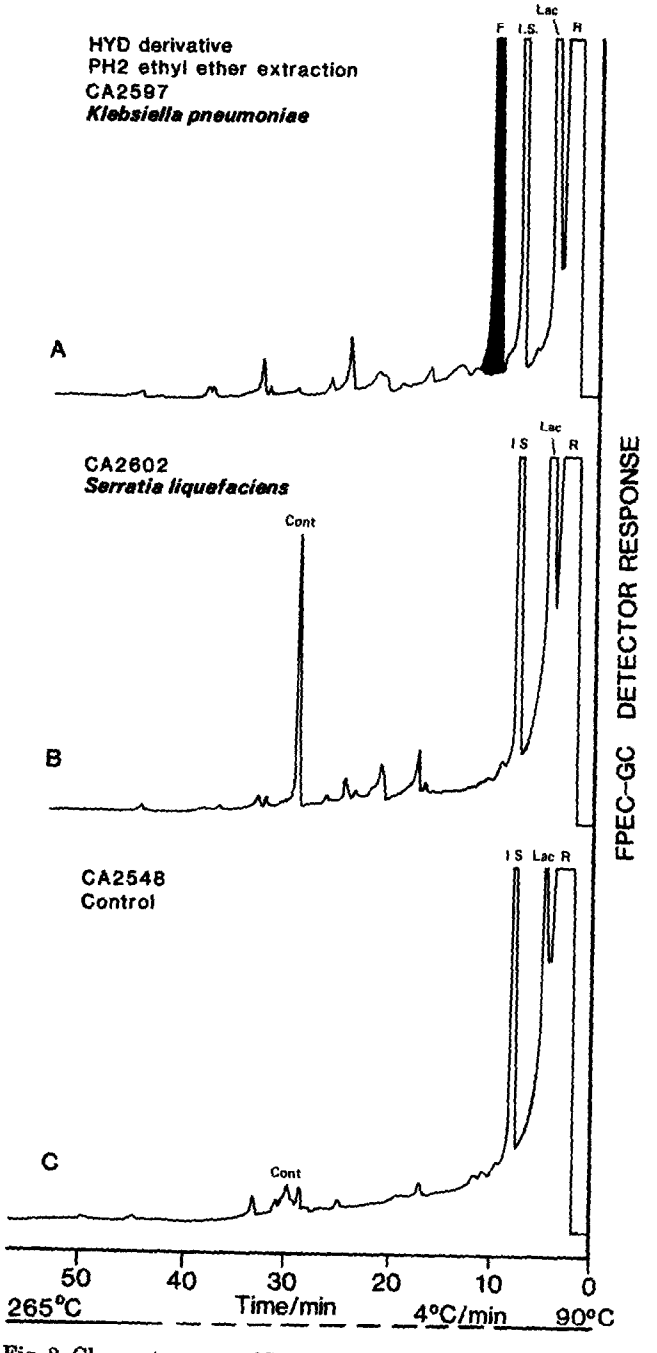

Fig. 2. Chromatograms of HFBA-ethanol (HYD) derivatives prepared from acidic ethyl ether extracts of stools. (A) Chromatogram from stool positive for *K. pneumoniae*; (B) chromatogram from stool positive for *S. liquefaciens*; (C) control stool. Analyses were performed on an OV-101 packed column. Cont. = control; I.S. = internal standard (2-hydroxyisovaleric acid); R = reagent.

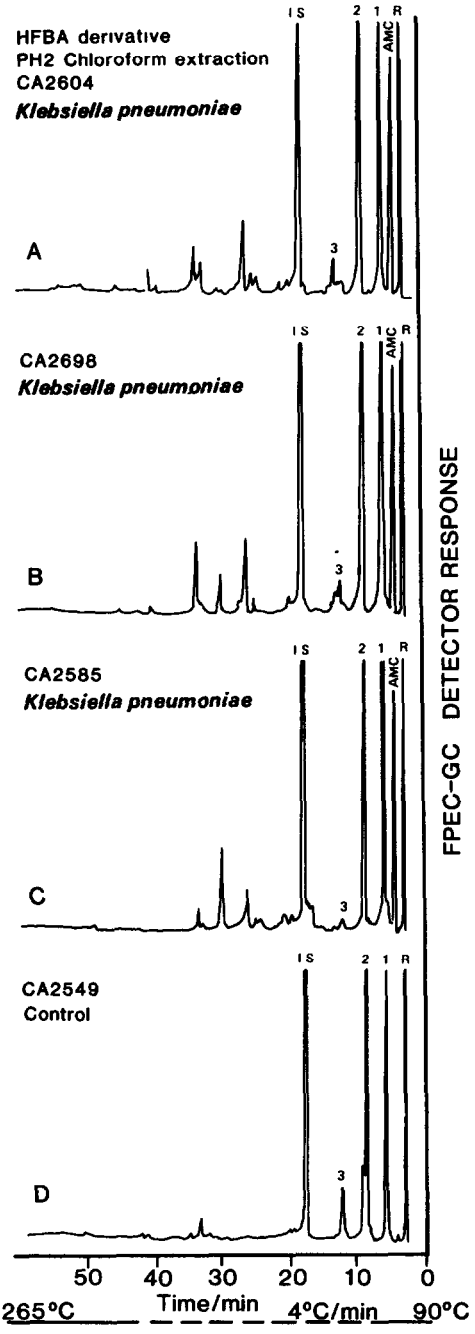

Fig. 3. Chromatograms of HFBA derivatives prepared from acidic chloroform extracts of stools positive for *K. pneumoniae* and control stool. Analyses were performed on an OV-101 packed column. AMC = acetoin; I.S. = internal standard (nonyl alcohol); R = reagent.

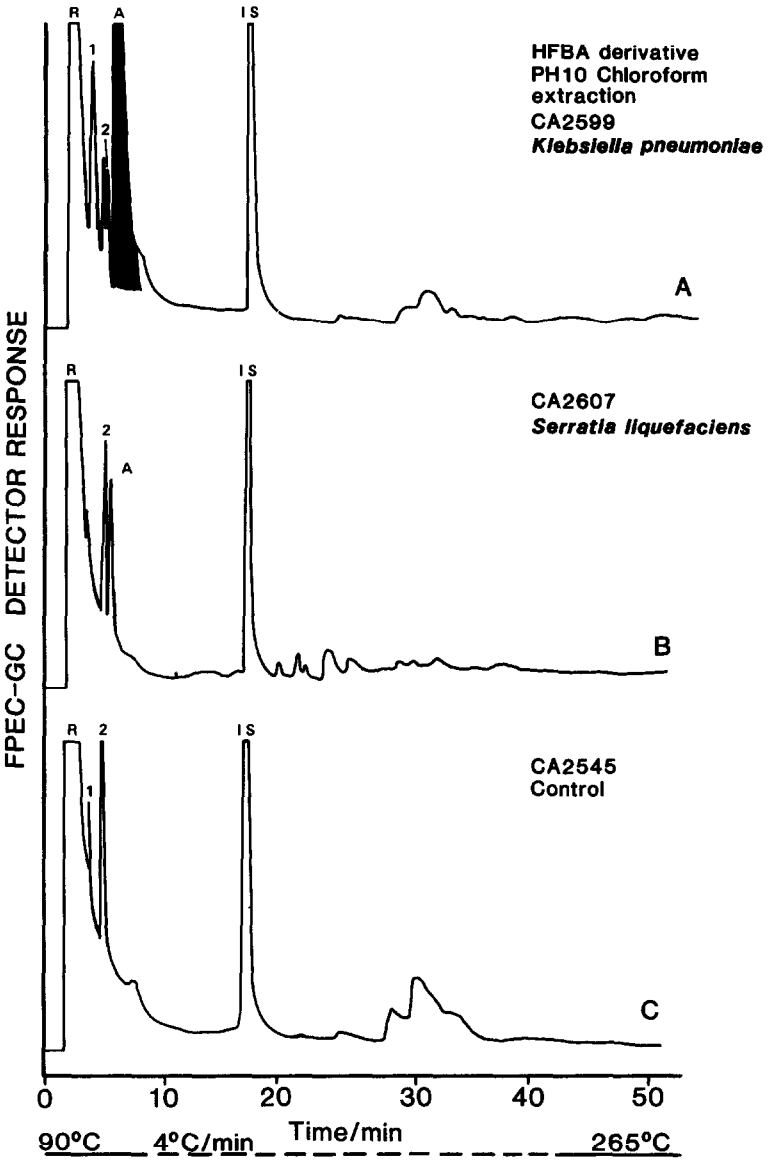

Fig. 4. Chromatograms of HFBA derivatives prepared from basic chloroform extracts of stools. (A) Chromatogram from stool positive for *K. pneumoniae*. (B) Chromatogram from stool positive for *S. liquefaciens*. (C) Chromatogram from control stool. Analyses were performed on an OV-101 packed column. I.S. is internal standard (di-*n*-butylamine) and R is reagent.

5A, B, and C) and control stools (Fig. 5D). The data show that two out of three cases studied (Fig. 5A and B) had full scale peaks of the short-chain fatty acids (C_2–C_5) and that the concentration of these peaks, with the exception of IC_5 and C_5, which were slightly reduced, was like that found in controls (Fig. 5D). The third case (Fig. 5C) had full scale peaks of the fatty acids C_2–C_4. The fatty acid C_5 was reduced in all cases except in one case (Fig. 5C) C_5 was missing and IC_5

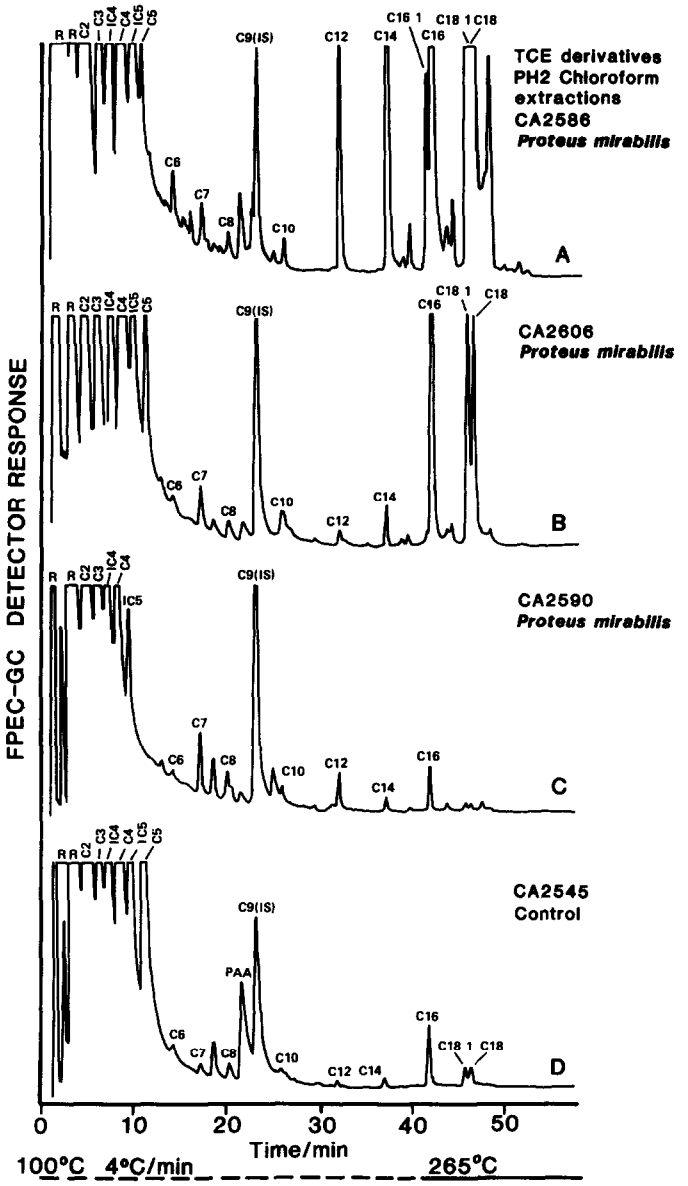

Fig. 5. Chromatograms of TCE-derivatized acidic chloroform extracts of stools positive for *P. mirabilis* and control stool. Analyses were performed on an OV-101 packed column. For an explanation of the notations see Fig. 1.

was reduced below full scale. The long-chain acids, C_{12} and higher, did not seem to bear a relationship with *P. mirabilis* infections.

The GC profile of heptafluorobutyryl ethyl esters (HYD) of hydroxy acids obtained by derivatization of acidic ethyl ether extracts of diarrheal stools containing *P. mirabilis* (Fig. 6A) differed from the profiles obtained from control stools (Fig. 6B). As shown in Fig. 6A and B, peaks B and D were detected in *P.*

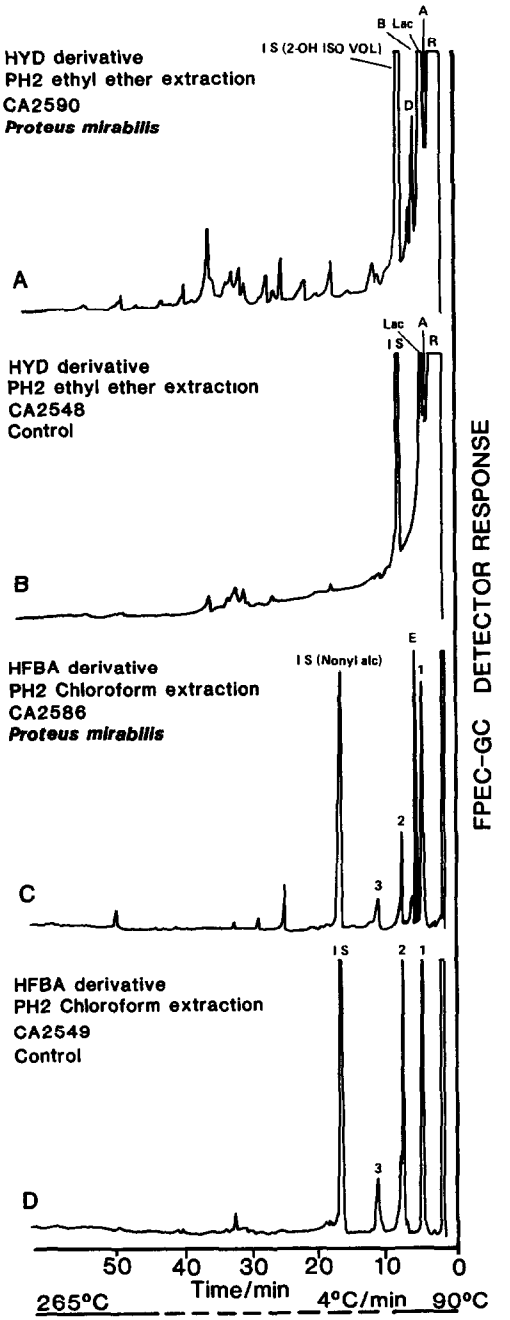

Fig. 6. Chromatograms obtained from stools analyzed on an OV-101 packed column. The types of extraction and derivative are indicated in the figure. (A and C) Chromatograms from stools positive for *P. mirabilis*; (B and D) chromatograms from control stools. For an explanation of the notations see Figs. 2 and 3.

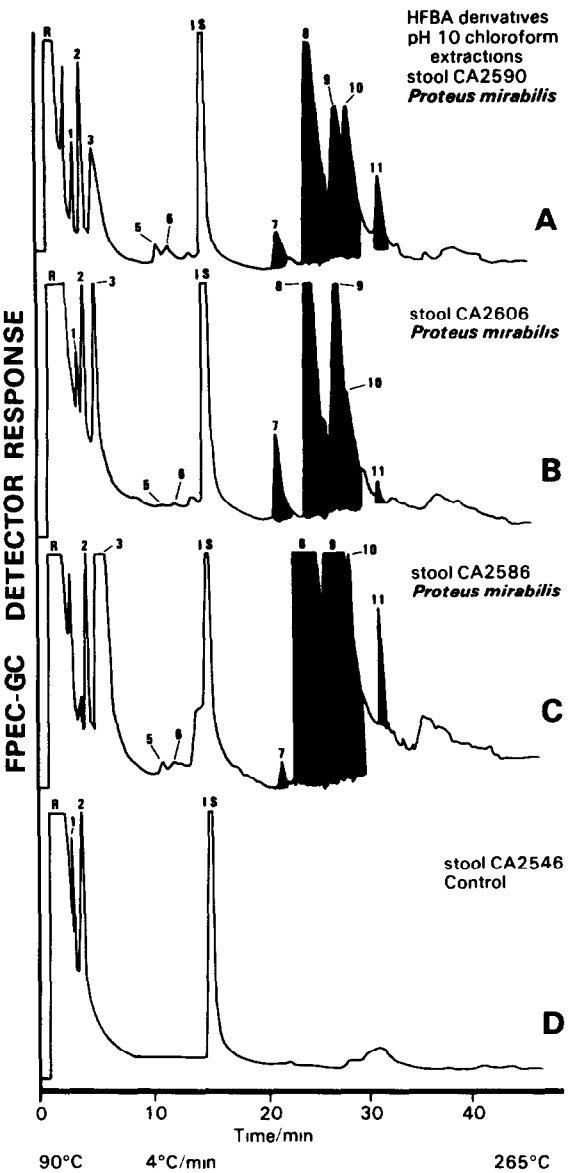

Fig. 7. Chromatograms of HFBA derivatives prepared from basic chloroform extracts of stools positive for *P. mirabilis* and control stool. Analyses were performed on an OV-101 packed column. For an explanation of notations see Fig. 4.

mirabilis-positive stools, but they were absent in control stools. Fig. 6 also shows FPEC–GC profiles obtained from HFBA derivatives of acidic chloroform extracts of stools positive for *P. mirabilis* (Fig. 6C) and a control (Fig. 6D). Alcohols and sometimes chloroform-extractable hydroxy acids were detected in these extractions. Peak E (Fig. 6C) was observed in each of the three cases of *P. mirabilis*-positive stools studied, but the peak was absent in controls.

The chromatograms in Fig. 7 (A, B, and C) show FPEC–GC profiles obtained from the analysis of HBA-derivatized basic chloroform extracts of stools positive for *P. mirabilis*. Fig. 7D shows the control profile. Peaks 5–11 (Fig. 7A–C) were consistently found in the cases of *P. mirabilis* studied. The small peaks 5 and 6 were also found in cases involving *E. coli* that produce LT, but peaks 5 and 6 were found in much greater quantities in diarrheal specimens containing *E. coli* LT [11]. Peaks 7–11 were found only in diarrheal stools containing *P. mirabilis*. The peaks have been blackened for quick observation. FPEC–GC profiles from three cases (Fig. 7A, B, and C) are shown to demonstrate reproducibility.

DISCUSSION

The results of this study and other studies [2,10,11] suggest that there are several reproducible differences in the FPEC–GC profiles obtained from the stools of patients with different causative agents of diarrhea. The data obtained thus far indicate that FPEC–GC analysis of stool specimens might be a way to rapidly distinguish the most common members of the *Enterobacteriaceae, Clostridium difficile,* and rotavirus associated with acute diarrhea in infants.

Although we cannot categorically state that the *K. pneumoniae, S. liquefaciens,* and *P. mirabilis* were the causative agents of the diarrheal disease, they were isolated from these patients in the absence of any other recognized pathogens and were not present in controls. Other workers [1,18,19] have recognized the potential of these organisms as pathogens for diarrheal disease in infants. In many instances the products detected by FPEC–GC analysis of stool specimens in this study are products known to be associated with these organisms and their detection adds support to our observations. The FPEC–GC markers that are of particular value for differentiation of stools containing *K. pneumoniae, S. liquefaciens* and *P. mirabilis* are shown (Figs. 1–7). Some of the most important markers of stools positive for *K. pneumoniae* are acetoin, hydroxy acid-labeled peak F, and amine peak A. AMC was a unique marker for *K. pneumoniae*-positive stools among the other members of the family *Enterobacteriaceae* studied by FPEC–GC. The presence of large amounts of amine peaks 7–11 in *P. mirabilis*-positive stools adds support to previous findings [20] of amine production in vivo and in vitro by *P. mirabilis*.

The potential agents of diarrheal disease isolated from diarrheal stools used in this and other FPEC–GC studies of *Enterobacteriaceae* [2,10,11] were characterized by extensive testing for metabolic products, observation of morphology, and in some instances by testing for toxins. In instances where interested scientists wish to repeat our findings, we strongly recommend the approach to characterization described [2,12,13]. Although a specific organism was not present in the gut in pure culture, it was present in large enough numbers to produce the detectable concentrations of metabolites. In instances where more than one potential pathogen was isolated, one type of FPEC–GC pattern usually predominated. Where an underlying disease is present, it is possible that additional peaks could be detected; however, only certain types of disease should present this problem.

Once the patient is cured of diarrhea, a control stool could be established for future reference.

The data obtained through this FPEC–GC study of the type diarrheal diseases reported in this study are in general agreement and warrant further investigation. The study of additional specimens, particularly those obtained from other parts of the world, is in order before conclusion can be made on the absolute merits of the FPEC–GC test.

Analysis time for the FPEC–GC test using dual columns is about 4 h. The cost of instrumentation is about $ 12 000.00 and with an automatic injector about $ 19 000.00. Data processors cost, which can speed data evaluation, range from about $ 3000.00 to $ 30 000.00. Neither an automatic injector nor a data processor is essential, but their use can cut the cost of analysis and make data interpretation simpler. Both analysis time and expense could be substantially reduced if further research shows that analysis for a few specific metabolites could suffice for identification. However, if detecting changes in gut metabolites during the diarrheal state is the major concern, the FPEC–GC analysis would have to be done for detection of the maximum number of metabolites. More recent studies (unpublished data) show that substitution of a large-bore (25 m×0.5 mm I.D.) OV-1 capillary column for a packed column can improve the resolution of peaks and avoid packing the column.

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