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Studies of Stools from Pseudomembranous Colitis, Rotaviral, and Other Diarrheal Syndromes by Frequency-Pulsed Electron Capture Gas-Liquid Chromatography

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Thirty-five patients with various diarrheal syndromes and 22 controls were studied. All stool samples were carefully cultured for *Clostridium difficile*, using selective isolation media. Cytotoxin assays with proper antitoxin neutralization were done in MRC-5 cells. The stool samples were extracted four times, three times at pH 2 and once at pH 10, using $CHCl_3$ or ether. Derivatizations of extracts were done with trichloroethanol, heptafluorobutyric anhydride, and heptafluorobutyric anhydride-ethanol, and all derivatives were analyzed by frequency-pulsed electron capture gas-liquid chromatography (FPEC-GLC). A dedicated computer was used to assist in both qualitative and quantitative data analysis. Isocaproic acid (iC_6) was always found in stool from which *C. difficile* was isolated and was absent in *C. difficile*-negative specimens. *p*-Cresol was found frequently in both persons with pseudomembranous colitis and controls. Tryptamine was found in stool containing *C. bifermentans*. The FPEC-GLC profiles of persons with acute diarrhea were very different from those of normal persons. Diarrhea associated with adenovirus and rotavirus, *Klebsiella* spp., and *Escherichia* spp. showed different FPEC-GLC patterns. Stools from well persons consistently contained full-scale peaks of pyruvic, acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids. In rotavirus stools isobutyric, isovaleric, and valeric acids were reduced in quantity from those found in control stools, whereas propionic and butyric acids were increased.

Clostridium difficile has been implicated in a gastrointestinal syndrome associated with antimicrobial therapy (2, 10, 12). In certain instances the organism has also been demonstrated to be a part of the healthy individual's intestinal flora (13, 15, 20). Our studies (unpublished data) indicate that *C. difficile* is present in approximately 12 to 15% of healthy individuals, with no record of antibiotic therapy for at least the preceding 2 weeks. On the other hand, the percentage of isolation in newborn babies under preventive antimicrobial treatment was 30 to 42% (13), and in patients with proven pseudomembranes on the rectal mucosa the percentage was as high as 89% (13). This increased isolation of *C. difficile* in patients with pseudomembranous colitis (PMC) has been seen by some workers as a possible association between this disease and *C. difficile* (12). Although procedures have been described (14, 18) for isolation of *C. difficile*, in a significant number of well-defined cases of PMC no isolates of *C. difficile* were obtained. A test that could detect the presence of *C. difficile* by analysis for specific metabolic products present in stool could be a valuable aid in the diagnosis and rapid treatment of PMC associated with *C. difficile*.

Gas-liquid chromatography (GLC) has been used to detect isocaproic acid in fecal material of patients with PMC and has been recommended for use in a screening test to detect *C. difficile* involvement in cases of PMC (19). The proposed method has been criticized for lack of sensitivity which resulted in 39% false negatives (3). In addition, the type of GLC test recommended (19), although easy to perform, lacks the specificity and resolution needed to establish confidence in the identification of isocaproic acid in such complex mixtures as feces.

Frequency-pulsed electron capture (FPEC) GLC, using high-resolution columns and specific functional-group electron-capturing derivatives, should provide the selectivity and sensitivity necessary for detection of isocaproic acid and other compounds present in fecal materials during a diseased state. These FPEC-GLC patterns may be useful for both rapid diagnosis of *C. difficile* involvement in PMC and rapid detection of other types of agents that cause diarrhea.

MATERIALS AND METHODS

Cultural procedures. A total of 66 stool specimens from 57 persons were studied by FPEC-GLC. The distribution of the 57 subjects is shown in Tables 1 and 2. Stools were collected and frozen at −20°C until FPEC-GLC analyses were made. All aerobic and facilitative microorganisms were identified by speciality laboratories within the Centers for Disease Control (Divisions of Bacterial Diseases and Viral Diseases).

C. difficile isolation was performed on selective media by using mannitol-cycloserine agar and mannitol-cycloserine-blood agar. The mannitol-cycloserine-blood agar medium consisted of the following: mannitol, 6 g; Trypticase (BBL Microbiology Systems), 30 g; yeast extract, 5 g; sodium chloride, 2.5 g; sodium sulfite, 0.1 g; L-serine, 0.2 g; L-leucine, 0.2 g; agar, 20 g; and distilled water, 1 liter. The mixture was autoclaved and cooled to 50°C, after which a solution containing 0.5 g of cyloserine dissolved in 50 ml of distilled water and, for mannitol-cycloserine-blood agar, 50% defibrinated sheep blood was added. For mannitol-cycloserine agar, 1.5 ml of a 1% solution of bromothymol blue was added. Each stool was treated with absolute ethanol for 1 h and then inoculated into the selective media, anaerobic blood agar, and chopped-meat–glucose broth as described previously (10). All isolates except *C. difficile* and *C. bifermentans* were cultured both anaerobically and aerobically in brain heart infusion broth for subsequent analysis by FPEC-GLC. *C. difficile* and *C. bifermentans* isolates were cultured anaerobically in Trypticase-yeast-salt broth as

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TABLE 1. Cases and controls studied for *C. difficile* isolation and toxin in stools

Type/condition	Test results		No. of individuals	iC_6
	Toxin[a]	Isolates		
Adults, PMC[b]	+	+	9	+
Adults, PMC	−	+	3	+
Adult, PMC	+	−	1	+
Adults, ulcerative colitis	−	−	2	−
Adults, acute (bloody) diarrhea	−	−	9	−
Adults, adult control healthy individual	+	−	6	+
Adult, control healthy[c] individuals	−	−	11	−
Adult, leukemic patient[d]	1/8	4/8	1	(see Table 2)
Adult, leukemic patient	−	−	1	−
Baby, acute diarrhea with adenovirus type 40 or 41	−	−	3	−
Baby, acute diarrhea with rotavirus	−	−	7	−
Baby control, healthy[c] babies	−	−	4	−
Baby control, rotavirus negative[e]	−	−	3	−

[a] *C. difficile* toxin.

[b] It is understood that PMC existed when pseudomembranes were carefully observed by endoscopy or surgery. All PMC patients were previously treated with antimicrobial agents.

[c] These individuals were considered healthy since they had no symptoms, their stools appeared normal, brown, and formed, and they had not received antimicrobial therapy for the previous 15 days.

[d] A leukemic patient from which eight samples were analyzed at different time intervals.

[e] These babies had diarrhea, but no bacteria, virus, or parasites were found.

described before (17). *C. difficile* toxin in stools was detected by using the MRC-5 cell line in flat-bottom microplates with specific neutralization with *C. difficile* antitoxin to confirm the specific nature of toxicity detected (16).

The stool specimens were prepared for FPEC-GLC analysis by taking 1 part (1 ml or 1 g) of stool, regardless of consistency, and mixing with 9 parts (9 ml) of distilled water. After mixing by shaking, the suspension was centrifuged at 5,000 rpm for 30 min, and then the supernatant was filtered through a 0.45-μm cellulose disk filter. Culture isolates were incubated for 3 days in brain heart infusion broth or Trypticase-yeast-salt broth and filtered, and the supernatant was used for FPEC-GLC analysis; 2 ml of the stool supernatant or 2 ml of the spent culture media was used for extraction and derivatization.

Extraction and derivatization procedures. A 2-ml portion of stool or culture supernatant fluids was placed in a 50-ml round-bottom centrifuge tube with a Teflon-lined screw cap. Nonanoic acid (3.15 nmol in 0.1 ml of distilled water, made basic [about pH 10] with NaOH to obtain solubility), 2-hydroxyisovaleric acid (2.68 μmol in 0.1 of distilled water), and di-*n*-butylamine (1.19 nmol in 0.4 ml of distilled water, made acidic [about pH 2] 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 detection of alcohols.

Next, the samples were acidified to about pH 2 with 0.1 ml of 50% (vol/vol) H_2SO_4, mixed by shaking, and extracted with 20 ml of nanograde chloroform (Mallinckrodt) by shaking for 5 min on a Burrell wrist action shaker at a setting of 10. To obtain the amines, the residual aqueous phase was made basic (about pH 10) with 0.3 ml of 8 N NaOH and reextracted with 20 ml of chloroform, as described for the acidic extraction. Then, the residual basic aqueous phase from the basic extraction was reacidified to about pH 2 with H_2SO_4 and extracted with 20 ml of ethyl ether (Fisher reagent grade stabilized with butylated hydroxytoluene) to obtain the hydroxy acids. The acidic chloroform extracts were derivatized with trichlorethanol (TCE)-HFBA to form TCE esters of carboxylic acids and HFBA esters of alcohols as described previously (1, 5) and 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-ethanol to form amides and esters, respectively, as described before (4, 6). After we prepared the TCE and HFBA derivatives of carboxylic acids, alcohols, and amines, the TCE derivatives were dissolved in 0.4 ml of xylene-ethanol (1:1) and the alcohol and amine HFBA derivatives were dissolved in 0.1 ml. The HFBA derivatives of hydroxy acids were dissolved in 0.1 ml of ethyl acetate. A 1-μl injection was used for 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 (5).

Apparatus. The derivatives were analyzed on a Perkin-Elmer model 900 gas chromatograph equipped with a 10-mCi ^{63}Ni FPEC. The glass column (0.2-cm inside diameter by 7.3 m in length), packed with 3% OV-101 on 80/100-mesh Chromosorb W H.P. (acid-washed dimethyldichlorosilane treated), was used under conditions previously described (1, 5, 6). The instrument was temperature programmed as shown in Fig. 1 through 7. The instrument was also equipped with an OV-101 fused-silica capillary column 50 m in length and a splitless injector. A switching valve permitted the use of either the capillary or the packed column. For analysis of TCE derivatives on the capillary column (not shown in figures), the derivatives were diluted in xylene-ethanol to 0.9 ml, and 1 μl was injected. The instrument was then operated isothermally at 100°C for 12 min and then programmed at 6°C/min to 265°C.

A Perkin-Elmer programmable processor (PEP-2) equipped with a modular software system (MS-16 revision B) accumulated data from the gas chromatograph, analyzed the data according to a stored program, and prepared a report. An internal standard analysis was performed on the data by using nonanoic acid, di-*n*-butylamine, 2-hydroxyisovaleric acid, and nonyl alcohol, which were added as internal standards to the samples being tested (1, 5), as described by the equation: concentration = (peak area/peak area of the

TABLE 2. Eight stool samples collected from a leukemic patient without diarrhea or antimicrobial treatment

No.	Date	Day difference	*C. difficile* isolates	*C. difficile* toxin	IC_6
1[a]	11-20-81		+	−	+
2	11-23-81	3	−	−	−
3	11-26-81	3	−	+	−
4	11-30-81	4	+	−	+
5	12-3-81	3	+	−	+
6	12-7-81	4	+	−	+
7	12-28-81	21	−	−	−
8	1-4-82	7	−	−	−

[a] This sample was also positive for *C. bifermentans* (Fig. 6D).

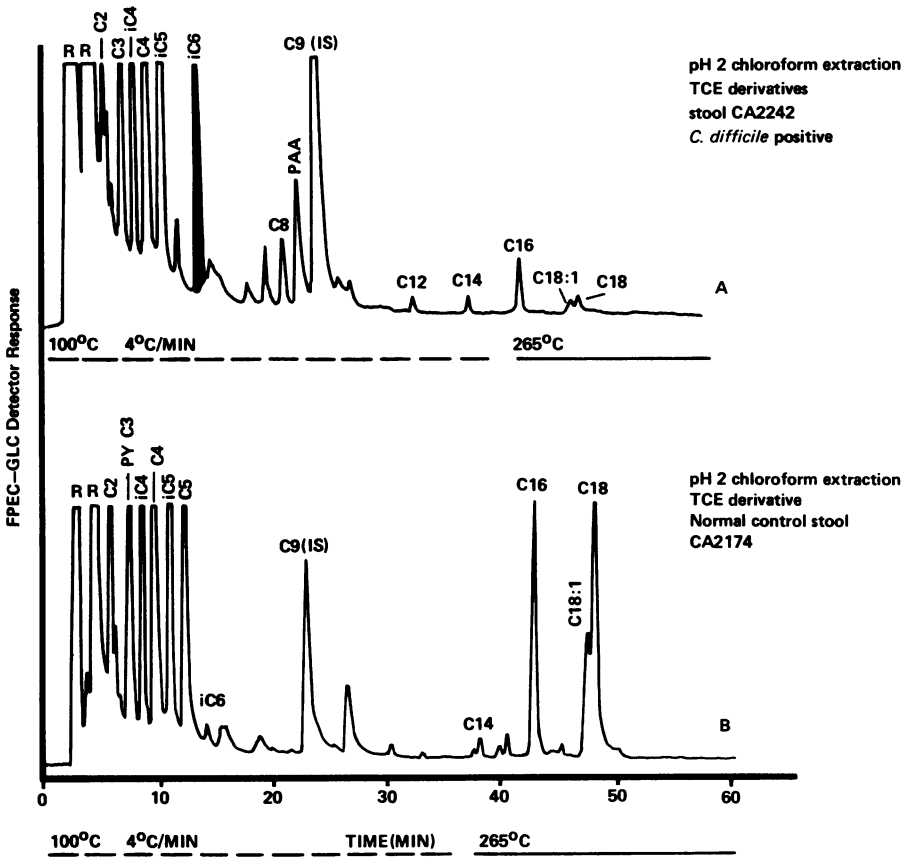

FIG. 1. Chromatograms of derivatized stool extracts. (A) TCE derivatives of an acidic chloroform extract from a stool from which *C. difficile* was isolated; (B) TCE-derivatized acidic stool extract of a stool from a normal healthy individual. The letter "C" followed by a number indicates a saturated straight-chain carboxylic acid with the number of carbons indicated by the number. The letter "i" is iso. The use of a colon between two numbers indicates unsaturation. R, reagent, IS, internal standard; PAA, phenylacetic acid; PY, pyruvic acid.

internal standard) × concentration of the internal standard.

Mass spectrometry. A DuPont model 21-491B mass spectrometer interfaced with a Varian 2700 gas chromatograph was used. The resolution was about 1,000, and the temperature of the ion source was 220°C. The instrument was operated in both the electron impact and chemical ionization modes. Methane was used as the reagent gas in the chemical ionization mode. The gas chromatograph was equipped with a glass column (2.4 m in length by 4 mm in inside diameter) with 3% OV-101 on Chromosorb W. The instrument was held isothermally for 8 min at 90°C and then programmed at a linear increase of 4°C/min to 225°C. Helium was used as the carrier gas with a flow of 36 ml/min.

RESULTS

Figure 1B shows fatty acids obtained by FPEC-GLC analysis of stool extracts from normal healthy controls (representing both infants and adults). A consistent pattern of full-scale peaks for carboxylic acids C_2 through C_5 was detected, and with the exception of phenylacetic acid, which was sometimes present, and small amounts of C_8, the stools were almost devoid of acids through C_{11}. The long-chain acids C_{12} and higher were present in normal controls 6 months of age and older above one-fourth scale about 25% of the time. The consistent presence of the acids through C_5, even after antibiotic therapy, may indicate that they are present as a result of body metabolism rather than microbial origin. Capillary column analysis and mass spectral confirmation of the carboxylic acids (TCE derivatives) from control specimens revealed that the peaks labeled C_3, C_4, and iC_5 actually contained two compounds each. The second peak found with C_3 was pyruvic acid. The peaks which coelute with C_4 and iC_5 may be keto acids, but their identities have not been proven. All of the carboxylic acid peaks labeled in the control stools were confirmed by chemical ionization and electron impact mass spectrometry by making a comparison between the fragmentation patterns of known and unknown compounds and by comparing retention data of known and unknown compounds on the 5-m capillary column.

The detection of additional carboxylic acids (Fig. 1A, 3B and C) and hydroxy acids (Fig. 2A, B, and C) and the selective removal of some or most of the carboxylic acids C_2 through C_5 (Fig. 4A and 5A) offer a means to differentiate between certain types of pathogens. Detection of isocaproic acid (iC_6) in *C. difficile* stools (Fig. 1A and 3B and C) along with detection of tryptamine in stools containing *C. bifermentans* (Fig. 6A, B, and C) offer a means to identify *C. difficile* even in the presence of *C. bifermentans* (7, 8). The rotavirus FPEC-GLC profile (Fig. 4A) showed that selective removal of carboxylic acids (iC_4, iC_5, and C_5, but not C_2, C_3, and C_4), which were found in all normal control stools, was highly reproducible in seven specimens (Table 3). This information may be useful to readily distinguish between rotavirus and *C. difficile* infections (Fig. 1A), controls (Fig. 1B and 5C), and also adenovirus infections in which an

TABLE 3. Concentration of short-chain carboxylic acids in rotavirus-positive and rotavirus-negative stools

Sample no.[a]	Concn (mol/liter)[b]					
	C_2	C_3	iC_4	C_4	iC_5	C_5
CA2213	17.99	112.61	12.38	297.82	34.41	102.64
CA2214	12.83	173.81	3.92	380.34	3.08	9.88
CA2215	13.19	164.18	32.03	334.96	83.59	14.72
CA2280[c]	20.57	376.84	81.26	161.24	25.39	3.99
CA2289	14.01	229.81	89.32	369.46	167.42	42.66
CA2390	29.08	268.39	30.15	281.81	39.31	44.93
CA2392	36.22	235.95	12.97	333.53	24.18	11.94
Avg[d]	20.55	223.08	37.43	308.45	53.48	32.96
CA2220	7.55	86.37	48.07	113.60	69.12	83.26
CA2221	13.13	110.05	65.02	127.09	86.45	81.89
CA2222	9.47	122.20	77.12	150.59	95.60	105.45
Avg[e]	10.05	106.21	63.40	130.43	83.72	90.20

[a] All specimens except CA2289 were taken about 1.5 days after onset of diarrhea. CA2289 was taken 3 h after onset of diarrheae.

[b] Concentrations of acids were determined by comparisons to the internal standard C_9 as described in the text.

[c] This specimen came from Cairo, Egypt.

[d] Average values for the concentrations of acids found in rotavirus-positive patient samples with diarrhea (first five samples in the table) listed above. No other viral, bacterial, or parasitic agent was found.

[e] Average values for the concentrations of acids found in rotavirus-negative cases (last three samples in the table) are listed below. These patients had diarrhea, but no viral, bacterial, or parasitic agent was found.

alteration of the normal C_2 to C_5 acid profile was not observed (Fig. 4B). Further, the hydroxy acid FPEC-GLC profile obtained from the adenovirus cases (Fig. 2A) was different from the hydroxy acid profile detected in other stools in that the adenovirus-infected stools contained peaks 1 to 6 and many other unlabeled small peaks which were absent in other stools (Fig. 2B and C). Adenovirus types 40 and 41 have only recently been reported (9) to be associated with diarrheal disease.

Figure 3 provides data obtained from the FPEC-GLC analysis of stools taken from a leukemic patient over a period of 6 weeks. Table 2 provides additional information on isolation of *C. difficile*, detection of its toxin, and production of iC_6 over a 6-week period. In Fig. 3, chromatogram A shows the analysis of a stool taken 7 days after isolation of *C. difficile*. Chromatograms B and C were obtained when *C. difficile* was isolated, and chromatogram D shows results of FPEC-GLC analysis of stool when the stool was again negative. Table 1 shows that FPEC-GLC analysis detected iC_6 in three persons with PMC who were toxin-negative and culture positive for *C. difficile* and in one healthy individual who was toxin positive and culture negative.

Figure 5A and B shows results obtained by FPEC-GLC analysis of stool samples taken from patients with diarrhea (Table 1, line 5) in which cytotoxin-producing *Escherichia coli* O157:H7 was suspected of being the causative agent (21). Chromatogram A shows results obtained from the more acute phase, and the diarrhea was less acute. Figure 5C shows results of FPEC-GLC analysis of a stool sample that was taken from the normal population of the same geographical area as the samples shown in Fig. 5A and B. In the acute phase (A) the carboxylic acids from C_2 to C_5 have been reduced or eliminated. Twenty-four hours later (B) the normal acids were present and a new peak labeled 1 was found. In the control sample (C) the concentrations of the long-chain acids C_{14} to $C_{18:1}$ were present in higher concentrations than were detected in other healthy controls.

Other types of compounds detected in the controls were essentially as shown in Fig. 2C, 6C, and 7C. *p*-Cresol (not shown) was present full scale in most control samples extracted at pH 2 and derivatized with HFBA, and peaks 11, 12, and 13 (Fig. 6C) were present in controls about 50% of the time. Different types of pathogens (Fig. 1, 2, 4 to 8) produced different types of FPEC-GLC profiles, and in the presence of two potential pathogens only one type of FPEC-GLC was found (Fig. 8B). Further, products detected in vitro in cultures were sometimes also detected in vivo in stools (Fig. 7A, B, and D). Note that peak 1 and putrescine were detected in both stools and spent media and that peaks 1, 3, and 6 were found in both spent media and stools (Fig. 8A, B, and C). A control individual (Fig. 2C) developed diarrhea, and *Klebsiella pneumoniae* was isolated as a suspected pathogen (Fig. 7D). Some of the peaks detected in vivo (Fig. 7D) were also seen in a *Klebsiella* species in vitro (Fig. 7A). Later the same individual had another bout with diarrhea, and the only organism isolated was *Pseudomonas aeruginosa*. Observe that no amines, or only trace amounts, were found in the stool from which *P. aeruginosa* was isolated (Fig. 7E), whereas large amounts of peak 1 and putrescine were detected in other cases involving *Klebsiella* species (Fig. 7B and D).

From Table 3 it is apparent that the carboxylic acids C_2, C_3, and C_4 were found in about twice the amounts found in the rotavirus-negative diarrheal cases from which no bacteria, viruses, or parasites were detected and that on the average iC_4, iC_5, and C_5 acids were found in smaller amounts than the full-scale peaks shown in controls (Fig. 1 and 5) or in the rotavirus-negative diarrheal samples. In only two instances (CA2280 and CA2389) was the iC_4 amount slightly above the average values of that found in rotavirus-negative samples, and in only one instance was the amount of iC_5 (CA2389) or C_5 (CA2213) above the rotavirus-negative levels. In no instance did iC_4, iC_5, and C_5, combined, exceed control averages. In one instance a rotaviral stool (CA2389) slightly exceeded the average rotavirus-negative values for iC_4 and greatly exceeded those for iC_5. The short course of the disease may have been responsible for these higher values. This sample was the only sample taken 3 h after onset of diarrhea.

DISCUSSION

The cause(s) of PMC is not clear. However, there is a substantial accumulation of data suggesting that *C. difficile* may be involved in many cases. There is a definite need for a reliable test to demonstrate the presence of *C. difficile*. Causative agents of diarrheal diseases in general often present problems in diagnosis. The reasons for some of the problems are that multiple potential pathogens are sometimes isolated from stools, no pathogens are isolated, or the involvement of certain organisms, such as viruses or even *C. difficile*, as causative agents is often hard to prove.

Several interesting observations that could have significant diagnostic value, as well as provide insight into changes in metabolites that take place during diarrhea, were observed during this study. First, acid metabolites were consistently detected in the control stools of all age groups 6 months or older. Consistency is important in the establishment of an FPEC-GLC diagnostic test. Second, metabolites such as iC_6 were reproducibly detected when a particular organism (i.e., *C. difficile*) was isolated. Reproducible FPEC-GLC patterns that are different for different pathogens indicate that the infecting organism is probably affecting the metabolic pattern. Third, normal fecal metabolites were removed or changed by infection with a particular

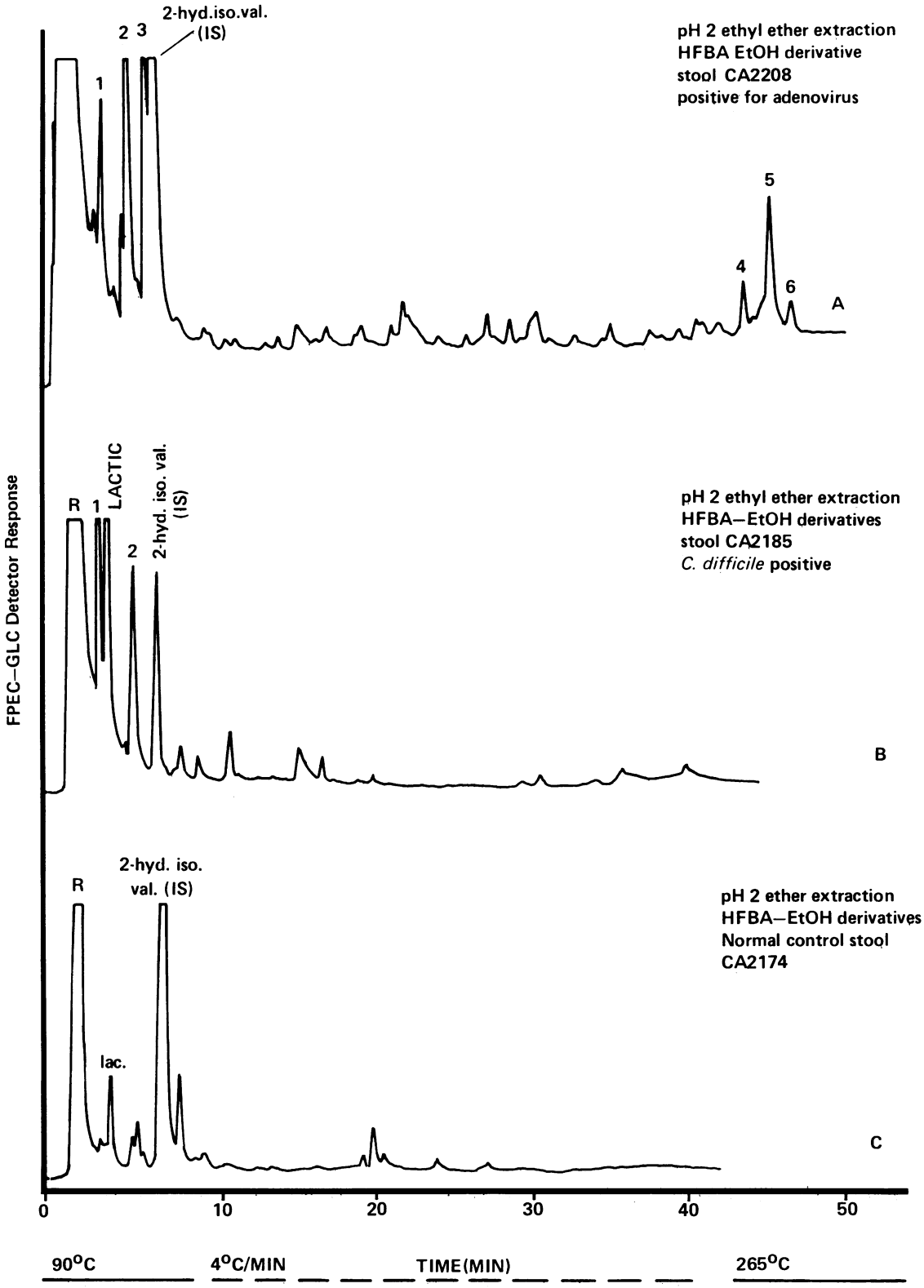

FIG. 2. FPEC-GLC chromatograms of HFBA-ethanol-derivatized acidic ethyl ether (third) extraction. (A) Chromatogram from a stool that was positive for adenovirus; (B) chromatogram from a stool that contained *C. difficile*; (C) chromatogram from a normal healthy control. Note that several one-fourth-scale peaks (unlabeled) were detected. These peaks appear to be significant. 2-hyd. iso. val., 2-Hydroxyisovaleric acid; Lactic and lac, lactic acid. For other abbreviations, see legends to Fig. 1 and 6.

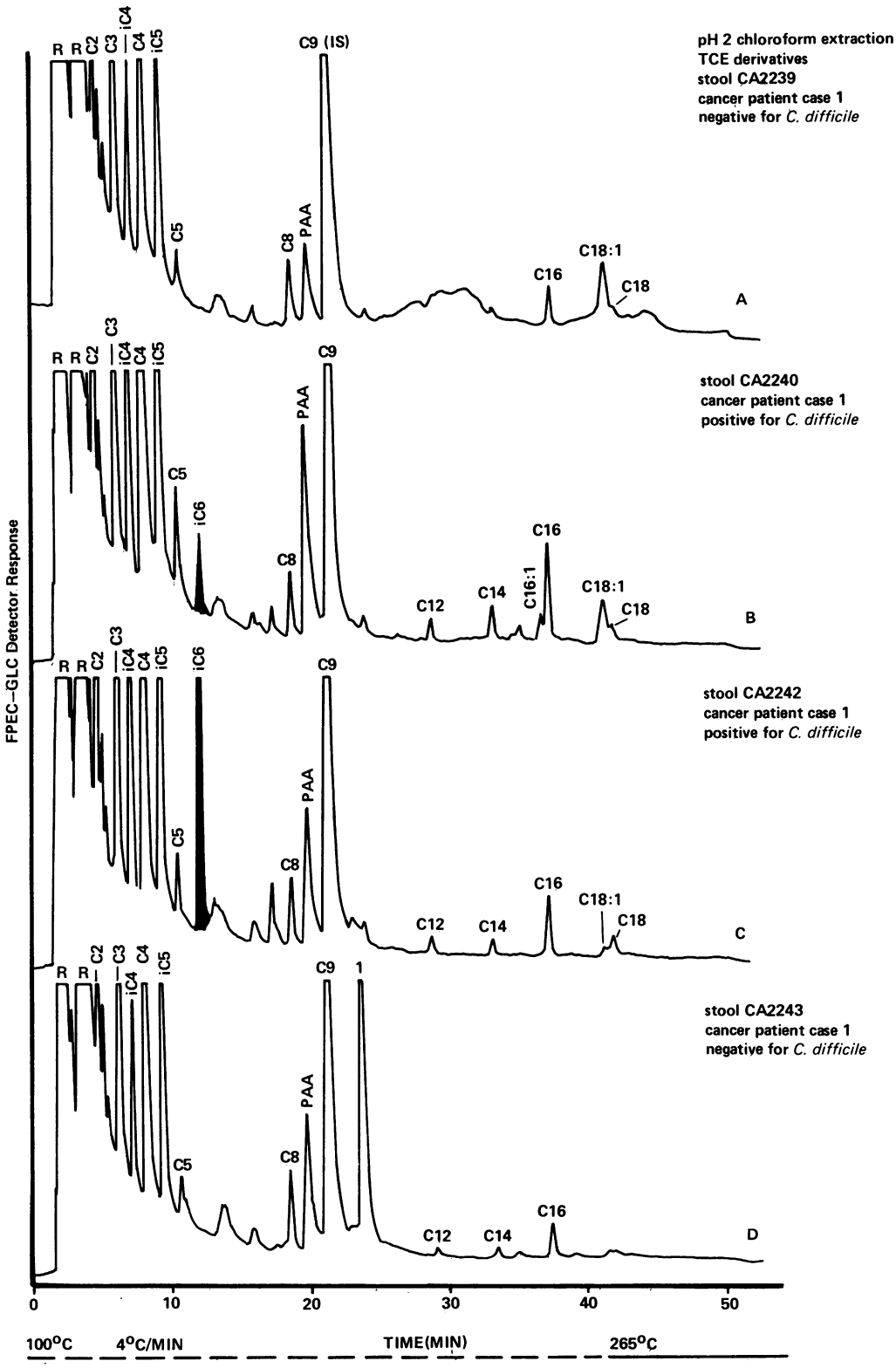

FIG. 3. FPEC-GLC chromatograms of the acidic chloroform TCE-derivatized extracts made from stools taken from a leukemic patient over a period of time (Table 2). Observe (Table 2) the detection of isocaproic acid when the stool is positive for *C. difficile*. Peak 1 is unidentified. For other definitions of labels, see legend to Fig. 1.

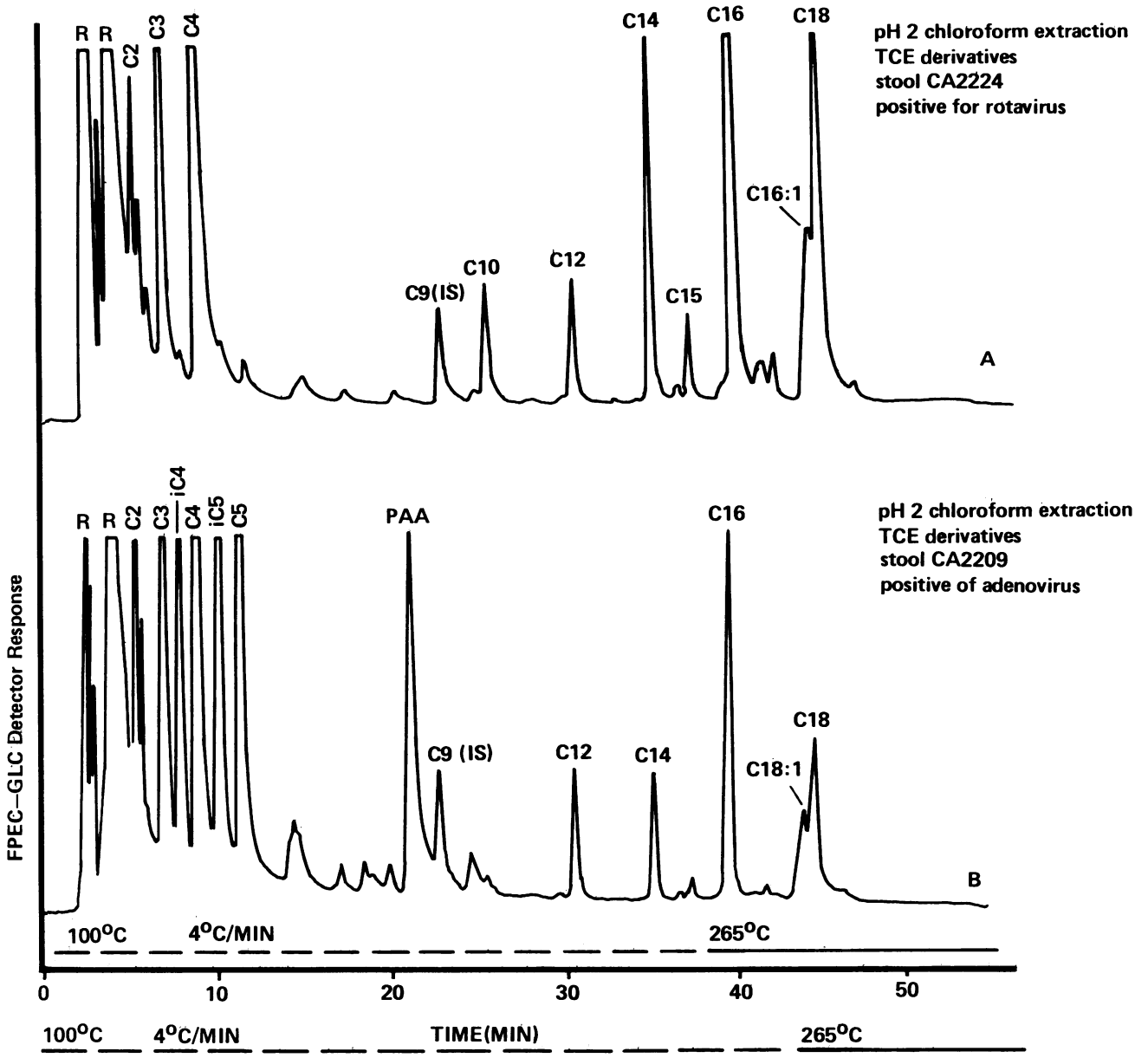

FIG. 4. FPEC-GLC chromatograms of derivatized stool extractions from patients with diarrhea. (A) TCE derivative of a stool extract from which rotaviruses were demonstrated. Note that part of the acids seen in the normal flora are missing, but that the relative amounts of C_3 and C_4 are unaffected. (B) TCE derivatives of a stool extract from which adenovirus was demonstrated. Note that the FPEC-GLC profile is almost like that from a normal person. Long-chain acids were always associated with viral diarrhea. Controls contained long-chain acids about 25% of the time. For abbreviations, see legend to Fig. 1.

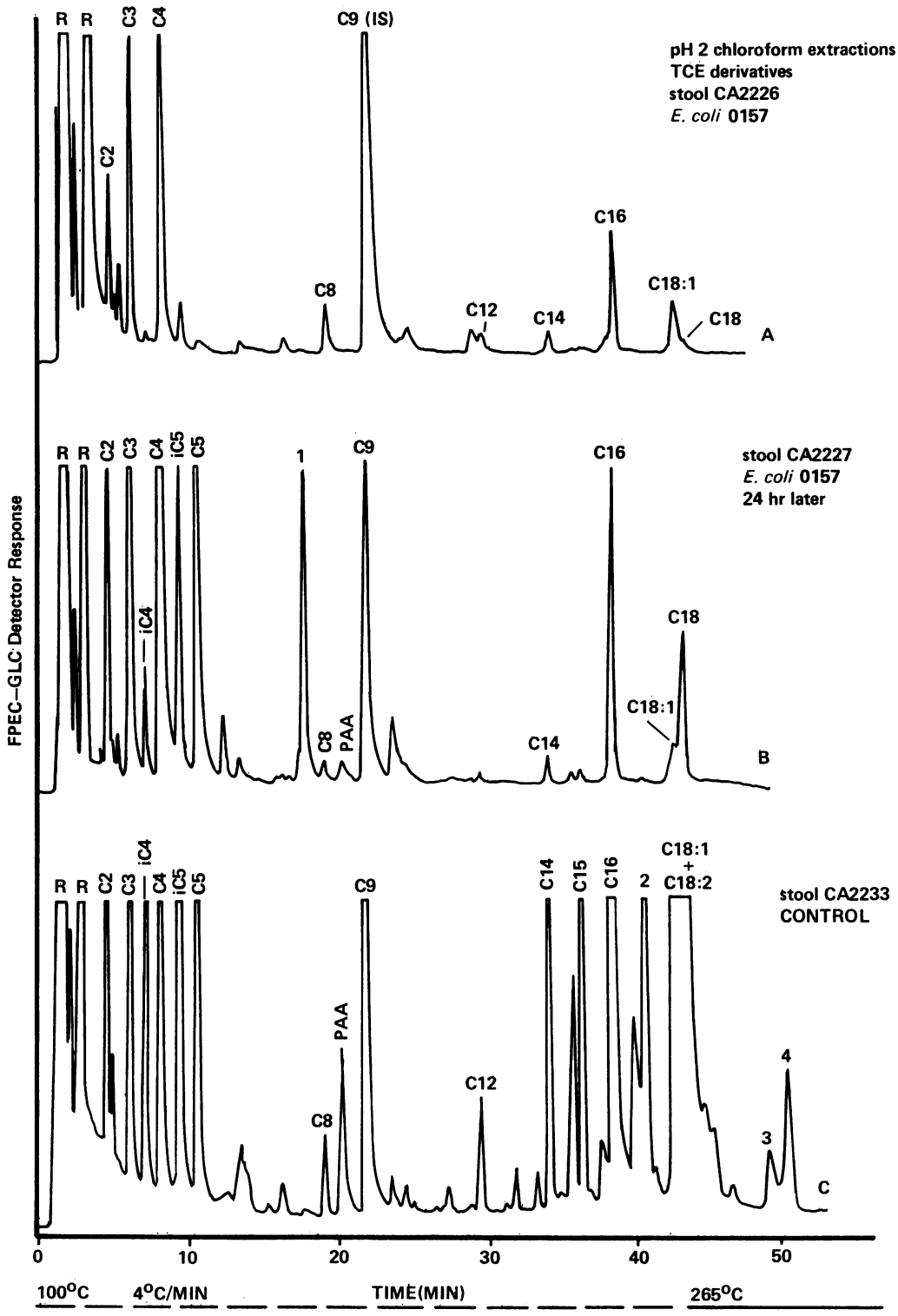

FIG. 5. FPEC-GLC chromatograms of TCE-derivatized extracts taken from stool. (A) Type of carboxylic acids from a patient with acute diarrhea from which *E. coli* O127 was isolated. (B) was made from a stool taken 24 h after the acute phase. Note that peaks C_2 to C_5 are beginning to return to the same size as in the control stool. (C) Chromatogram made from a control individual from the same geographic area as (B). Observe that the patterns in (C) have high concentrations of long-chain acids C_{14} to $C_{18:1}$. For abbreviations, see legend to Fig. 1.

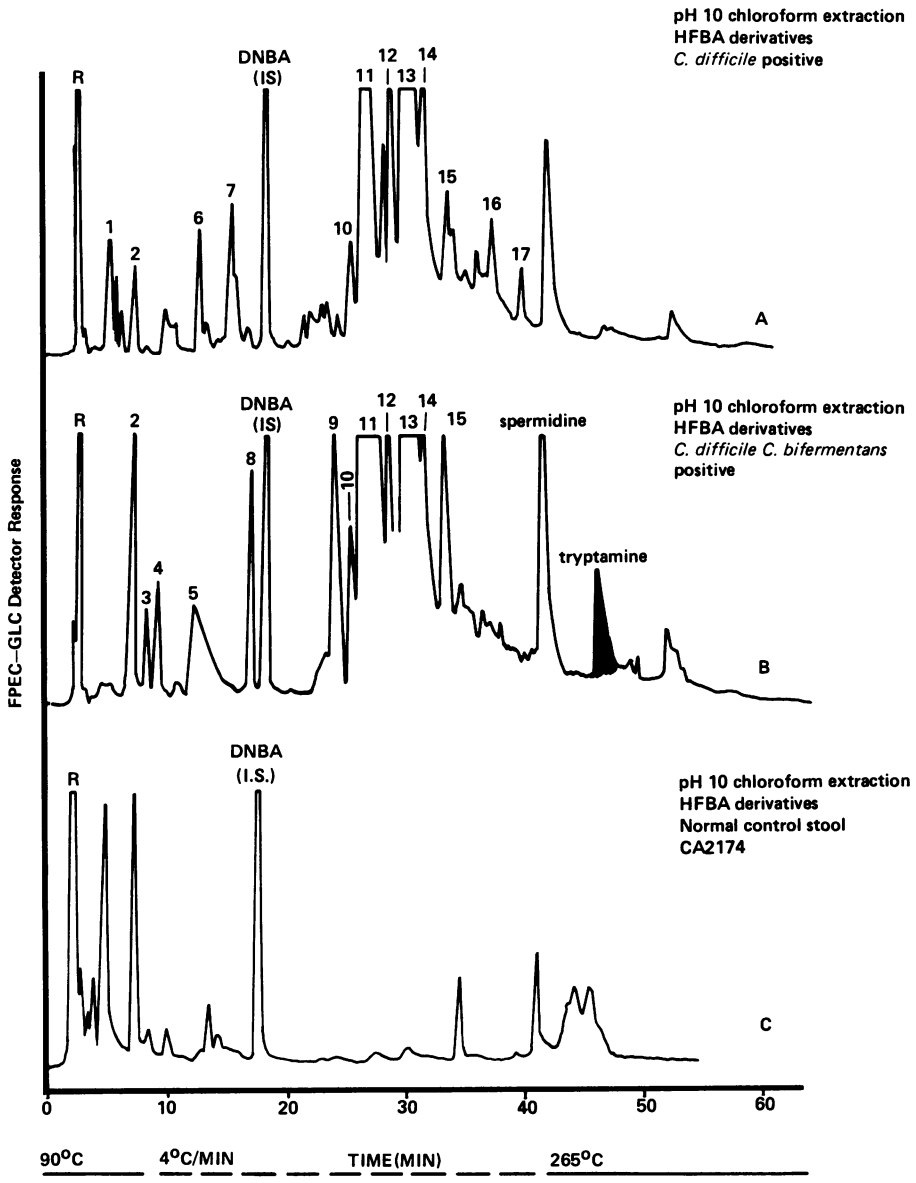

FIG. 6. FPEC-GLC chromatograms of the HFBA of derivatized basic chloroform extractions from stools. (A) Chromatogram from a stool from which *C. difficile* was isolated; (B) chromatogram from a stool which both *C. difficile* and *C. bifermentans* were isolated; (C) chromatogram of normal control stool. Note the presence of tryptamine in (B). DNBA, Di-*n*-butylamine. For other definitions, see legend to Fig. 1.

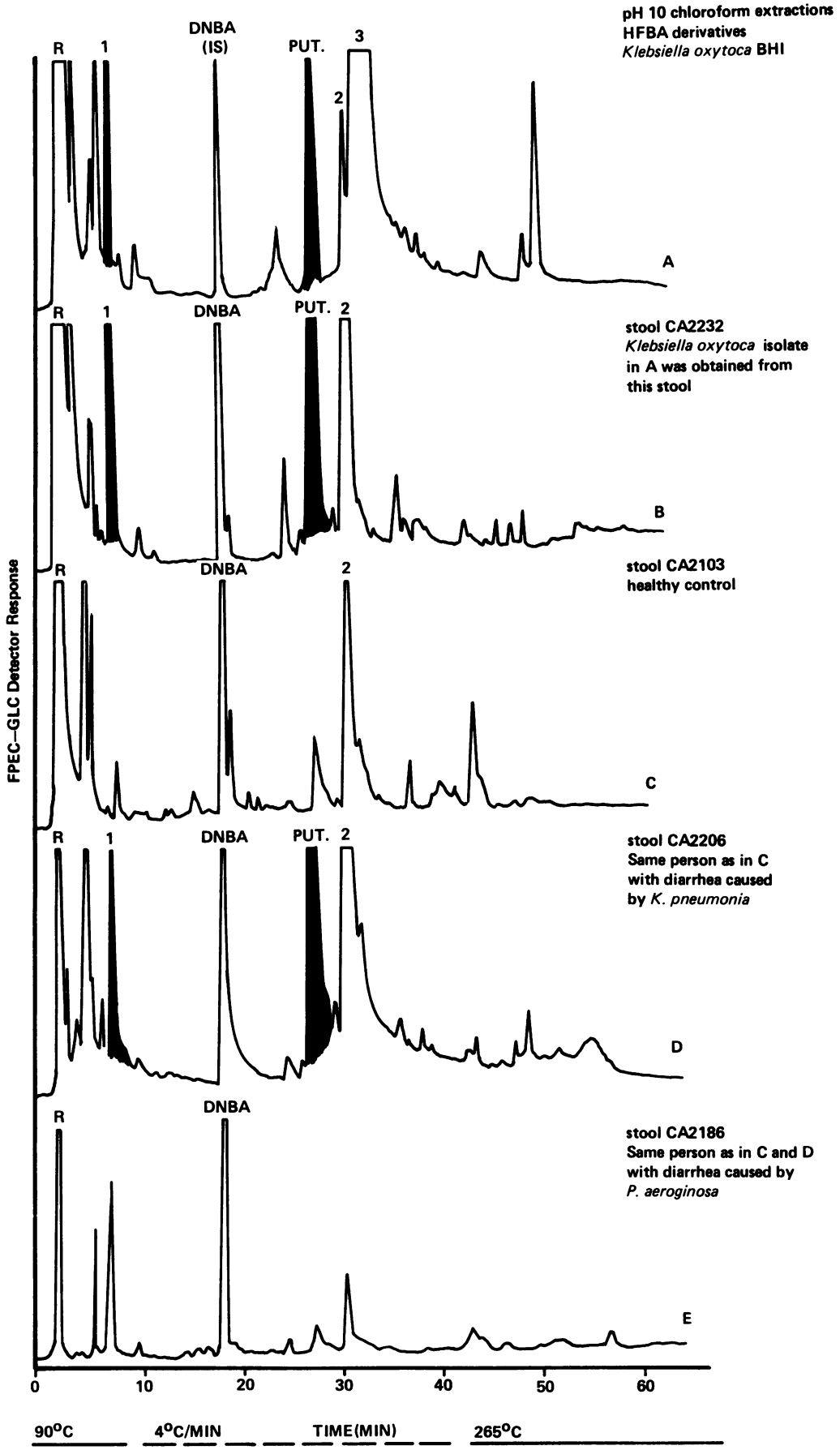

FIG. 7. FPEC-GLC chromatograms of HFBA-derivatized stool extractions made under basic conditions. (A) shows the amine products obtained in brain heart infusion broth from an isolate obtained from stool which is shown in (B) and identified as *K. oxytoca*. Note that peak 1 and putresceine (PUT) were detected in both brain heart infusion broth and stool. (C) Chromatogram taken from a healthy control. (D) is the same individual with diarrhea from which *K. pneumoniae* was isolated. (E) Chromatogram made from the same person (C and D), but in this case *P. aeroginosa* was isolated. For other abbreviations, see legend to Fig. 1.

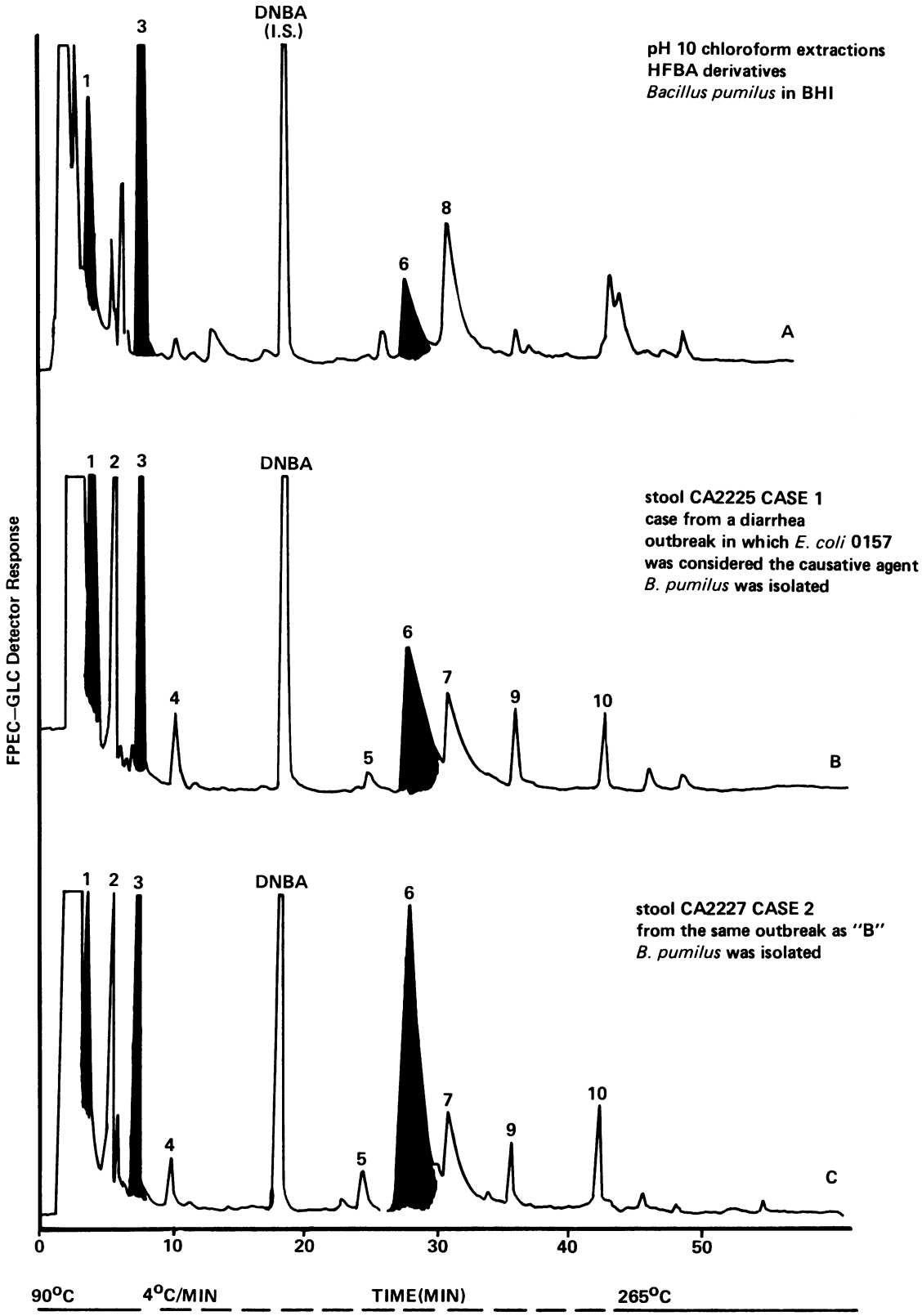

FIG. 8. FPEC-GLC chromatograms of HFBA-derivatized stool extractions made under basic conditions. The stools were obtained from the same diarrhea outbreak as the one shown for *E. coli* in Fig. 5 except in these cases *Bacillus pumilus* was isolated. (A) Isolate grown in brain heart infusion broth. Observe the similarities of the two cases shown in (B) and (C) and the similarities in products found in culture (A). For abbreviations, see legend to Fig. 1.

pathogen in a reproducible manner. Fourth, in the situation where a mixed group of potential pathogens was isolated, a predominant pattern characteristic of one particular pathogen was present. A predominant FPEC-GLC pattern from stool containing mixed pathogens possibly indicates that the principal organism responsible for the disease is producing the pattern.

Further, the data indicate that *C. difficile* produces iC_6 in vivo as it has been shown to do in vitro. In a study just completed (unpublished data) we found that enrichment of Trypticase-yeast-salt broth with amino acids affected some products of *C. difficile* in spent media, but had little effect on production of iC_6. Criticism concerning the possibility of *C. bifermentans* and *C. sporogenes*, which are occasionally found in stool, producing iC_6 and confusing the identification of *C. difficile* (3), could in all probability be eliminated by testing for amines and other products that have already been reported to be produced by these organisms (7, 8; Fig. 6A and B). Criticisms concerning the insensitivity, lack of resolution, and specificity of the technique described (19) could probably be overcome by use of the FPEC-GLC methods described above. It should be emphasized that sensitivity without both resolution and specificity would yield false-positive results.

FPEC-GLC profiles obtained by testing of stools taken over a period of time from a well-characterized person who developed bouts of diarrhea possibly caused by different agents indicate that different types of causative agents produce or affect the production of different types of metabolites in stool. Reproducible FPEC-GLC patterns from controls and different causative agents producing diarrhea provide a potential means for rapid identification of several potential causative agents of diarrhea. Rotavirus-induced diarrhea is the most common cause of diarrhea in infants and young children throughout the world (11). It has also been shown (11) to affect adults. Adenoviruses (9) have also been recently demonstrated to be involved in diarrhea outbreaks. Tests for these agents which require electron microscopy, culture, or use of immunological reagents, and which are difficult to produce commercially in high quality, are less than satisfactory. The data presented here on a limited number of specimens show a high degree of reproducibility for the specimens tested and could, with further study, be defined as a very effective detection method for distinguishing rotavirus and adenovirus infections. Further studies now in progress will add additional support to these findings and may provide additional FPEC-GLC profiles for identification of other causative agents involved in diarrheal outbreaks.

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