Differentiation and Characterization of *Klebsiella pneumoniae* Strains by Pyrolysis–Gas-Liquid Chromatography–Mass Spectrometry

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Nine coded duplicate strains of capsular nontypable *Klebsiella pneumoniae* were analyzed by pyrolysis–gas-liquid chromatography–mass spectrometry. All duplicate strains were correctly matched, and individual strains, including seven nontypable strains, were clearly distinguishable from one another. The addition of mass spectrometry to the analysis has aided the process of identification and has provided chemical structural information on *K. pneumoniae* strains. Application of this technique to the identification of some disease outbreaks or nosocomial problems could be of epidemiological importance, especially when conventional methods do not identify the epidemic strain.

Since 1965, when the first report on differentiation of pathogenic bacteria by the pyrolysis–gas-liquid chromatography (PGLC) method appeared (12), the technique has gained wide acceptance not only in the biomedical field but also in the areas of geology, energy, industry, and environmental science (6, 8).

The PGLC technique is simple in concept. If one takes an involatile sample, heats it in an inert atmosphere under carefully controlled conditions, and separates the gaseous degradation products by gas-liquid chromatography, one observes a series of peaks recorded on a strip chart. The series of peaks, or pyrochromatogram, is analogous to a “chemical fingerprint,” since it reflects the individual chemical character of a cell line. A number of pyrochromatographic studies dealing with bacteria have been reported, including the enteric bacteria (18, 26), mycobacteria (17, 20, 21), salmonellae (4, 10, 19), streptococci (12, 25), clostridia (2, 13, 16), pseudomonads (14), and *Vibrio* (5), as well as other studies in the fields of mycology and parasitology.

The hallmarks of the PGLC technique are simplicity of sample preparation, rapidity of analysis, precise PGLC duplication of like cells (reproducibility), and differentiation of unlike cells. Moreover, by elucidating the chemical structure of some or all of the peaks by means of mass spectrometry, we gain a new dimension in the process of identifying the infectious agent. It was these outstanding features of the PGLC technique which led us to consider it for an investigation of *Klebsiella pneumoniae*. This species has been associated with nosocomial outbreaks and as such is an important candidate for epidemiological study.

MATERIALS AND METHODS

**Strains.** Nine biochemically defined strains of *K. pneumoniae* were used to determine the PGLC profiles of capsular nontypable isolates. A list of isolates from the various sources is given in Table 1. All strains were isolated by the Hospital Infections Laboratory, Center for Disease Control, Atlanta, Ga. Capsular types were determined by the quellung method as described by Edwards and Ewing (3). Isolates were analyzed for antibiotic susceptibility by the Kirby-Bauer technique (1). The antibiotic disks used were nitrofurantoin, chloramphenicol, streptomycin, cephalothin, tetracycline, gentamicin, nalidixic acid, ampicillin, kanamycin, carbenicillin, and colistin.

**Preparation of PGLC samples.** Each strain was grown overnight on Warfel-Ferguson agar (Difco Laboratories, Detroit, Mich.). A heavy suspension of cells from each of the strains was harvested and suspended in 5 ml of sterile water for injection. The harvested cell suspension was washed three times in 5 ml of sterile water for injection and then resuspended in 5 ml of the same diluent. The washed cell suspension was divided into equal portions and coded for PGLC testing. Each suspension was frozen and lyophilized.

**PGLC.** Pyrolysis was carried out with a Chemical Data Systems Pyroprobe 100 unit linked via a heated interface to the inlet system of a Varian 3700 gas chromatograph. The capillary column used in this investigation was a glass support-coated open tube. It was 43 m long and had an inside diameter of 0.5 mm.

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The liquid phase was Carbowax, 20 M; such a column has been in use for about 1 year. It has a resolution capability of 37,600 effective plates.

**PGLC operating parameters.** The operating parameters for the pyrolysis system are as follows: temperature of pyrolysis interface, 180°C; temperature of pyrolysis sample, 800°C for 10.0 s. The gas-liquid chromatography conditions are: temperature program, hold at 65°C for 4 min, raise temperature by 6°C/min to 165°C and hold to approximately 70 min total recording time; carrier, gas, helium at a velocity (g) of 38.5 cm/sec; recording, Varian CDS-111 strip chart operating at 1 mV and 1 cm/min; detection, flame ionization detector operating at electrometer-amplifier setting of 1012 A. A full scale; temperatures, inlet at 250°C, detector at 250°C.

**Mass spectrometry.** A Varian MAT 112-S mass spectrometer, of reverse Nier-Johnson geometry, was coupled to the output of the Varian 3700 gas chromatograph through a direct open-split coupling. The temperatures of the interface and ion source were 250°C. Mass spectra of all the eluents were obtained by using an electron beam of 80 eV and 1.5 mA. An ion-accelerating voltage of 850 V was used, and the spectrometer was operated at 700 resolution with an exponential scanning range of 1 s per mass decade in the M/z range 35 to 350. Mass spectra were acquired and stored with the Varian MAT SS200 data system composed of a microprocessor-controlled interface coupled to a PDP 11/34 CPU and a Control Data Corp. Hawk dual disk drive. Documentation of the procedures used for identification of the compounds has been presented elsewhere (15). This procedure makes use of low-resolution electron impact spectra for identification purposes. In addition, this procedure has been extended to include exact mass determinations of significant ions in each individual spectrum at 2,000 resolution. Chemical ionization spectra at 700 resolution were also obtained, using isobutane as the reagent gas for molecular M+ and (M+1)+ ion formation (11).

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**RESULTS**

The strains of *K. pneumoniae* were biochemically the same but had different antimicrobial susceptibility patterns. All strains were susceptible to chloramphenicol, streptomycin, cephalothin, gentamicin, and nalidixic acid except those shown in Table 1. Capsular types were nontypable except for C2 and C7 (capsular type 4) and C8 and C10 (capsular type 44). The nine coded strains, each of which had been divided into two portions, were individually pyrolyzed and subjected to PGLC-mass spectrometry. The resultant pyrochronograms of duplicate coded samples were easily matched. Each one of the nine individual strains offered a characteristic profile of peaks (fingerprint). Figure 1 shows the profiles, i.e., detector responses versus elution time, of three strains. Each profile is arranged with a common time ordinate with peaks of a given identity having the same retention time. By simple visual inspection, the profiles in Fig. 1 are distinguishable. The patterns differ quantitatively (height or area of peaks) from one another throughout the profiles. For example, the large peaks indicated by the arrows are present in Fig. 1A and B, but are not present in any great quantity in Fig. 1C. This component was identified by mass spectrometry as acetic acid. Also, the unresolved components shown in the bracket in Fig. 1B are essentially absent in Fig. 1A and C. The last two major peaks, with 25- and 28-min retention times, have approximately identical heights, in Fig. 1C, in contrast to the corresponding peaks in Fig. 1A and B. In addition, the patterns of peaks in the 40- to 60-min range show clear differences between the pyrochronograms in Fig. 1.

In like manner one can clearly distinguish the
three nontypable strains C12, C15, and C17 on the basis of the profiles in Fig. 2.

The pyrochromatograms for isolates C2, C6, and C1 are presented in Fig. 3. We draw attention to the arrow and bracketed areas as prominent features that distinguish one strain from another.

The paired, coded samples C3 and C6 were analyzed and readily identified as identical isolates. The profiles were essentially the same (Fig. 4).

Mass spectrometry was used to identify the chemical compounds which constituted the major peaks. These compounds included acetonitrile (peak 1), toluene (peak 2), ethylbenzene (peak 3), pyridine (peak 4), pyrrole (peak 5), furfuryl alcohol (peak 6), phenol (peak 7), and cresol (peak 8).

**DISCUSSION**

Etiological agents are characterized by various marker systems during an epidemiological inves-
Investigation. Often, limited marker systems are available for the particular organism, such as biotyping, antimicrobial susceptibility patterns, and serotyping. However, some of the strains causing infection are serologically nontypable. It was considered that the nontypable strains possessed distinguishing characteristics, but perhaps required another method of identification. PGLC was used as an alternate method of grouping *K. pneumoniae* serologically nontypable strains. It was hypothesized the PGLC would give a distinct and detailed fingerprint of the nontypable strain, reflecting its chemical structure. The PGLC analysis could then be compared with those of other nontypable strains.

PGLC has been used as a means of rapidly classifying various species and strains of microorganisms. Studies confirm results and demonstrate that the distribution of pyrolytic products is characteristic of the microorganism and is directly or uniquely related to its chemical structure.

Some of the more predominant compounds formed by pyrolysis of the *K. pneumoniae*
strains have been numbered in Fig. 1 through 4. The compounds toluene, pyrrole, phenol, and cresol were predominantly formed from the breakdown of proteins (24), whereas it has been suggested (9) that furfuryl alcohol results from carbohydrate decomposition, and it has been found (23) as a product of polysaccharide pyrolysis. The above-listed compounds are among the 15 most abundant products found in the pyrolysis of Micrococcus luteus, Bacillus subtilis subsp. niger, and Streptomyces longisporoflavus (9). Acetonitrile, ethylbenzene, and pyridine, protein decomposition products, have also been found by previous investigators (9, 24) in relatively high yields from bacterial pyrolysis. These numbered peaks in Fig. 1 through 4 have also been observed in the pyrolysis-field ionization mass spectrometry of Pseudomonas putida (22). It is to be noted that we do not find acetamide as one of the major pyrolysis components from Klebsiella, in contrast to the earlier bacterial pyrolysis work of Simmonds and co-workers (9, 24) involving M. luteus, B. subtilis subsp. niger, and S. longisporoflavus. The large peak appear-
ing at 12 min (arrows in Fig. 1 and 3), which serves as an important marker for differentiating several Klebsiella strains, is due to acetic acid. Acetic acid has been used as one of the key compounds for the pyrolysis-mass spectrometric differentiation of capsular polysaccharides of Neisseria meningitidis (J. Haverkamp, H. L. C. Meuzelaar, E. C. Beuvery, P. M. Boonekamp, and R. H. Tiesjema, Anal. Biochem., in press). Lipid decomposition products are noticeable by their absence as predominant products, a fact consistent with previous pyrolysis work (9, 22, 24) and the relatively low fat content in many bacteria (7). In contrast, pyrolysis products from human tissues, viz., liver, spleen, kidney, and brain, contain appreciable quantities of compounds of lipid origin (15). These compounds are characterized by a series of alkenes and their corresponding saturated homologs. It has been hypothesized that the mechanism of their formation is decarboxylation of the corresponding fatty acid. A more complete analysis of the full range of pyrolysis products and delineation of possible marker peaks by means of mass spectrometry-computer techniques is an ongoing project.

The prospect of solving nosocomial problems in rapid fashion by PGLC with but a small investment of sample is well within the capabilities of the system described in this paper and should be considered when further differentiation of an etiological agent is required.

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LITERATURE CITED


